UNIVERSITEIT VAN PRETORIA
UNIVERSITY OF PRETORIA
YUNIBESITHI YA PRETORIA

The Kisspeptin signalling pathway and its role in breast cancer biology

By

Udochi Felicia Azubuike

Submitted in partial fulfilment of the requirements for the Doctor of Philosophy degree in Human Physiology

in the

Department of Physiology

Faculty of Health Sciences

UNIVERSITY OF PRETORIA

2022

SUMMARY

Kisspeptin is a neuropeptide that was first identified as a metastasis suppressor in human melanoma. It is the endogenous ligand for the G protein-coupled receptor, Kisspeptin 1 receptor (KISS1R). In addition to melanoma, Kisspeptin has since been shown to inhibit metastasis in pancreatic, lung, bladder, and ovarian cancers. However, in breast cancer, some data suggest that it may have a pro-metastatic effect, at least in oestrogen receptor-negative (ER^-) breast cancers. However, the exact mechanisms of how this is achieved, and if this effect is universal for all ER^- breast cancers, remains unclear. This study aimed to shed light on the ability of Kisspeptin to induce cell signalling related to metastasis in two triple-negative breast cancer (TNBC) cell lines. The non-metastatic BT-20 and the metastatic MDA-MB-231 TNBC cell lines were selected as they have very different migratory and metastatic characteristics. Real-time quantitative polymerase chain reaction (RT-qPCR) analysis was performed to assess *KISS1R* mRNA expression while Western blot analysis was used to investigate extracellular signal-regulated kinase 1 and 2 (ERK1/2) and protein kinase B (Akt/PKB) phosphorylation, and β-arrestin1/2 expression. Calcium signalling was measured using Fluo-3 AM, cell proliferation was measured using resazurin, and cell migration was assessed using an Oris™ migration assay. It was found that both cell lines express endogenous *KISS1R* mRNA. However, ERK1/2 and Akt phosphorylation and calcium mobilisation occurred in the BT-20 cell line after Kisspeptin-10 (KP-10) stimulation. ERK1/2 phosphorylation occurred late, in a β-arrestin-dependent manner. In contrast, only Akt phosphorylation and calcium mobilisation occurred in MDA-MB-231 cells after stimulation with KP-10. KP-10 increased migration in a calcium-dependent manner in the MDA-MB-231 cell line. KP-10 did not increase cell proliferation in either cell line. These data suggest that in these two related cell lines different signalling and physiological outcomes are initiated after KP-10 stimulation. This means that beyond the presence of Kisspeptin and KISS1R or Kisspeptin signalling, it is likely that other specific internal cellular signalling components also need to be present for Kisspeptin to act in a pro-metastatic manner in ER^- breast cancer. The data also suggest that Kisspeptin and KISS1R may not play a pro-metastatic role in all ER^- breast cancers.

Key words: Akt, β-arrestin1/2, Breast cancer, BT-20, Calcium, Cell migration, Cell signalling, ERK1/2, Kisspeptin, KISS1R, MDA-MB-231, TNBC.

DECLARATION

I declare that this thesis I am submitting for the Doctor of Philosophy in Human Physiology, is my work and where I used other sources, I referenced them properly. This thesis has not been previously submitted by me for another degree either at the University of Pretoria or another university.

Udochi Felicia Azubuike

ACKNOWLEDGEMENTS

This project would not have been possible without the help of God Almighty ("For from Him and through Him and for Him are all things. To Him be the glory forever. Amen." Romans 11:36), my supervisor, Dr Iman Van den Bout, co-supervisor, Dr Claire Newton, my parents, and siblings.

I would also like to thank my good friend Dr Mrs Ané Pieters for her advice.

I am indebted to my supervisors, Dr Iman van den Bout and Dr Claire Newton.

I also thank my parents, Mr and Mrs Timothy Azubuike, my best friend and twin brother Felix, Uncle Abraham, my siblings, Emmanuel, Victor and Benefit, members of the CNE and my friends.

This project was supported by a bursary from the National Research Foundation and the University of Pretoria Doctoral bursary.

I would also like to thank Dr Aron Abera for allowing me to use their facility at Inqaba Biotechnical Industries for RT-qPCR.

TABLE OF CONTENT

TABLE OF FIGURES

LIST OF APPENDIX FIGURES

LIST OF TABLES

LIST OF ABBREVIATIONS

Akt	Protein kinase B
BAPTA-AM	1,2-bis-(o-aminophenoxy)-ethane-N,N,N',N'-tetra-acetic acid, tetra-acetoxymethyl ester
B.C	Before Christ
DAG	Diacylglycerol
EMT	Epithelial-to-mesenchymal-transition
ER	Endoplasmic reticulum
ER^-	Oestrogen receptor-negative
ER^+	Oestrogen receptor-positive
ERK1/2	Extracellular signal-regulated kinase1/2
g	Gram
GDP	Guanine nucleotide diphosphate
GTP	Guanine nucleotide triphosphate
Gαβγ	G-alpha beta gamma
GPCR	G protein-coupled receptor
$G\alpha_q$ and $G\alpha_{11}$	G protein $G\alpha_q$ and $G\alpha_{11}$ subfamilies
hrs	Hours
IP_3	Inositol-1,4,5-triphosphate
IP_3R	Inositol-1,4,5-triphosphate receptor
kD	Kilodalton
KISS1R	Kisspeptin 1 receptor
KP-10	Kisspeptin-10
µl	Microliter
µM	Micromolar
M	Molar
min	Minute
ml	Millilitre
MMP	Matrix metalloproteinases

PI3K	Phosphoinositide 3 kinase
PIP_2	Phosphoinositol-4,5-bisphosphate
PIP_3	Phosphoinositol-3,4,5-triphosphate
PR^+	Progesterone receptor-positive
PKA	Protein kinase A
PKC	Protein kinase C
PLC	Phospholipase C
secs	Seconds
TMH	Transmembrane helix
TNBC	Triple-negative breast cancer

Chapter 1 LITERATURE REVIEW

1.1. Cancer

Cancer is a heterogenous group of diseases that is characterized by uncontrolled cell growth in one or more body organ.[1] Cancer was discovered in fossilised dinosaurs and the bones of humans from primeval times.[2] The first cancer growths in humans were discovered in Egyptian mummies and the first written record of the cancer disease was described in 3500 B.C in two Egyptian papyri, the Edwin Smith and Ebers papyri. In the Edwin Smith surgical papyrus, about 48 cancer cases were described and 8 of them were similar to breast cancer. In one of the breast cancer cases, case 39, breast cancer was described as a protruding head in a patient's breast, and it was reported to be an untreatable disease.[3] In another case, case 45, breast cancer was described as a disease that is cool to touch, untreatable because it had metastasized, and a disease with a bulging head.[2] The George Ebers papyrus was more detailed than the Edwin Smith papyrus and it described cancer as swollen tumour vessels, and he provided the diagnosis, prognosis and treatment options and also gave the various treatment methods including mechanical, magical and pharmacological treatment.[4] Over time, more suggestions and descriptions of the cancer disease were made. In 460 B.C, the father of modern medicine, Hippocrates (460-370 B.C), described cancer as a humoral disease. He predicted that the body has four different humours: phlegm, blood, black and yellow bile, and he suggested that breast cancer was due to black bile in the breast, and it should not be treated but should be allowed to harden and rupture so that the black bile can flow to other parts of the body.[2] He was the one that called cancer "Karkinos", which is a Greek word that means crab, to describe its crab-like appearance in that the tumour had a central body and protruding extensions. He also said that surgery on breast tumour can be life-threatening and should therefore be avoided.[4] Although cancer is an ancient disease, its prevalence has increased in current years due to the increase in the aging population and presence of many carcinogens in the environment resulting from modernisation and industrialisation.[2]

The cancer disease is a global burden, and it is predicted that its burden will continue to grow for at least the next twenty years.[5] Presently, after cardiovascular diseases cancer is the next leading cause of death in the world,[6] with 8.29 million people dying from cancer in 2010 and 10.0 million people in 2019, and it is predicted that there will be an increase in the number to 13.1 million by 2030.[7] In 2020, there were 19.3 million

cancer cases diagnosed and 10 million cancer related deaths, with over half of the deaths (58.3%) occurring in Asia, 19.6% deaths in Europe, 14.2% in America and 7.2% in Africa.[8] In the USA alone, it is estimated that there will be 1.9 million cancer cases and an estimated six hundred thousand deaths in 2022,[9] with prostate cancer being the leading cancer type in males and breast cancer the leading cancer type in females.[9] The distribution of the cancer cases and deaths worldwide for the ten cancers that are common in the world is shown in Figure 1.1.

Although disease incidence is increasing, there have also been global increases in cancer research, and this has caused an improvement in the 5-year survival rates of cancer patients. This improvement is attributed to better cancer detection techniques and screening. Due to improvements in screening, cancer can be detected at its early, treatable stage. However, despite this improvement majority (90%) of cancer related deaths still led to the development of metastasis and there is currently no preventative therapy for metastasis.[10]

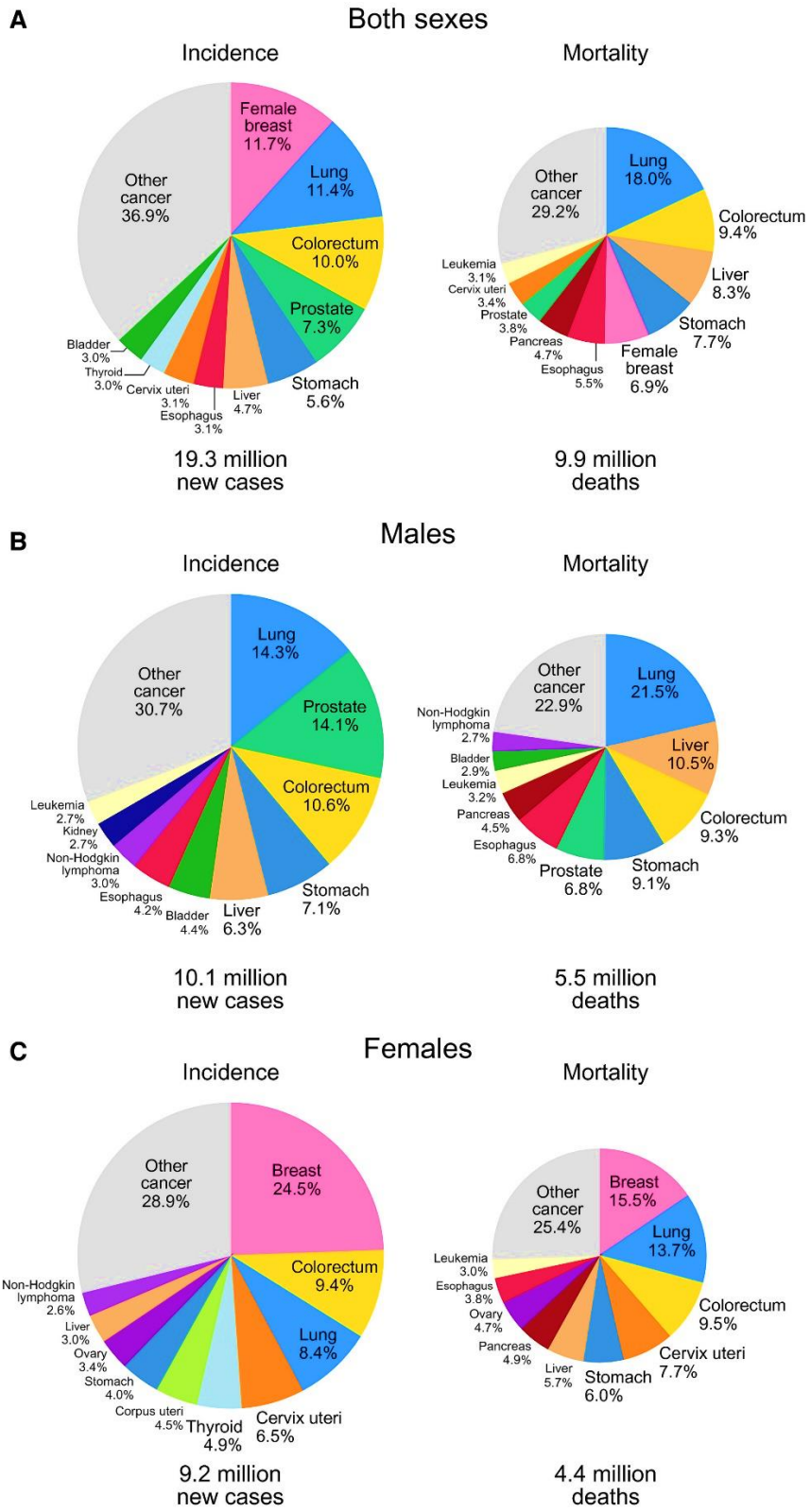


Figure 1.1: The global distribution of cancer incidence, and mortality for the 10 most common cancers in 2020.

Global cancer incidence and mortality of the 10 most common cancers are presented as a percentage of all cancers in both genders (A), in males (B) and in females (C).[8] (reproduced with permission from Cancer Journal of Clinicians,[8] see Appendix 6.10 for permission confirmation).

1.2. Cancer biology

Tumours exhibit aberrant cell proliferation. In normal cells, cell proliferation, survival, and differentiation occur in a meticulously regulated manner. But with cancer cells these regulated processes are disrupted so that cells begin to grow uncontrollably.[11] Cancer is a group of diseases that is characterised by genetic and cellular changes that affect genes that control apoptosis and cell cycle. In cancer, genes that promote cell cycle/cell growth and proliferation and/or inhibit apoptosis are affected either through mutation, chromosomal deletion or translocation, and aberrant activation of signalling pathways.[12] Some of the genetic changes can be inherited mutations in genes that are involved in the repair of DNA such as the breast cancer associated genes 1 and 2 (*BRCA1* and *BRCA2*). Other changes can be somatic mutations caused by exposure to environmental factors such as smoke from cigarettes, and radiation.[6] Apart from these genetic alterations, there are also epigenetic alterations (changes in gene expression that are not caused by alterations in the DNA sequence) such as methylation that can cause carcinogenesis even in cells that do not have any mutations. The silencing of tumour suppressor genes via epigenetics promotes tumour progression and inhibits apoptosis.[12]

Genetic changes observed in cancer mostly affect three types of genes: the proto-oncogenes, the tumour suppressor genes and DNA repair genes. The proto-oncogenes are genes that regulate normal cell growth and division. However, when they acquire gain-of-function mutations they become constitutively active and become cancer promoting genes (oncogenes). Due to these mutations in these genes, cells grow uncontrollably.[13] One example is the proto-oncogene, called *MYC*. Under normal physiological conditions this gene codes for transcription factors that regulate genes that are involved in cellular processes such as cell cycle progression and metabolism, cell survival, proliferation, and differentiation. However, in cancerous cells genetic aberrations (due to retroviral gene insertion, chromosomal amplification or translocation, activation of super-enhancers in the gene and mutation in the upstream

signalling pathway to enhance its stability), the *MYC* gene gets abnormally activated so that it promotes cancer progression.[14] As another example, a gain-of-function mutation in the proto-oncogene *HER2* can permanently activate the protein due to a loss of regulatory motifs, resulting in increased cell proliferation. Tumour suppressor genes, such as *TP53,* prevent uncontrolled cell growth. However, when they acquire inactivating mutations, their function is impaired, and this leads to uncontrolled cell growth.[13,15]

DNA repair genes or stability genes such as the *BRCA1* and *BRCA2* genes that repair damage in the DNA, such as excised nucleotides and mismatched nucleotide pairs, which happens during normal DNA replication or when cells are exposed to mutagens. Therefore, when they acquire inactivating mutations, the mutation rate of a cell is increased resulting in the accumulation of mutations that can induce cancer progression.[13,16] For example, an inherited mutation in *BRCA1* and *BRCA2* accounts for 5-10% of breast cancers.[17] *BRCA1* and *BRCA2* play a role in controlling cell division, DNA repair, and induce apoptosis in compromised cells. Mutations in these genes inactivate them resulting in loss of cell division suppression leading to uncontrolled growth.[13,15]

Tumours exhibit aberrant cell proliferation and can be either benign or malignant.[11] In cancer pathology, it is important to be able to distinguish between benign and malignant tumours. Benign tumours are tumours that do not invade surrounding tissues and, they remain confined to their original location. They also can be treated through surgery, chemotherapy, or radiotherapy and when treated, they do not grow back. However, compared to benign tumours in other parts of the body, benign brain tumours can be life threatening due to the resultant effects that the presence of the tumours can have on vital brain functions.[11,18] On the other hand, malignant tumours can invade surrounding tissues and spread through the lymphatic and circulatory system to other parts of the body.[11,18] Malignant tumours are the only tumours referred to as cancers and they grow more quickly compared to benign tumours.

Both benign and malignant tumours are classified based on the type of cell that they arise from. Most types of cancer fall into one of the three main groups; carcinomas, sarcomas, and leukaemias/lymphomas.[11] Carcinomas arise from epithelial cells,

sarcomas from connective tissues, and lymphomas and leukaemia's from cells of the immune system and blood forming cells, respectively.[11]

The cancer disease consists of many molecular components that are constantly evolving. Therefore, explaining the events that drive cancer genesis and progression is still a major challenge for clinicians and researchers.[19] In 2000, Hanahan and Weinberg gave a detailed logical framework for studying cancer. They demonstrated six characteristics that cancer cells acquire during tumorigenesis and tumour development. These were called the 'hallmarks of cancer'. These hallmarks of cancer differentiate cancer cells from normal cells. These first six hallmarks include: sustaining proliferative signalling, evading growth suppressors, resisting cell death, enabling replicative immortality, inducing angiogenesis, and activating invasion and metastasis.[20] In 2011, Hanahan and Weinberg extended the original six hallmarks, as they identified two additional characteristics, inflammation, and genome instability, that are important for the acquisition of the six hallmarks. Other characteristics that have since emerged as hallmarks of cancer include the deregulation of cellular energetics and avoidance of immune destruction.[21]

1.3. Breast cancer

Breast cancer is a group of heterogeneous tumours that mostly originate from the epithelial cells that line the breast ducts (tracks that bring the milk from the gland to the nipple) or lobules (the gland that secretes milk).[22] In the breast ducts and lobules there are two types of epithelial cell lineages that are derived from a common origin, luminal epithelial cells (that surround a central lumen and are responsible for secretion of the milk) and the basal or myoepithelial cells (that are contractile and aid in milk ejection).[23]

In 2020, breast cancer was the most diagnosed cancer overall, the fifth leading cause of cancer related death overall (685 000 people, which accounts for 18.4% of total cancer death) and the leading cause of cancer related death in women (Figure 1.1).[24] Each year breast cancer accounts for 23% (1.38 million women) of cancer diagnoses and 14% (458 000 women) of cancer deaths.[25]

Breast cancer has been shown to have a higher incidence in developed countries compared to developing countries, even though half of the diagnoses and over 50% of breast cancer deaths occur in developing countries.[4]

Some of the most important risk factors for breast cancer are: gender (it affects both males and females but females are at a higher risk of the disease), age (breast cancer incidence increases with age), late or no childbearing, short breastfeeding time, use of birth controls or contraceptives (women who use hormonal contraceptives are more prone to contracting breast cancer compared to women that have never used hormonal contraceptives [26]), race (Caucasian women are more at risk of developing breast cancer although African women are more likely to die from the disease), obesity, excessive alcohol consumption, and hormone replacement therapy after menopause.[17] Also, a person with a mother, sister or daughter that has been diagnosed with breast cancer is at a higher risk of developing breast cancer due to the potential for inheriting cancer-related gene mutations.[17] As previously mentioned, in Edwin Smith's Papyrus, eight breast cancer cases were identified, and breast cancer was described as an untreatable disease.[4] Fortunately, our understanding of breast cancer and its treatment has greatly evolved over centuries from being an untreatable systemic disease to a heterogeneous group of diseases that can be cured if diagnosed and treated early. Since 1989, there has been a decline in breast cancer mortality in premenopausal women.[27] This is most likely because of early detection and improved treatments in developed economies.[27]

Hippocrates described breast cancer as a black bile with crab-like tumours that should be allowed to harden, rupture, and release the black bile to the different parts of the body.[4] By 203 C. E, a Greek physician known as Galen suggested that it should be treated using sulphuric acid, castor oil, and opium. He also suggested that the affected breast should be removed using hot cautery and from the Middle Ages, breast cancer was treated through surgery by either lumpectomy (removal of only affected breast cells or tumours) or mastectomy (removal of the affected breast).[4,28] Many of the advances in breast cancer treatment were made in the 20th century, when it was identified that surgery was not the only treatment option for breast cancer, especially after the tumour cells have disseminated to other parts of the body.[28] More treatment options such as radiotherapy and chemotherapy, were identified. Such treatments include chemotherapy using doxorubicin, which has shown great response with limited

resistance.[27] It has also been found that using a combinational therapy consisting of lumpectomy surgery, radiation therapy, and chemotherapy was more effective in treating breast cancer than using only radical mastectomy or either one of the treatments alone.[27]

Oestrogen is a steroid hormone that regulates the growth and development of the reproductive system. In the 20th century tamoxifen was discovered as an anti-oestrogen drug that can be used as a more targeted therapy to treat ER^+ breast cancers.[4] By the 19th century, breast cancer was recognised to be a hormone (Oestrogen) dependent disease.[4] The first indication of this was observed by Galen in 203 C.E., who described breast cancer as a disease that is common in menopausal females and females with abnormal menstrual cycles and later, in the 18th century, through epidemiological studies, it was observed that breast cancer was more common in nuns than in married women and this observation was linked to childbearing.[4] Therefore, it was theorized that breast cancer was caused by childlessness as well as depressive mental disorders and breast inflammation filled with pus.[4] In the 19th century, it was observed that in premenopausal women with breast cancer, due to the high level of oestrogen, the growth of the tumour was faster, and it fluctuated with the menstrual cycle.[4] While in postmenopausal women, since they have low oestrogen the tumour growth was slower.[4] In addition to the use of oestrogen modulators such as tamoxifen, other targeted therapies for the treatment of breast cancer have been developed, for example trastuzumab (Herceptin), was discovered as a monoclonal antibody drug that can be used to treat breast cancers that overexpress HER2+.[4]

With respect to advances in breast cancer diagnosis, during the 20th century, the discovery of X-rays led to the development of mammography as a novel detection tool for breast cancer which is more accurate compared to the physical examinations such as palpation and nipple inversion and retraction, which were previously used to detect or diagnose breast cancer.[4]

By the 20th century, it was also established that in addition to hormonal and environment factors, breast cancer can also be inherited. An interesting discovery was that there is an increased risk of breast cancer in individuals with family history of mutations in the breast cancer tumour suppressor genes: *BRCA1* and *BRCA2*.[4] In the

21st century, with the advent of the human genome being sequenced in 2003, more methods of studying breast cancer as well as new therapeutic and diagnostic tools have been, and are still being, discovered.[4] One such tool is The Cancer Genome Atlas (TGCA), which uses bioinformatics and large-scale genome sequencing to find cancer-related mutations.[4] These tools have helped to further the understanding of the molecular basis of cancer and have enabled breast cancer classification based on gene expression profiling. Moreover, various genetic tests have been developed such as the Oncotype DX Breast Cancer Assay that can stratify breast cancers to allow more specific or personalised treatment options and to determine which patients would benefit more from particular treatment options.[4]

Breast cancer is a heterogenous group of diseases with different clinical, molecular, cellular, and histological characteristics. There are two types of tumour heterogeneity: intra-tumour and inter-tumour heterogeneity, and breast cancers display both types of heterogeneity.[22] This heterogeneity has made its treatment challenging. However, attempts at classification/stratification have been made.

Based on the histopathological features of the primary tumour and/or molecular profiling, breast cancer can be classified into at least six subtypes based on the presence or absence of the ER, PR, and on the overexpress *HER2* oncogene and levels of the cell proliferation marker, Ki-67. These subtypes are Luminal A, Luminal B, $HER2^+$, basal-like or triple-negative breast cancer (TNBC), normal-like breast cancer and claudin-low breast cancer (Table 1.1).[17] This system of classification helps to determine the optimal treatment options for each patient. For instance, a patient with ER^+ breast cancer will be given anti-oestrogen therapies such as tamoxifen, while a patient with $HER2^+$ breast cancer will be given anti-HER2 therapies such as Herceptin.[25]

Table 1.1: Classification of breast cancer based on molecular profiling.

Subtype	Features	Description
Luminal A breast cancer	ER^+, PR^+, $HER2^-$, low level of Ki-67, and high	Accounts for 50%-60% of all breast cancers. Grows

	levels of cytokeratin 8 and 18.	slowly and responds to hormone therapy.[17,29,30]
Luminal B breast cancer	ER^+ or PR^+, $HER2^+$, high level of Ki-67 and low levels of cytokeratin 8 and 18.	Accounts for less than 15%-20% of all breast cancers. Grows faster than Luminal A cancer and has a slightly worse prognosis. Resistant to endocrine hormone therapy.[17,29,30]
HER2 positive breast cancer	ER^-, PR^-, $HER2^{++}$, high level of Ki-67 and low levels of cytokeratin 8 and 18.	Accounts for 15-20% of breast cancers patients. Grows faster than the Luminal cancers and has a worse prognosis. However, it can be treated with targeted therapies like Herceptin, which target the HER2 protein. Over 40% of the patients with $HER2^+$ breast cancer have mutations in the *TP53* gene.[17 29,30]
Basal-like breast cancer or triple-negative breast cancer (TNBC)	ER^-, PR^-, $HER2^-$, high level of Ki-67, low levels of cytokeratin 8 and 18.	Accounts for 20% of all breast cancers.[17] Often diagnosed in women below 50 years of age.[31]
Normal-like breast cancer	ER^+, PR^+, $HER2^-$, low level of Ki-67 and, low levels of cytokeratin 8 and 18.	Accounts for 5%-10% of all breast cancers. Has similar features to Luminal A but its prognosis is worse. [17,29,32]

Claudin-low breast cancer	ER^-, PR^-, $HER2^-$, low level of Ki-67 and, low levels of cytokeratin 8 and 18.	Accounts for 7%-14% of all breast cancers.[33,34]

$^+$, present; $^{++}$, overexpressed; $^-$, absent; ER, oestrogen receptor; PR, progesterone receptor; HER2, Human epidermal growth factor receptor 2. Ki-67 is a marker for cell proliferation and cytokeratin 8 and 18 are epithelial cell markers.

Although it accounts for only 20% of breast cancers, TNBC is an aggressive type of breast cancer that has a short progression time and metastasises very early. It also cannot be treated with targeted anti-oestrogen or anti-HER2 therapies. As a result of these factors, patients with TNBC have a poor overall survival. There are currently no targeted hormone therapies for TNBC, although about 20% of the patients are responsive to standard chemotherapies.[35] Therefore, lots of research is currently ongoing to find a specific therapy for TNBC.

1.4. Breast cancer metastasis

Breast cancer metastasis is the cause of almost all breast cancer-related mortality. In the late 18th century, the French surgeon Henry LeDran first theorized that breast cancer was a localized disease that has the potential to spread through the vasculature to other parts of the body as the disease progressed.[28] Metastasis is a multi-step process that involves a series of events or adaptations that occur sequentially, but are interrelated, leading to the movement of cancer cells away from the primary site and the establishment of secondary tumours in distant organs.[36,37] There are seven processes that are necessary for metastasis to occur: tumour cell detachment from the primary site, invasion and migration to the vasculature, intravasation into the circulation, circulation through the lymphatic or circulatory system, extravasation at a distant site, survival in the tissue and expansion to form a secondary tumour (Figure 1.2).[38] Metastatic breast cancers are also called stage 4 or advanced breast cancers, and they can be detected in lymph nodes around the armpit and in distant organs including the lung, liver, bone, and brain.[17,39] The different steps in the metastasis cascade will be discussed in the next few paragraphs.

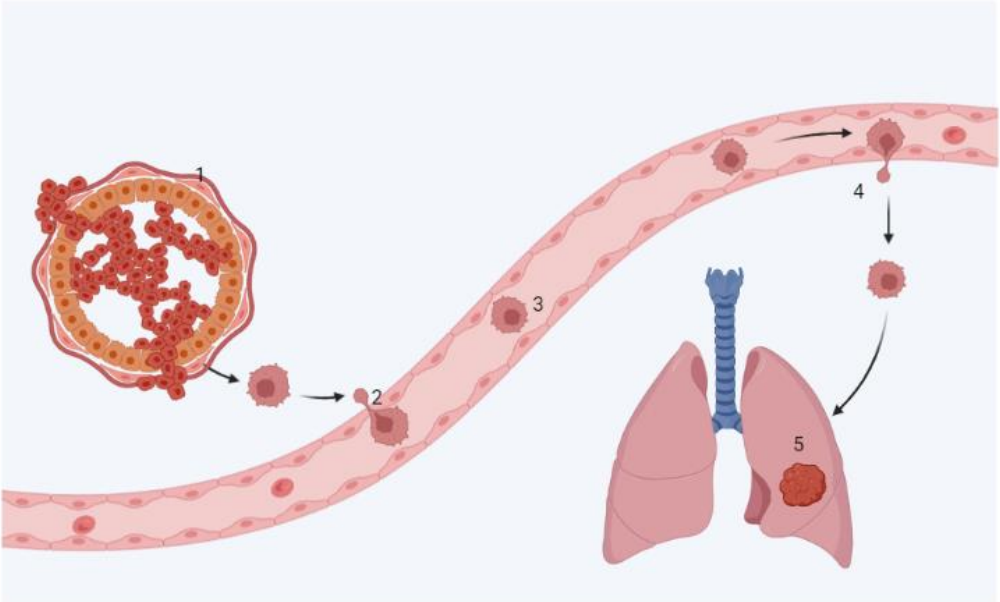


Figure 1.2: Summary of the process of metastasis process.

During metastasis, (1) cancer cells that have undergone epithelial-mesenchymal transition detach from the primary tumour and invade the basement membrane and migrate to the vasculature, (2) the cells intravasate across the endothelial barrier to the blood and lymphatic vessel, (3) they circulate in the blood or lymphatic vessel, evading attack by the immune system, (4) they extravasate at distant capillary beds, (5) they invade and colonise the distant organ (the lung) and form a microenvironment that facilitates angiogenesis and proliferation, leading to a macroscopic, malignant, secondary tumour.[37,38] (Image adapted from "Tumour metastasis (Layout 2), by Bio-render.com.[40]).

1.4.1. Epithelial-to-mesenchymal transition (EMT)

Most solid tumours or carcinomas originate from epithelial cells (carcinomas). These epithelial cells are separated from the underlying stroma by a basal lamina. When a tumour progresses from an *in situ* to an invasive carcinoma, epithelial cells decrease their cell-to-cell interactions and acquire the ability to invade the basal lamina. Invasion of surrounding tissue is dependent on epithelial-to-mesenchymal transition (EMT) of cancer cells.[41] The process of breast cancer metastasis begins when cancer epithelial cells of the primary tumour undergo EMT. During EMT, epithelial cells that were initially

well organised, differentiated, and polarized, transition into cells that are undifferentiated, isolated and mesenchymal, with the ability to migrate and invade the basement membrane.[36] These mesenchymal cells have the phenotype of being elongated and fibroblast-like in shape and they secrete enzymes such as MMPs that degrade the ECM and allow the cells to invade the basement membrane and the surrounding connective tissue.[32]

EMT is an essential first step in metastasis. It is activated by transcription factors such as Twist, Snail, Zeb1 and Slug, as well signalling pathways such as Notch, transforming growth factor β (TGF-β), and Wingless-related integration site (WNT) signalling pathways.[42] During EMT there is a change in cell-to-cell adhesion and cell-ECM adhesion. Cell-to-cell adhesion is mediated by a family of transmembrane glycoproteins called cadherins.[43] There are at least twenty members of the cadherin family and they function in a homotypic manner, for example E-cadherins in epithelial cells only recognise and bind to other E-cadherins on neighbouring cells in or E-cadherin on the same cells.[44] The E-cadherins are down-regulated or lost during the process of EMT and this allows the cells to survive after being separated from their cell-to-cell junctions.[45] While the expression of E-cadherins is down-regulated, the expression of the mesenchymal marker N-cadherin is up-regulated.[46] In addition to N-cadherins, there are other mesenchymal markers that are expressed by the mesenchymal cells after EMT, including vimentin, desmin, alpha smooth muscle actin (α-SMA), and fibroblast-specific protein 1 (FSP1).[47] Therefore, at the end of the EMT process, the epithelial cells lose their apical-basal polarity to gain a front-rear polarity, they lose their cell junctions, their cytoskeleton architecture is reorganised, and epithelial genes are down-regulated while mesenchymal genes are up-regulated. All these changes increase their migratory and invasive behaviour.[48]

1.4.2. Invasion and migration

After the cancer cells have transitioned from epithelial to mesenchymal cells at the local site, the next step in process of metastasis is invasion. This is the process by which the tumour cells infiltrate the basement membrane and migrate either collectively or individually through the ECM to their surrounding tissue or distant sites. Therefore, invasion and migration are prerequisites for metastasis. EMT increases the

invasiveness of cancer cells.[42] For example, up-regulation of α5β1 integrin has been correlated to cancer invasiveness during cancer metastasis.[36] Some integrins also promote invasion by activating proto-oncogenes such as the *Src*.[49] *Src* encodes for non-receptor tyrosine kinase, which when activated releases MMPs which degrade the basement membrane and ECM, allowing the primary tumour cells to invade surrounding tissue.[50]

Cancer cell invasion and migration is affected by parameters in the tumour microenvironment such as: the stiffness of the ECM, hypoxia, chemoattractants, and nutrient depletion. Tumour cells can migrate using similar mechanisms and migration modes as seen in normal (non-cancer) cells during physiological processes such as leukocyte and fibroblast migration during immune defence [36,43] and collective migration of epithelial and endothelial cells during early development.[51] Cell migration can be divided into two different categories: single-cell migration and collective/ coordinated migration.[36,43] In single cell migration the epithelial cells that have undergone EMT to become single/individual cells migrate independently. Whereas, in collective cell migration, a group of cells remain attached to each other, and they migrate as a group or sheet. In normal non-cancer cells, collective migration occurs mostly in epithelial and endothelial cells while single cell migration occurs in leukocytes and fibroblasts during inflammation.[36,43] Metastatic tumour cells use both collective and single cell migration during invasive growth and metastasis.[51]

During single cell migration, the tumour cells can migrate in either of two ways: (1) protease-dependent mesenchymal migration or (2) protease-independent amoeboid-like migration.[36] In protease-dependent mesenchymal migration, the mesenchymal cells take advantage of their elongated, fibroblast-like shape to move through the ECM that has been degraded by MMPs secreted by the cells.[36,43] While in protease-independent amoeboid migration, cells have a similar behavioural and movement pattern as the single-celled organism, amoeba. Amoeboid tumour cells only weakly attach to the ECM through non-integrin adhesion using selectin instead of integrin-based adhesions.[51]

During collective migration cells migrate as a group that are connected to each other through adherens junction formed around cadherins.[51] The group of moving cells are polarised so that they have a leading edge, containing integrins and proteases.

Studies have shown that there is a difference in gene expression and morphology of cells in the leading edge and those following them, which are called the trailing edge cells.[51] The cells at the leading edge are usually mesenchymal-like with weaker cell-cell adhesions and protrusions adhering to the ECM via pseudopodia and invadopodia while the trailing edge cells collectively form rosette-like tubular structures with tight junctions between them.[51]

1.4.3. Intravasation

After tumour cells have evaded surrounding tissues, they need to cross the endothelial barriers to get to the circulatory system, and this process is called intravasation. Intravasation does not only occur in tumour cells, it also occurs in normal cells during development and the immune response where cells are directed by growth factors and tissue injury, respectively, to cross blood vessels.[52] The tumour cells usually cross the endothelial barrier of either the blood or lymphatic vessels, although intravasation through lymphatic vessels can also occur, most of it occur via the blood vessels.[52] The intravasation through the blood vessels can be either a passive or active process depending on the type of tumour, the structure of the blood vessel and the tumour microenvironment.[52] There is also a change in the cytoskeleton of the tumour cells as integrins and other adhesion molecules are upregulated to allow the tumour cells attach to the endothelial barrier.[52] As they enter cross the barrier, shear forces from the blood movement can kill most of the cells before they enter the bloodstream.[52] If intravasation occurs via the lymphatic vessels, the tumour cells encounter several lymph nodes before converging at the thoracic duct where they are drained into the blood.[52] The tumour cells survive better in the lymphatic vessel than the blood vessel because the shear force is lower in the lymphatic vessel due to the slow flow of the lymph.[52]

1.4.4. Circulation

The circulatory step is usually the most difficult step for most intravasated cancer cells in terms of cell survival. Thus, the circulatory environment is the largest bottleneck for

successful metastasis and most tumour cells do not survive here. The tumour cells in the circulation are referred to as called circulating tumour cells (CTCs).[53] The CTCs either move in the circulation as clusters or as single cells.[53] The clustered cells consist of CTCs, stromal cells and some immune cells carried from the original microenvironment, and the heterogenicity of these clusters improves their survival in the circulation.[53] For this reason, cells that move as clusters are more likely to metastasize to distant organs than those that move as single cells.[53] CTCs survive by being resistant to anoikis (death of cells in a programmed manner due to lack of adhesion to the ECM), resisting shear forces of the blood flow, and evading the immune system.[53] One way in which they are also able to protect themselves from shear forces and detection by the immune system is by interacting with the components of the circulatory microenvironment to form small emboli, which consists of circulating tumour cells covered by platelets.[32] The circulating tumour cells that survived the harsh conditions of the vascular environment extravasate into the target tissue or organ, by arresting in the endothelial capillaries and either extravasate through trans-endothelial migration or by growing within the vessel before extravasating and colonising the target organ.[53]

1.4.5. Extravasation to a distant organ

Extravasation occurs when tumour cells exit the vasculature. CTCs get entrapped when they pass through small capillaries, resulting in forced extravasation and microvascular rupture.[53] For organs such as the liver and bone with very permeable vessels CTCs are more likely to metastasize to these organs.[53] For other organs, such as the lungs and brain, the CTCs must transmigrate through tight barriers and basement membranes, and this may require genetic modifications and changes in endothelial architecture including receptor expression and cell-to-cell contacts.[53,54]

Tumour cell extravasation requires three sequential steps. Firstly, there is a weak interaction where the cells attach loosely to the vascular endothelial cells through an interaction between the cell surface ligands such as lectins found on the cancer cell and selectins, which are protein glycoproteins or receptors expressed on the endothelium. Following the initial weak interaction, integrin-dependent binding ensues allowing the cancer cell to spread on the endothelium and produce protrusions that

invade the cell layer. Integrins also direct the site of extravasation and colonization by mediating anchorage-independent survival.[53,54]

The tumour cells do not migrate randomly to any organ. They migrate to specific organs in a process known as organotropism.[53] Organotropism was observed by Paget in 1889,[55] who noticed the preferential dissemination of tumour cells 'seeding' to target organs 'soil'.

1.4.6. Survival and outgrowth

The CTCs that have made it to the target site are exposed to harsh conditions which they must survive for metastasis to happen. The tumour cells in the new microenvironment must adapt to the new environment, which has stroma components, tissue organization and matrix composition that are different from their original site.[32] They also express certain chemokine receptors such as chemokine receptor 4, which binds to locally produced CXCL12 and activates pro-survival and proliferation pathways.[32]

In order to survive in their new environment, they induce the production of new blood vessels through which they can get nutrients from the blood through a process known as angiogenesis. Angiogenesis is regulated by the balance between pro-angiogenic and anti-angiogenic factors.[56] Angiogenesis can be induced by tumour cell expression of angiogenic proteins in response to hypoxia and nutrient depletion.

Thus, cancer progression and especially metastasis is a process largely driven by adaptation to changing environments and survival in hostile ones. Many of the receptors that detect such changes are important targets of research to investigate whether modulation of these can inhibit the metastatic success of breast cancer cells. Many of the cell surface receptors that have been implicated in cancer metastasis belong to the family of cell surface receptors known G protein-coupled receptors (GPCRs).[57]

1.5. G protein-coupled receptors (GPCRs)

GPCRs are the largest family of cell surface receptors in mammals.[58] Structurally, they consist of a transmembrane spanning domain comprised of seven transmembrane helixes (TMH 1-7), which are connected by three intracellular and three extracellular loops.[59] They also have an extracellular N-terminus and intracellular C-terminus. They are called GPCRs because of their interaction with intracellular guanine nucleotide binding regulatory proteins (G proteins).[60] GPCRs respond to extracellular stimuli by changing their conformation and transducing the extracellular signal to an intracellular response through this interaction.[60]

Currently, there are ~800 GPCRs that have been identified in the human genome. They can be classified into five families based on their phylogenetic trees: Glutamate, Rhodopsin, Adhesion, Frizzled/Taste and Secretin.[61] The Rhodopsin family comprises of 80% of the human GPCRs. The majority of the members of this family that have their ligand-binding domain in their transmembrane region, between the helices, which is either close to the extracellular surface or buried in the membrane. They react to light, hormones, and neurotransmitters.[61,62]

The G proteins are proteins that bind to guanine nucleotides (guanine nucleotide diphosphate (GDP) and guanine nucleotide triphosphate (GTP)), and they have intrinsic GTPase activity. Human G proteins can be classified into two groups; the small G proteins which are monomeric, and the heterotrimeric G proteins.[63] It is the latter group with which GPCRs interact to transduce intracellular signalling. These heterotrimeric G proteins consist of Gα, Gβ and Gγ subunits with the latter two mostly existing as an obligate dimer. There are four subclasses of G proteins (Figure 1.3), and these are categorised based on their component the Gα subunit: $G\alpha_s$, $G\alpha_{i/o}$, $G\alpha_{q/11}$, and $G\alpha_{12/13}$.[58] In its inactive state, Gα is bound to the Gβγ dimer and GDP. When a ligand binds to a GPCR it adopts a conformation that allows it to activate the heterotrimeric G proteins. The ligand-receptor-G-protein-complex catalyses the exchange of GDP for GTP on the Gα subunit thereby promoting the dissociation of the GTP bound Gα subunit from the Gβγ subunit. The dissociated Gα-GTP and Gβγ proteins activate downstream effector molecules as shown in Figure 1.3. The free active receptor can bind and activate another G protein complex thereby resulting in signal amplification.[62] $G\alpha_{q/11}$ activates phospholipase C, which hydrolyses phosphatidylinositol-4,5-bisphosphate (PIP_2) to produce the second messengers, inositol-1,4,5-trisphosphate (IP_3) and diacylglycerol (DAG). DAG activates different

isoforms of protein kinase C (PKC) while IP_3 binds to the IP_3 receptors (calcium channels) on the endoplasmic reticulum to release calcium into the cytosol which regulates cell processes such as cell proliferation, contraction, secretion, gene transcription, and apoptosis.[64,65] $G\alpha_s$ activates adenylyl cyclase enzyme, which produces the second messenger molecule, cyclic AMP (cAMP). cAMP activates protein kinase A (PKA). PKA phosphorylates activates several intracellular proteins that regulate different cellular processes to modulate their activity. $G\alpha_{i/o}$ inhibits adenylyl cyclase enzyme thereby reducing cAMP production. Lastly, $G\alpha_{12/13}$ activates the small GTPase RhoA.[66]

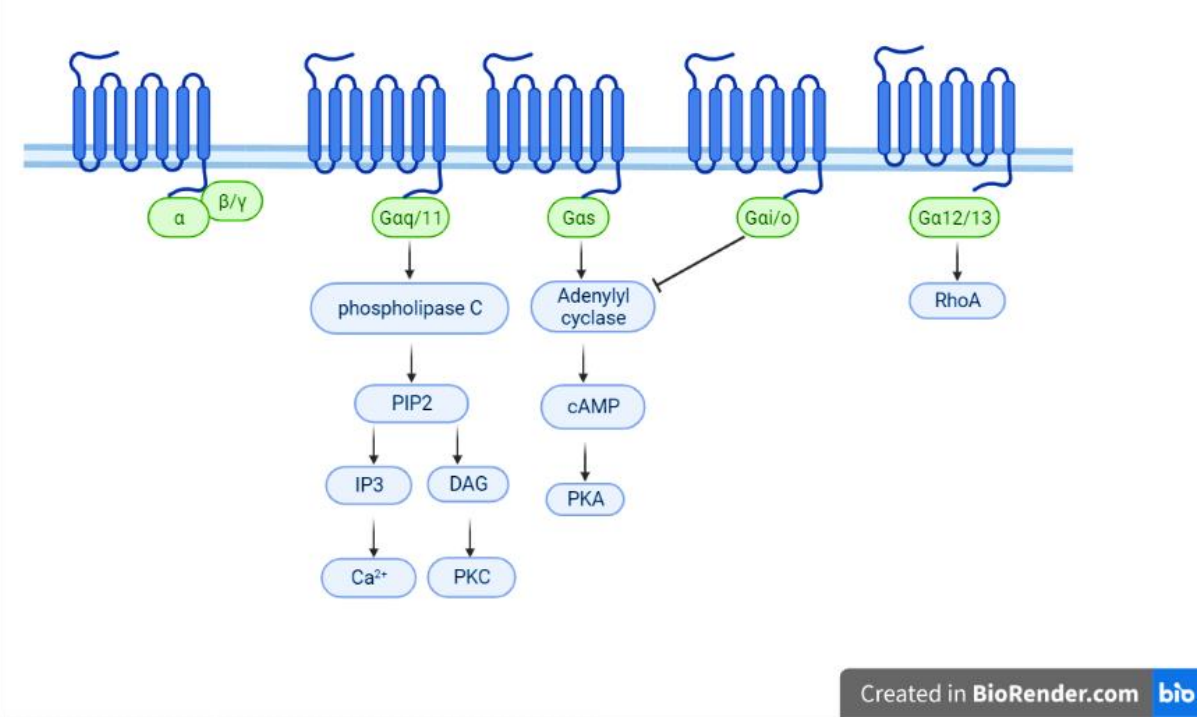


Figure 1.3: G protein-coupled receptors (GPCRs), G protein and its sub-classes.

The GPCRs are receptors that span the membrane seven times and transduce extracellular signals into intracellular responses via interaction with intracellular heterotrimeric G proteins. G proteins comprise of three subunits (αβγ). In their inactive state, the heterotrimeric αβγ protein are together. The Gα subunit is classified into four sub-classes: $G\alpha_{q/11}$, $G\alpha_s$, $G\alpha_{i/o}$, or $G\alpha_{12/13}$. The $G\alpha_{q/11}$ activates the enzyme phospholipase C (PLC), which cleaves phosphatidylinositol-4,5-bisphosphate (PIP_2) to inositol-1,4,5-triphosphate (IP_3) and diacylglycerol (DAG), which are second messengers. The IP_3 binds to IP_3 receptors on the endoplasmic reticulum (ER) and causes Ca^{2+} release. Whereas the DAG activates protein kinase C (PKC). The $G\alpha_s$ activates the enzymes adenylyl cyclase which produces the second messenger, cyclic adenosine monophosphate (cAMP) from adenosine triphosphate (ATP) and this

cAMP activates protein kinase A (PKA). $G\alpha_i$ inhibits the activity of adenylyl cyclase and therefore reduces the production of cAMP and activation of PKA. $G\alpha_{12/13}$ activates RhoA which is a family of GTPases that exchange guanosine diphosphate (GDP) for guanosine triphosphate (GTP).[66] (Image adapted from "GPCR effector pathway", by Bio-render.com.[67])

GPCR signalling is regulated tightly to ensure that the required degree of signal transduction is elicited. Thus, continued signalling by activated GPCRs is inhibited by receptor desensitization.[62] Desensitization is a two-step process that involves GPCR cytoplasmic phosphorylation and the binding of β-arrestin which then leads to receptor internalisation.[66] GPCRs are phosphorylated on serine and threonine residues in the third intracellular loop and in the carboxy-terminal tail. Heterologous desensitization is mediated by proteins such as PKC and PKA that are activated by second messenger molecules. PKC and PKA phosphorylate amino residues at in the receptor's third intracellular loop and C terminus to inhibit further G protein coupling resulting in the inhibition of signalling. This heterologous desensitization can occur regardless of the activation state of the GPCR. Conversely, homologous desensitization only affects activated receptors. This method is desensitization is mediated by a family of kinases known as GPCR receptor kinases (GRKs).[66] GRKs recognise activated GPCRs and mediate receptor phosphorylation of serine and threonine residues within their carboxyl C terminus. Regardless of the method of phosphorylation, phosphorylation of GPCRs mediates β-arrestin binding, which prevents further G protein interaction signalling and promotes receptor internalisation.[66]

Due to the ability of GPCRs to control various physiological responses and due to their relevance in various diseases they have been an important source of drug targets in the pharmaceutical industry. Approximately 36% of drugs on the market target GPCRs.[57] Based on the notion that GPCR activation is in two states and its activation is dependent on its association with heterotrimeric G proteins, most available therapeutic targets of GPCRs include agonists (which activate the receptor), partial agonists (which only partially activate the receptor), and antagonists (occupy the receptor and inhibit activation by endogenous ligands).[62,68]

Recent studies have shown that GPCR signalling is more complicated than the dualistic active/inactive on-and-off switch model that was the previous dogma. This is because it has been shown that activation by different ligands can induce different signalling responses e.g. coupling to different G proteins or altered signalling via other

adaptor proteins that are not dependent on G protein coupling. For example, protease activated receptor 1 (PAR1) is able to couple to $G\alpha_{i/o}$ when activated by elastase, and couple to $G\alpha_q$ and $G\alpha_{12/13}$ when activated by Factor Xa.[69] Therefore, it is now believed that they can adopt many different active states, each with specific downstream signalling preferences.

Furthermore, it has become evident that receptor oligomerisation (either homo-oligomerisation of like receptors or hetero-oligomerisation of different receptors) can affect receptor pharmacology, with respect to their activation, signalling, and internalisation. The clustering of receptors in membrane microdomains has also been postulated to affect receptor signalling and trafficking.[68] Additionally, complexity in signalling is further increased due to the ability of a GPCR to couple to different heterotrimeric G proteins to elicit unique signalling in a cell-type specific manner. For example, β_2-adrenergic receptor is a GPCR that has been shown to couple to $G\alpha_s$ in a healthy heart and $G\alpha_i$ in an unhealthy heart. [70]

1.6. GPCR signalling pathways implicated in cancer

The altered expression of several GPCRs has been linked to tumorgenicity. Due to their involvement in cancer initiation and progression, a few of them have been exploited to develop drugs that can inhibit signalling pathways associated with cancer.[57] For example, in colon cancer when the GPCR, called formylpeptide receptor 2 (FPR2), is highly expressed it leads to rapid growth and cell migration.[71] Also, FPR2 signalling through Akt pathway (an intracellular signalling pathway involved in regulating apoptosis) leads to drug resistance in colon cancer.[72] In prostate cancer, a GPCR known as prostate G protein-coupled receptor (PSGR) is expressed at low levels in normal prostate tissues but highly expressed in prostate cancer tissues, where it increases metastasis. Another GPCR called G protein-coupled receptor family C group 6 member A (GPRC6A) increases prostate cancer cell migration and invasion through ERK1/2 signalling.[57,73] In breast cancer the overexpression of GPR161 in TNBC has been linked with increased cell proliferation and invasion.[57,74] Another GPCR called the G protein-coupled oestrogen receptor (GPER), which is a GPCR that is known to also activate oestrogen signalling, induces Ca^{2+} mobilization when it is activated by its ligand and this leads to the regulation of expression of genes that are

involved in breast cancer cell migration and growth.[75] In melanoma, GPR55 has been linked with tumour development where it causes tumour cell proliferation, invasion and anchorage independent growth.[57,76] Therefore, GPCRs are important as potential targets in treating cancer, and, in some cases, this has been exploited to develop drugs that can inhibit signalling pathways associated with cancer.[57] Some of these signalling pathways, which were also assessed in this study will be discussed further in the next few sections.

1.6.1. ERK1/2 signalling (ERK1/2)

Extracellular signal regulated kinase 1/2 (ERK1/2) is a member of the mitogen activated protein kinase (MAPK) family. These kinases that transmit extracellular signals from cell surface receptors to intracellular targets.[77] There are four different MAPKs they are; i. ERK1/2, ii. c-Jun N-terminal kinase (JNK), iii. ERK5, and iv. p38 MAPK (p38).[78] The ERK1/2 pathway is activated when cell surface receptors are activated by growth factors, while JNK, p38 and ERK5 are activated by stress and growth factors.[79] The ERK1/2 pathway is the prototypical MAPK/ERK pathway and is made up of a group of core kinases that sequentially phosphorylate each other starting with rapidly accelerated fibrosarcoma (RAF), then mitogen-activated protein kinase kinase (MEK) and lastly ERK1/2.[80]

The ERK1/2 signalling pathway is activated as a downstream result of activation of by either receptor tyrosine kinases (RTKs) or and GPCRs.[80] The activation of the ERK1/2 pathway by these receptors results in a change in the conformation of the Rat sarcoma virus (RAS) GTPase.[80] RAS is a central small GTPase that acts as a central present as an initial regulator of pathways such as the PI3K/Akt, and ERK1/2 pathways.[78] So far, four types of RAS have been identified, they are: Harvey Rat sarcoma virus (HRAS), neuroblastoma Rat sarcoma virus (NRAS), Kirsten Rat sarcoma virus 4A (KRAS4A) and Kirsten Rat sarcoma virus 4B (KRAS4B).[81] KRAS4A and KRAS4B are products of the same gene. Under normal physiological conditions, when cell surface receptors get activated by their ligands, RAS is switched from its inactive GDP bound state to its active GTP bound state.[80] However, when they acquire certain mutations they become constitutively active and therefore stimulate continuous cell growth or differentiation.[81,82] In fact 19% of human tumours have been shown to harbour RAS

mutations.[83] Patients with tumours containing RAS mutations show poorer prognosis and a shorter overall survival times.[81] RAS activation is mediated by the binding of an adaptor protein called growth factor receptor bound protein 2 (Grb2), which after receptor stimulation binds and interacts with the guanine nucleotide exchange factor called son of sevenless (SOS). Once bound, SOS activates RAS by displacing the bound GDP to induce Ras GTP binding leading to RAF activation.[84]

RAF is an oncogene, and it is found as 3 isoforms: RAF-1 (also known as C-RAF), B-RAF, and A-RAF. Mutations in RAF-1 and A-RAF are quite rare, while many activating mutations have been detected in the B-RAF isoform. Indeed, mutations in B-RAF have been identified in 100% of cell leukaemia, 50-60% of melanoma and 40-60% of thyroid cancer.[85] RAF is the direct effector protein of RAS and the activator of the dual mitogen-activated protein kinase kinase 1 and 2 (MEK1 and MEK2).[78] RAF has three conserved regions. The conserved region 1 (CR1) consists of the RAS-binding domain, which interacts with RAS and with membrane phospholipids that are needed to recruit the RAF protein to the membrane. The second conserved region (CR2) has phosphorylation inhibitory sites that negatively regulate RAS binding and RAF activation. The third conserved region (CR3) contains the kinase domain, which is necessary for initiating kinase activity.[86,87] In its inactive state, RAF is present in the cytosol.[86] Upon activation by RAS-GTP, it moves to the plasma membrane and it changes its conformation through the dephosphorylation of the negative regulatory sites by phosphatases and phosphorylation of different domains by either protein kinase B or protein kinase C.[86,88] After binding to RAS, RAF can phosphorylate MEK1 and 2.[77]

MEK1 and MEK2 are 85% homologous to one another. They contain a catalytic kinase domain where phosphorylation occurs, a regulatory N-terminal domain for ERK1/2 binding, and a shorter C-terminal region around it. MEK1 and 2 are activated by the phosphorylation of two serine residues. When they are activated, they act as dual specific kinases and phosphorylate the threonine and tyrosine residues of ERK1 and ERK2.[89] MEK2 mediates cell morphology and survival while MEK1 mediates cell proliferation.[90]

ERK1 and 2 are 44 and 42 kDa proteins, respectively, that have 85% sequence homology to one another and are expressed ubiquitously.[84] When ERK1/2 gets

activated it either gets translocated from the cytoplasm to the nucleus where it phosphorylates transcription factors such as c-Fos, Elk-1, c-Jun and c-Myc, which are protooncogenes that promote cell survival, growth and proliferation, or it phosphorylates molecules in the cytoplasm such as ribosomal S6 kinases and cytoskeletal proteins, which then regulate cell movement, metabolism and adhesion.[80] It also plays an important role in regulating apoptosis through the post-translational phosphorylation of regulatory molecules such as Bad, Bim and caspase 9 that promote apoptosis.[88] ERK1/2 is also involved in cell motility and is found in adherens junctions and focal adhesions, and cell-cell and cell-matrix contact sites.[91] The subcellular localization of ERK1/2 is important for their physiological function and regulation. Their accumulation in the nucleus after being stimulated have been shown to be necessary for inducing proliferation.[92]

The ERK1/2 signalling pathways are activated by several stimulating factors such as viruses, growth factors, and GPCR ligands.[77] Moreover, intracellularly, ERK1/2 can be activated through calcium release, receptor tyrosine kinase RAS activation, and PKC activation.[77] ERK1/2 activation has many different effects in different cellular compartments. When stimulated, ERK1/2 can relocate to specific sites such as the nucleus, plasma membrane, and cytoplasmic microtubules.[91]

Activation of ERK1/2 by mitogens results in a biphasic ERK1/2 response, with a peak in phosphorylation of the protein peaking 5 to 10 min after stimulation followed by a second peak of ERK phosphorylation which is lower but more sustained lasting up to 2 hrs and yields a ERK1/2 cytoplasmic accumulation.[93]

1.6.2. PI3K/Akt signalling

The phosphoinositide 3 kinase (PI3K)/Akt (also known as or protein kinase B; (PKB)) is a serine/threonine protein kinase that plays an important role in various physiological processes such as metabolism, migration, cell cycle progression, and survival.[94] There are three isoforms of Akt, Akt1, Akt2, and Akt3.[95] These three isoforms of Akt have different and sometimes opposing functions in breast cancer. In breast cancer, the Akt1 isomer is necessary for the initiation and growth of the primary tumour while the Akt2 isomer plays an important in tumour progression so that metastasis can

occur.[96,97] The Akt3 isomer inhibits metastasis but promotes proliferation and acts as an oncogene in some cell types.[97] In fact, Akt3 has been shown to be highly expressed in TNBC where it promotes cell proliferation.[98]

The PI3K consists of three classes, class I, II, and III, which differ in terms of their structure and function. They are heterodimeric enzymes that consist of the catalytic subunit called p110 (it consists of three isoforms: β, γ and δ) and the regulatory subunit, which could either be p85 or p101.[99] Akt consists of three domains. An N-terminal pleckstrin homology (PH) domain that binds to phosphoinositide molecules, a central kinase domain, which contains a regulatory threonine residue Thr308, and C-terminal domain that contains a regulatory serine residue known as Ser473, which is the phosphorylation site.[95]

The Akt signalling pathway is usually activated in response to extracellular signals that activate integrins, RTKs or GPCRs.[80] Downstream of the receptors RAS is activated to induce the translocation and activation of PI3K subunit p110. The activated PI3K phosphorylates PIP_2 to phosphatidylinositol-3,4,5-triphosphate (PIP_3) in the plasma membrane. PIP_3 recruits phosphoinositol dependent kinase (PDK1) and binds to the PH domain of Akt to localise it at the plasma membrane where it is phosphorylated on Thr308 by PDK1.[80] In order for Akt to be fully activated, several kinases such integrin-linked kinase (ILK) phosphorylate Akt at Ser473.[95] Akt activation is terminated through dephosphorylation by protein phosphatase 2 A (PP2A), and by removal of PIP_3 through the activity of phosphatase and tension homolog (PTEN), which dephosphorylates PIP_3 to PIP_2.[97] Activated Akt dissociates from the plasma membrane and re-localises to the nucleus where it inhibits the transcription of genes that promote apoptosis such as the Forkhead box O (*FOXO*) and *Bad* genes, and in this way cell survival is enhanced. When Akt phosphorylates the *FOXO* transcription factors they become inactive and move from the nucleus to the cytoplasm where they are degraded.[95] Akt can also phosphorylate proteins on the plasma membrane or in the cytosol to further regulate cell survival.[95]

GPCRs can activate the PI3K/Akt pathway through their Gα and Gβγ subunits.[58,94] Gβγ binds to and directly activates PI3K heterodimeric proteins that contain either the p110β or the p110γ subunits.[58,94] The activation of PI3K by Gα has not been observed directly, but Akt activation also occurs as a result of crosstalk of GPCR signalling with

integrins, RTKs and other growth factor receptors.[94] β-arrestins have also been shown to mediate PI3K/Akt signalling.[66]

1.6.3. Calcium signalling

Calcium is the fifth most abundant element in the earth's crust, and it belongs to the alkaline earth metal group in the periodic table.[100] Calcium ions (Ca^{2+}) play an important role in many biological processes, as a second messenger or as a mediator of protein binding. Calcium signalling is mediated through changes in the concentration of calcium inside or outside the cell and this is regulated by a network consisting of calcium channels and pumps, and exchangers.[101] Calcium signalling has an impact on cell survival and function because it controls cellular processes such as gene transcription, cell death, metabolism, and secretion.[102] It was first shown to be important in cellular function in a study by Sydney Ringer in 1883, where he showed that saline solution that was made from tap water (and thus contained calcium) allowed contraction in isolated frog hearts, while the saline made from distilled water (which did not have calcium) did not support frog heart contraction.[103]

Ca^{2+} signals have spatiotemporal properties.[102] In terms of time, Ca^{2+} concentrations in the cytoplasm can often only be increased for a short time only, while it remains for longer periods inside organelles such as the ER and mitochondria. Spatially, Ca^{2+} can be released intracellular from the ER or exported through the plasma membrane. Inside the ER, Ca^{2+} is necessary to mediate molecular chaperone activity,[102] while Ca^{2+} is also present in the mitochondria. Ca^{2+} homeostasis in the mitochondria is required to maintain mitochondria function, dynamics, and metabolism.[104] Spatially, Ca^{2+} can be released into the cytoplasm intracellularly from organelles such as the ER or can be im-/exported through the plasma membrane.[102]

In resting cells, the concentration of intracellular or cytosolic Ca^{2+} is around 100 nM but this can increase to approximately 1 µM due to calcium mobilisation from either the ER or, sarcoplasmic reticulum (SR), and also to some extent, lysosomes or Golgi apparatus.[105] Cytosolic calcium is increased by intracellular calcium store release via IP_3, which activates the IP_3 receptor (IP_3R) on the ER and through the ryanodine receptor (RYR) of the SR.[101,106] IP_3R is expressed ubiquitously while RYR is

expressed only in skeletal muscles, cardiac muscle and some regions of the brain.[101,106] Thus, Ca^{2+} is released from these cytoplasmic stores to the cytosol. Cytosolic calcium can also be derived from the extracellular fluid.[106] For Ca^{2+} to be imported from the extracellular fluid, three major classes of plasma membrane Ca^{2+}-permeable channels are involved. They are the voltage-gated calcium channels (they couple membrane depolarization for calcium to enter the cell), transient receptor potential channels and the calcium release-activated Ca^{2+} channels (they function through the mechanism of store-operated calcium entry).[106]

When GPCRs or RTKs are activated, they can cause release of IP_3 through the activation of phospholipase C β (PLCβ) and PLCγ, respectively.[101] Upon GPCR activation, the PLCβ enzyme is activated which hydrolyses PIP_2 to IP_3 and DAG. The IP_3 binds to the IP_3R on the ER and result in an increase in cytosolic Ca^{2+}.[102] Apart from increasing cytosolic [Ca^{2+}], the release of Ca^{2+} from the ER also leads to an increase in [Ca^{2+}] in the mitochondria and lysosomes as well as other organelles.[102]

Following Ca^{2+} release into the cytoplasm, the Ca^{2+}-ATPases move the cytosolic Ca^{2+} back to the extracellular fluid or to the ER. This is done to maintain a low cytosolic Ca^{2+} concentration.[101]

Calcium signalling plays an important role in different cellular processes as described above (Section 1.5). It has also been implicated in cancer. In many cell-types, calcium signalling has been found to play a role in cell migration. For example, it has been shown that in RAW macrophages stimulated with growth factors, Ca^{2+} influx regulates the contraction of the trailing edge in migrating cells. Also, the inhibition of extracellular Ca^{2+} leads to a loss of PI3K activity at the leading edge of migrating cells, the disassembly of actin filament and ruffling (formation of protrusions that are rich in actin) cessation.[107] It has been shown that the leading edge of the macrophages is enriched with Ca^{2+}-sensitive protein kinase Ca (PKCa), and its enrichment is affected by Ca^{2+} influx, PI3K inhibition or F-actin depolymerization.[107] In intestinal epithelial cells Ca^{2+} signalling has been shown to regulate chemokine ligand (CXCL12) induced cell migration in a PLCβ dependent manner.[108] Indeed, in ovarian cancer, Ca^{2+} has been shown to modulate cell motility and invasion.[109] Furthermore, the transfer of Ca^{2+} between the ER and mitochondria induces apoptosis through the p53 pathway. The p53 is a protein that promotes apoptosis, and one of the mechanisms through which

it promotes apoptosis is through a Ca^{2+}-dependent mechanism. The p53 protein interacts with the Sarco/ER Ca^{2+} ATPase pumps increasing Ca^{2+} load to the mitochondria. This causes a change in the morphology of the mitochondria and induction of apoptosis. As a result of the increased Ca^{2+} levels mitochondrial depolarization occurs, and this leads to a decrease in ATP synthesis and subsequent cell death.[104] Therefore, disruption of these signalling pathways can impact cell survival in cancer cells.

1.7. β-Arrestin mediated signalling and signalling bias

There are four arrestin proteins, which can be grouped into two subfamilies; the visual arrestins which consist of arrestin-1 and arrestin-4, and β-arrestins or non-visual arrestins, which consist of β-arrestin1 and β-arrestin2 (also known as arrestin 2 and 3, respectively). Arrestin 1 is expressed in the rod and cone cells of the retina, while arrestin 4 is only expressed in cone cells. β-arrestin1 and 2 are expressed ubiquitously in most cell types and mammalian tissues, and they have 78% amino acid sequence homology.[110]

The main proteins that have been shown to transduce signals in a G protein independent manner are β-arrestin1 and 2. As described in Section 1.5, β-arrestin1 and 2 bind to phosphorylated GPCRs and stop heterotrimeric G protein coupling while mediating receptor endocytosis.[68] β-arrestins link with clathrin and clathrin adaptor protein 2 (AP2), which are components of the endocytic machinery, thereby targeting the receptor to the clathrin coated pit resulting in receptor internalisation. These internalised receptors first move to the early endosomes where they are either recycled back to the plasma membrane or moved to lysosomes where they are degraded. It has been shown that internalised receptors can activate downstream pathways in the endosomes resulting in sustained signalling.[66] Apart from desensitizing GPCRs, they have also been shown to act as scaffolds that bring members of various signalling cascades closer to one another so that the signalling pathway activated can function with more strength and efficiency.[111] β-arrestin mediated ERK1/2 activation mediates antiapoptotic signalling through the regulation of BCL2-associated agonist of cell death (BAD) phosphorylation. β-arrestin-mediated

ERK1/2 signalling is slower and sustained.[66] Apart from the ERK1/2 signalling pathway, β-arrestins also activate PI3K/Akt signalling.[66]

β-arrestins were first discovered to be activators of GPCR signalling in 1998, in a study by Daaka *et al*., where they showed that in β2-adrenergic receptor (β2AR) the activation of MAPK was halted when cells were transfected with dominant-negative mutants of β-arrestin. They have since been shown to mediate signalling downstream of many other GPCRs, including, proteinase activated receptor 2 (PAR-2), angiotensin II type 1 receptor (AT1R), neurokinin-1 receptor, vasopressin receptor 2 (V2R), parathyroid hormone 1 receptor (PTH1R), chemokine receptor type 4 (CXR4) and chemokine receptor type 7 (CCR7).[68,111] For example, β-arrestins are able to interact with tyrosine kinase c-Src, resulting in the formation of receptor-Src complexes and activation of ERK signalling.[112]

β-arrestins are also required for the activation of various proteins such as MAPK, the small GTPase RhoA, the actin filament severing protein cofilin, and the inhibitor of nuclear factor kappa B (NF-κB), LIM domain kinase (LIMK), and nuclear factor of kappa light polypeptide chain gene enhancer in B-cells inhibitor (IκB). The role of β-arrestin in these processes has been determined through siRNA knockdown, use of dominant negative mutants, and genetic deletion. Both β-arrestins can act as inhibitors or activators of some signalling pathways. For example, β-arrestin1 is known to both facilitate and inhibit the PI3K pathway.[68] Whereas with ERK1/2 signalling one of the β-arrestins is known to activate while the other one inhibits. In some GPCRs β-arrestin1 inhibits ERK1/2 while β-arrestin2 activates ERK1/2.[113]

Apart from the PI3K/Akt and ERK1/2 signalling pathways, the p38 and JNK pathways are also known to be activated in a β-arrestin dependent manner. In some cases, the activation of the signalling pathways by β-arrestin results in its sequestration to the cytosol. β-arrestin dependent ERK1/2 activation can occur in the presence or absence of receptor internalisation.[68]

1.8. Kisspeptin/KISS1R

Kisspeptin is a neuropeptide that is encoded by the *KISS1* gene. The *KISS1* gene was first discovered as a metastasis suppressor gene by Lee *et al*. in 1996.[114] They showed through Northern blotting that the *KISS1* mRNA was expressed in non-metastatic melanoma cells but not in metastatic melanoma cell lines. They proved that *KISS1* was an anti-metastasis gene by xenografting athymic nude mice with human metastatic melanoma cancer cell lines, C8161, and MelJuso, overexpressing *KISS1* cDNA and, they observed that metastasis was suppressed in these mice.[114]

The *KISS1* gene is located on chromosome 1q32, it has four exons, with two spanning the open reading frame. The initial product of the *KISS1* translation is a 145 amino acid peptide, called prepro-Kisspeptin. Following synthesis, a signal peptide at the amino-terminal of prepro-Kisspeptin directs the nascent peptide to the secretory pathway. Cleavage of this signal peptide during processing results in the release of pro-Kisspeptin which is then proteolytically cleaved further by furin or prohormone convertases to produce different shorter active peptides, designated by their respective amino acid lengths: Kisspeptin 54 (KP-54), Kisspeptin 14 (KP-14), Kisspeptin 13 (KP-13) and Kisspeptin 10 (KP-10) as shown in (Figure 1.3).[115,116]

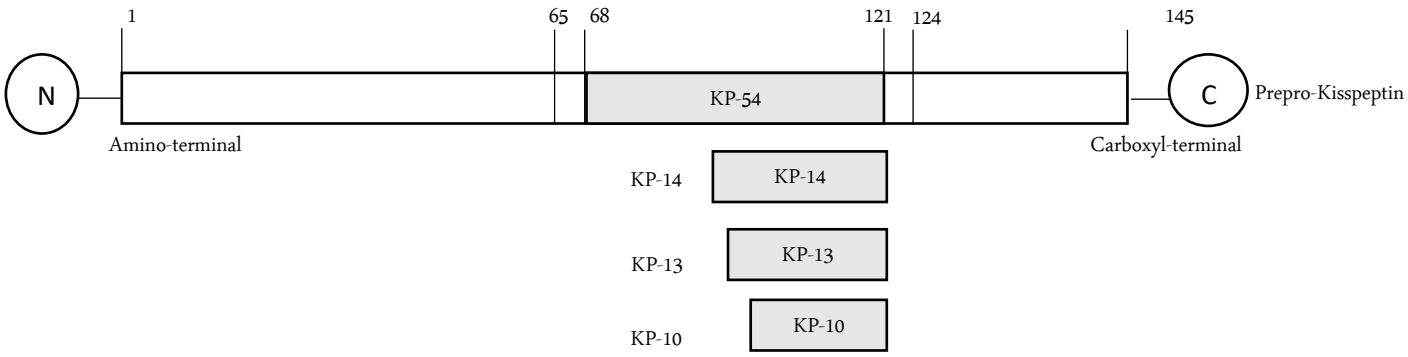


Figure 1.4:KISS1 and Kisspeptin peptides.

The *KISS1* gene gets transcribed to the KISS1 mRNA and the mRNA gets translated to prepro-Kisspeptin, which is a 145 amino acid peptide that gets cleaved to, Kisspeptin-54 (KP-54), Kisspeptin-14 (KP-14), Kisspeptin-13 (KP-13) and Kisspeptin-10 (KP-10). Kisspeptin-10 is the minimum peptide that is needed to activate the Kisspeptin receptor. (Image created in Microsoft PowerPoint).

The shorter peptides (KP-14, KP-13, KP-10) are thought to be degradation products of Kisspeptin-54 since there are no cleavage sites to explain their presence. All the Kisspeptins share the same ten amino acids at their carboxy-terminus, and it is the minimum length that is required to activate the Kisspeptin receptor.[117] All 4 four peptides are collectively called Kisspeptins, and they all have the same affinity and efficacy for the Kisspeptin receptor *in vitro*,[116] although they do exhibit differences in activity *in vivo*.[116,118] They belong to the RF amide (Arg-Phe-NH_2) family of peptides. The RF amide is a superfamily of neuropeptides that end with the sequence Arg-Phe-NH_2.[119]

Under normal physiological conditions, *KISS1* is expressed in various tissues in the body such as the testes, ovaries, pancreas, pituitary gland, hypothalamus, and the intestine.[120] It is also highly expressed in the placenta where it regulates placental growth, migration, and invasion. Kisspeptin is the endogenous ligand of the G protein coupled receptor, Kisspeptin receptor (KISS1R; previously termed GPR54 and known as AXOR12, metastin receptor and hOT7T175).[120]

KISS1R is located on chromosome 19p13.3 in humans.[117] It consists of five coding exons and four introns and an open reading frame that is 1197 base pairs long, which translates to a 398 amino acid sequence protein. Like all GPCRs, it has seven hydrophobic transmembrane domains connected by three intracellular and three extracellular loops and an extracellular N-terminus and a cytoplasmic C-terminus.[119]

KISS1R was discovered in 1999 by Lee *et al*.[121] Through phylogenetic analysis, the receptor was classified as a member of the galanin receptor sub-family of Rhodopsin GPCRs. However, it does not show specific binding with galanin, and only shares 45% homology to galanin receptor. Thus, since its ligand was not known (it was an orphan receptor), it was designated called GPR54.[121] However, in 2001, three independent groups; Kotani *et al*. 2001,[122] Ohtaki *et al*. 2001,[123] and Muir *et al*. 2001,[124] showed that Kisspeptin is the endogenous ligand for GPR54, hence it being named the Kisspeptin receptor/KISS1R. Through Northern blotting and *in situ* hybridization analyses, it was shown that KISS1R is expressed in various areas of the brain such as the amygdala, hippocampus, hypothalamus, midbrain, pons, thalamus, cortex, and striatum as well as peripheral tissues such as the liver, pancreas, and the intestine.[114]

Upon the binding of Kisspeptin to KISS1R coupling occurs with $G\alpha_{q/11}$ (see Section 1.5 for $G\alpha_{q/11}$ activation).[119] Figure 1.4 shows the process of KISS1R signalling through the $G\alpha_{q/11}$ pathway.

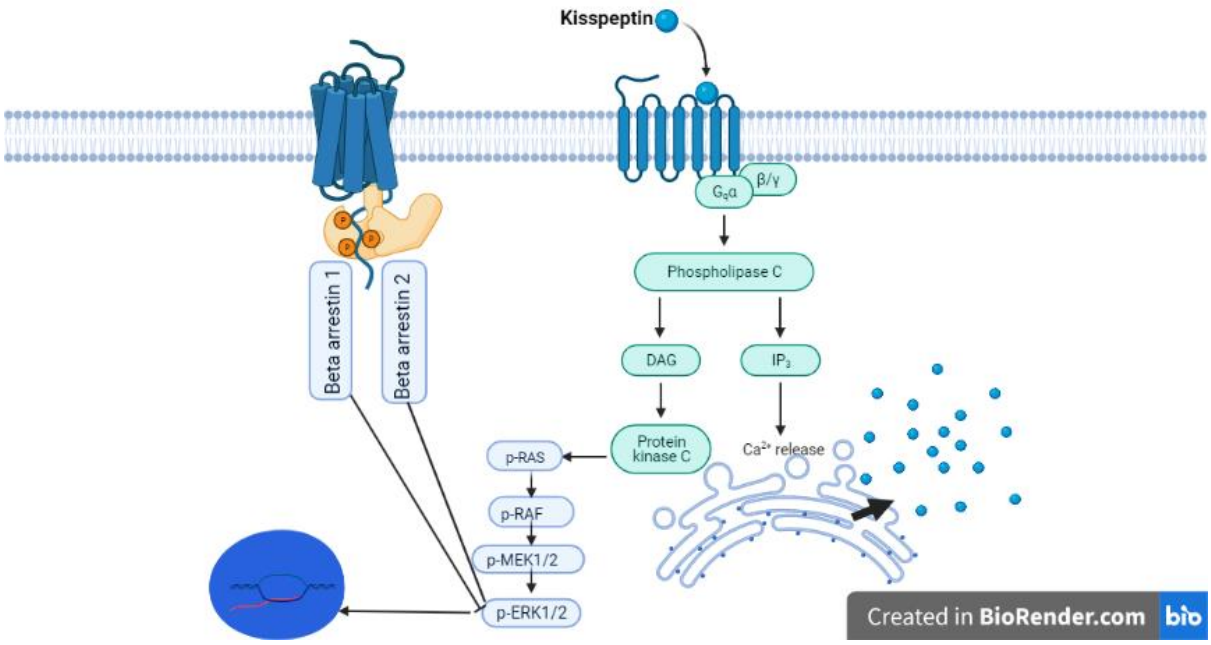


Figure 1.5: Activation of KISS1R by its endogenous ligand, Kisspeptin.

Binding of Kisspeptin to KISS1R results in coupling through $G\alpha_{q/11}$, which activates the phospholipase C (PLC) enzyme which results in the production of the second messenger molecules, 1,4,5-triphosphate (IP_3) and diacylglycerol (DAG) through the cleavage of phosphatidylinositol-4,5-bisphosphate. The IP_3 binds to the IP_3 receptor on the endoplasmic reticulum resulting in the release of calcium ion (Ca^{2+}) from the endoplasmic reticulum. The DAG activates the protein kinase C (PKC) enzyme, which phosphorylates the cascade of proteins leading to ERK1/2 phosphorylation. In cancer, the phosphorylated ERK1/2 regulates the transcription factors of genes that are responsible for cell proliferation and survival. The coupling of Kisspeptin to KISS1R is stopped through a two-step process. First, the KISS1R gets phosphorylated by G protein coupled receptor kinases. Secondly, the phosphorylated receptors attract β-arrestin1 and 2, which bind to the phosphorylated receptor and stops signalling. Although β-arrestin1 and 2 can also activate the ERK1/2 signalling pathway in a G protein-independent manner. (Image adapted from “GPCR effector pathway”, by Bio-render.com,[67]).

Activation of KISS1R by Kisspeptin results in the phosphorylation and activation of ERK1/2 in a G protein-dependent manner via PLC but also in a G protein-independent manner via β-arrestin1/2. Moreover, activation of Akt pathway via either Gα or Gβγ subunits, reorganization of stress fibres in the cell and inhibition of cell movement by inducing focal adhesion kinase has been shown to occur.[115,117]

Although Kisspeptin was first discovered as a metastasis suppressor, its normal physiological function is in the regulation of gonadotropin-releasing hormone (GnRH) secretion from the hypothalamus during puberty, thereby controlling the hypothalamic-pituitary-gonadal (HPG) axis.[115] It is secreted along with other neuropeptides such as neurokinin B and dynorphin and together these three neuropeptides regulate the release of the gonadotrophin releasing hormone (GnRH) from the hypothalamus.[115] The GnRH in turn stimulates the release of gonadotrophic hormones, luteinizing hormone (LH) and follicle stimulating hormone (FSH) from the anterior pituitary gland. The gonadotrophin hormones then stimulate the gonads (ovaries and testes), to facilitate gametogenesis and secretion of the steroid sex hormones, oestrogen progesterone and testosterone. It may also act directly on the pituitary and the gonads.[115] The importance of the Kisspeptin/KISS1R system in reproduction was proven in mice and humans where it was shown that inactivating mutations in *KISS1/KISS1R* led to hypogonadotropic hypogonadism, characterised by a lack of pubertal maturation and sterility.[125] While mice lacking *KISS1R* had idiopathic hypogonadotropic hypogonadism (IHH), which was characterised by small testes in male mice, and in female mice delayed vaginal opening and lack of follicular development.[126] *KISS1* has also been shown to play an important role in spermatogenesis, ovulation, and folliculargenesis. It was proven to control folliculargenesis and ovulation because female mice lacking a functional *KISS1* had a defective oestrus cycle, thread-like uteri, small ovaries, and no mature Graffian follicles.[125] It was shown to control spermatogenesis as male mice lacking a functional *KISS1* gene had rudimentary testes and incomplete spermatogenesis because of its arrest at the early haploid spermatid stage.[125]

1.8.1. Kisspeptin and cancer

In pathology, Kisspeptin and KISS1R have been shown to suppress metastasis in several other cancers including colorectal,[127] bladder,[128] oesophageal,[129] prostate,[130] ovarian,[131] and pancreatic cancer [132]. In colorectal cancer, Ji *et al.* (2014), showed that *KISS1* and *KISS1R* mRNA expression decreased in higher grade tumours.[127] Also, there was a significant decrease in expression of *KISS1R* mRNA in tumour tissues compared to normal background tissues. The study also showed that in patients

undergoing a combination of chemotherapy and radiotherapy there was higher *KISS1R* expression compared to those patients without therapy. While *KISS1* expression correlated negatively with tumour metastasis and invasion.[127]

Upon the activation of KISS1R by binding of Kisspeptin, coupling occurs with $G\alpha_{q/11}$ G proteins (see Section 1.5), which results in increased intracellular Ca^{2+}, and downstream activation of PKC. In tumours, activation of PKC results in the activation of the RAS-mediated MAP kinase pathway that leads to the phosphorylation and activation of ERK1/2 (which can result in altered transcription of selected genes, such as MMP-9, via NF-κB and p38 MAP kinase. Indeed, Kisspeptin signalling has been shown to suppress metastasis in these cancers by repressing matrix metalloproteinase 9 (MMP-9) activity thereby inhibiting cancer cell invasion and migration.[133]

1.8.2. Kisspeptin/KISS1R in breast cancer

In breast cancer, the role of Kisspeptin and KISS1R is not yet clearly understood. Studies by Stark and colleagues (2005)[134] showed that *KISS1* mRNA expression was lower in the brain metastases of breast cancer patients compared to the primary tumours. However, studies by Martin *et al*. (2005),[135] Cvetkovic *et al.* (2013),[136] Goertzen *et al*. (2016),[137] and Blake *et al*. (2017),[138] show that KISS1 and KISS1R protein are highly expressed in metastatic breast cancer cells compared to primary tumours. This high expression has been correlated with aggressive behaviours such as increased invasion and migration in metastatic breast cancer cells *in vitro* and resistance to chemotherapy.[136] In breast cancer cell lines, activation of KISS1R by Kisspeptin has been shown to promote EMT and invadopodia formation (F-actin rich protrusions that are formed by tumour cells to degrade the matrix), which allow increased migration and invasion.[137,138] One issue here is that most studies are done with cell lines exogenously overexpressing KISS1R. One study suggests that the loss of the ER alpha (ERα) in breast cancer cells allows the pro-metastatic signalling of KISS1R to occur.[138] One of the hallmarks of cancer is metabolism reprograming and this feature enables the cancer cells to proliferate and survive in an environment with fluctuating nutrients. In a study by Dragan *et al*. 2020, KISS1R signalling was shown to reprogram a KISS1R transfected ER^- breast cancer cell line to be glutamine-

dependent during tumorigenesis.[139] In a previous study from our laboratory (unpublished data), we showed, through scratch assays, that Kisspeptin-10 (KP-10) could increase cell migration in the MDA-MB-231 breast cancer cell lines, thereby suggesting that Kisspeptin plays a pro-migratory role in breast cancer.

There is therefore still such uncertainty regarding if/how Kisspeptin/KISS1R promotes or inhibits metastasis in breast cancer and one issue is that most studies are done with cell lines exogenously overexpressing KISS1R. Therefore, the aim of this the present study was to delineate the Kisspeptin signalling pathways and its role in breast cancer biology utilising cell lines that endogenously express KISS1R. Our hypothesis was that Kisspeptin signalling results in the activation of signalling pathways such as ERK1/2, Akt and calcium signalling pathways and one or more of these signalling pathways is associated with the pro-migratory effect which we observed previously in the MDA-MB-231 cells stimulated with KP-10.

AIM

This study aimed to identify the signalling pathways that are activated in response to Kisspeptin signalling in breast cancer cell lines, and to determine the role of these pathways in breast cancer cell behaviour.

OBJECTIVES

The objectives of this research study were:

1. To assess whether the intracellular signalling pathways: ERK1/2 and Akt/PI3K, are activated in response to Kisspeptin signalling using Western blot analysis of protein phosphorylation and to determine the role of β-arrestin1/2 in activation of this pathway.
2. To assess whether the second messenger, calcium, is released in response to Kisspeptin signalling through confocal microscopy.
3. To inhibit the Kisspeptin any signalling pathways identified in objectives 1 and 2 using chemical inhibitors, and to determine the role of these pathways in breast cancer cell proliferation and migration.

Chapter 2 MATERIALS AND METHODS

2.1. Cell culture

2.1.1. Materials for cell culture

Table 2.1: List of equipment and materials used for cell culture that are commercially available.

Item name	**Catalogue number/serial number**	**Supplier**
Dulbecco's Modified Eagle Medium, high glucose, GlutaMAX™ supplement (DMEM)	10566016	Thermo Fisher Scientific, Waltham, Massachusetts, USA.
Dulbecco's Modified Eagle Medium-F12 (DMEM-F12)	11320033	Thermo Fisher Scientific, Waltham, Massachusetts, USA.
Minimum Essential Media (MEM)	11095080	Thermo Fisher Scientific, Waltham, Massachusetts, USA.
Foetal bovine serum (FBS)	10270106	Thermo Fisher Scientific, Waltham, Massachusetts, USA.
BT-20	HTB-19™	ATCC, Manassas, Virginia, USA.
MDA-MB-231	HTB-26™	ATCC, Manassas, Virginia, USA.
Cell culture flask, 50 ml, 25 cm^2 (T-25 cell culture flask)	690940	Greiner Bio-one, Kremsmunster, Austria.
Cell culture flask, 250 ml, 75 cm^2 (T-75 cell culture flask)	658940	Greiner Bio-one, Kremsmunster, Austria.
Cell culture flask, 650 ml, 175 cm^2 (T-175 cell culture flask)	661940	Greiner Bio-one, Kremsmunster, Austria.

GlutaMAX™ supplement	35050061	Thermo Fisher Scientific, Waltham, Massachusetts, USA.
Trypsin-EDTA (0.25%), phenol red	25200072	Thermo Fisher Scientific, Waltham, Massachusetts, USA.
Glass Pasteur Pipette Unplugged	GLAS2P20M230	Lasec, Randburg, South Africa.
Trypan blue	T10282	Thermo Fisher Scientific, Waltham, Massachusetts, USA.
Countess™ Cell Counting Chamber Slides	C10228	Thermo Fisher Scientific, Waltham, Massachusetts, USA.
Countess™ Automated Cell Counter	AMQAX1000	Thermo Fisher Scientific, Waltham, Massachusetts, USA.
Cryogenic vials (Cryo vials)	126280	Greiner Bio-one, Kremsmunster, Austria.
Nalgene® Mr Frosty Cryo 1ºC freezing container	5100-0001	Thermo Fisher Scientific, Waltham, Massachusetts, USA.
5 ml serological pipette	606180	Greiner Bio-one, Kremsmunster, Austria.
10 ml serological pipette	607107	Greiner Bio-one, Kremsmunster, Austria.
25 ml serological pipette	760107	Greiner Bio-one, Kremsmunster, Austria.
15 ml falcon tube	188161	Greiner Bio-one, Kremsmunster, Austria.
50 ml falcon tube	227250	Greiner Bio-one, Kremsmunster, Austria.
ZEISS Primo vert inverted microscope	3842003871	ZEISS, Jena, Germany.
Centrifuge 5702	5702FG240241	Eppendorf, Hamburg, Germany.

Table 2.2: List of materials used for cell culture that were prepared in house.

Item name	**Components**
1× phosphate buffered saline (PBS)	• 137 mM sodium chloride (NaCl) • 2.7 mM potassium chloride (KCl) • 10 mM Disodium hydrogen phosphate (Na_2HPO_4) • 1.8 mM Potassium dihydrogen phosphate (KH_2PO_4) • pH: 7.4
Cryopreservation media	• 10% Dimethyl sulfoxide (DMSO) • 40% Foetal calf serum (FCS)

2.1.2. Methods for cell culture

The human TNBC cell lines, BT-20 and MDA-MB-231, were purchased from ATCC. The MDA-MB-231 cells were cultured in DMEM media supplemented with 10% (v/v) FBS. While the BT-20 cells were cultured in either DMEM-F12 or MEM media supplemented with 10% (v/v) FBS. The MEM media was supplemented with 1% (v/v) of GlutaMAX. Both cell lines were maintained in a humidified incubator at 37ºC, with 5% CO_2 and 95% relative humidity (RH) and in either T-25, T-75, and T-175 tissue culture flasks. The cells were passaged at least twice a week when they were about 80% confluent. To passage the cells, the old media was removed by aspiration followed by two gentle washes with 10 ml of 1× PBS, using a 10 ml serological pipette, to remove any FBS that might interfere with trypsinisation. Thereafter, the cells were detached from the tissue culture flasks by trypsinisation for 8 min at 37ºC in a humidified incubator and inspected under the Zeiss Primo vert inverted microscope to ensure that the cells are rounded up and were detached from the surface. Trypsinisation was stopped by adding 9 ml of complete media to the cells in trypsin using a 10 ml serological pipette and the cells in suspensions were transferred to 15 ml falcon tubes and centrifuged at 1 000 ×*g* for 3 min. Afterwards, the supernatant was

aspirated, and the pellet was resuspended in 3 ml of DMEM-F12 or MEM complete media for BT-20 and 10 ml of DMEM complete media for MDA-MB-231 cells. Using a 5 ml serological pipette, 1 ml of the cells in suspensions was transferred to T-75 flasks containing 12 ml of complete media. And the flasks were checked under the Primo vert inverted microscope to ensure that cells were present. Thereafter, the cells were grown in a humidified incubator at 37ºC with 5% CO_2 and 95% RH.

Stocks of each cell were cryopreserved in liquid nitrogen. Cryopreservation was performed by first mixing 1 ml of the cells in suspension (achieved following resuspension of cell pellets during cell passaging) with 1 ml of the cryopreservation media and transferring 1 ml of the resultant mixture into cryovials and the cryovials were placed into a Mr Frosty Cryo 1ºC freezing container. The freezing container with the Cryovials inside were stored at -80ºC overnight. The following day, the cryovials were removed from the freezing container and transferred to liquid nitrogen.

In order to thaw the frozen cells and bring them back for culturing, the frozen vials of cells were taken from the liquid nitrogen and thawed at room temperature. When they were fully thawed, the contents of the vials were transferred into 15 ml falcon tubes and complete media was added to dilute the DMSO in the cryopreservation media. Thereafter, the cells were centrifuged at 1 000 ×*g* for 3 min, to remove the DMSO-containing media. Subsequently, the supernatant was aspirated, and the cell pellet was resuspended in 5 ml of complete media and the entire volume was transferred into T-25 tissue culture flasks. Thereafter, the cells were grown in a humidified incubator at 37ºC with 5% CO_2 and 95% RH.

In order to seed the cells for the different experiments, after trypsinization, 10 µl of the cells in suspension (achieved following resuspension of cell pellets during cell passaging) was mixed with 10 µl of trypan blue and 10 µl of this mixture was pipetted into a Countess™ counting chamber slide, and the number of viable cells was counted using the Countess™ automated cell counter. The Countess™ automated cell counter is an instrument that counts the number of viable cells by using trypan blue staining and light. After counting, the cells were diluted in complete media at a density suitable for each experiment and in different tissue culture plates as required by the experiment.

2.2. Western blotting to determine the expression of β-arrestin 1 and 2 protein

2.2.1 Materials for Western blotting

Table 2.3: List of materials, equipment, and reagents used for Western blotting that are available commercially.

Name	Catalogue number	Supplier
Pierce™ BCA Protein Assay Kit	23225	Thermo Fisher Scientific, Waltham, Massachusetts, USA.
Phosphatase inhibitor cocktail Tablets	04906837001	Roche, Basel, Switzerland.
cOmplete™, Mini Protease inhibitor cocktail	04693124001	Roche, Basel, Switzerland.
Novex™ Wedgewell™ 4-20% Tris Glycine Gel 1.0 mm 15 well	XP04205BOX	Thermo Fisher Scientific, Waltham, Massachusetts, USA.
Novex™ Wedgewell™ 4-20% Tris Glycine Gel 1.0 mm 10 well	XP04200BOX	Thermo Fisher Scientific, Waltham, Massachusetts, USA.
Tween® 20 for Synthesis	9005-64.5	Merck kGaA, Darmstadt, Germany.
Amersham™ Hybond™ P 0.45 PVFD blotting membrane	10600023	Cytiva, Marlborough, Massachusetts, USA.
Western blot filter paper (thin)	GB003	Cytiva, Massachusetts, USA.
Clarity™ Western ECL substrate	170-5061	Bio-Rad, Hercules, California, USA.
Sigma 1-14K centrifuge	162104	Sigma Laborzentrifuge GmbH, Osterode, Germany.

Mini Gel Tank	A25977	Thermo Fisher Scientific, Waltham, Massachusetts, USA.
Mini Trans-Blot Wet transfer apparatus	153 BR 100953	Bio-Rad, Hercules, California, USA.
Roller mixer	VM-370B	Gemmy Industrial Corp, Taipei, Taiwan.
ChemiDoc™ MP imaging system	73BR02464	Bio-Rad, Hercules, California, USA.

Table 2.4: List of materials used for Western blotting that were prepared in house.

Name	**Composition**
RIPA buffer (radioimmunoprecipitation buffer) supplemented with protease inhibitors	• 150 mM NaCl • 1% NP-40/Igepal • 0.5% sodium deoxycholate • 0.1% sodium dodecyl sulphate (SDS) • 50 mM Tris-HCl, pH 8.0 • cOmplete™, Mini Protease inhibitor cocktail Protease inhibitor (1 tablet per 10 ml)
10× Tris-Glycine-SDS running buffer	• 250 mM Tris base • 1.9 M glycine • 1% sodium Dodecyl sulphate (SDS) • pH adjusted to 8.5
10× Towbin buffer	• 250 mM Tris base • 1.9 M glycine • pH adjusted to 8.3
1× Tris-Glycine-SDS running buffer	• 25 mM Tris base • 190 mM glycine • 0.1% SDS • pH adjusted to 8.5

1× Towbin buffer	• 25 mM Tris base • 190 mM glycine • pH adjusted to 8.3
1× PBS	• 137 mM NaCl • 2.7 mM KCl • 10 mM Na_2HPO_4 • 1.8 mM KH_2PO_4 • pH adjusted to 7.4
1× PBS+	• 137 mM NaCl • 2.7 mM KCl • 10 mM Na_2HPO_4 • 1.8 mM KH_2PO_4 • 0.9 M Calcium chloride ($CaCl_2$) • 0.5 M Magnesium chloride ($MgCl_2$) • pH adjusted to 7.4
4× Loading buffer	• 8% (w/v) SDS • 20% (v/v) 2-mercaptoethanol • 40% (v/v) glycerol • 0.008% (w/v) bromophenol blue • 0.2 M Tris-HCl • pH adjusted to 6.8
0.2% Tween® PBS	• 0.2% (v/v) 2 ml of Tween® 20 in 1 × PBS
5% blocking solution 1	• 5% of skimmed (w/v) milk in 10 ml of 0.2% (v/v) Tween® PBS buffer
5% blocking solution 2	• 5% bovine serum albumin (BSA) in 10 ml of 0.2% (v/v) Tween® PBS buffer

Table 2.5: List of primary and secondary antibodies used for Western blotting.

Antibody	**Catalogue number**	**Dilution**	**Manufacturer**
Anti-β1-Arrestin mAb	A47520	1:1 000	Transduction Laboratories™/BD Biosciences, New Jersey, USA.
Goat pAb to Beta Arrestin2	Ab31294	1:1 000	Abcam, Cambridge, UK.
Monoclonal Anti-β-tubulin antibody produced in mouse	T5293-2 mL	1:1 000	Sigma-Aldrich, St. Louis, Missouri, USA.
Goat Anti-Mouse HRP conjugate	1705047	1:10 000	Bio-Rad, Hercules, California, USA.
Alexa Fluor® 555 Donkey anti-goat IgG (H + L)	A21432	1:5 000	Invitrogen, Waltham, Massachusetts, USA

2.2.2. Method for Western blotting to determine the expression of protein expression

The expression of β-arrestin 1 and 2 protein was assessed in the BT-20 and MDA-MB-231 cells via Western blotting. The BT-20 and or MDA-MB-231 cells were seeded in a 6-well tissue culture plates at densities of 8×10^5 cells/well and 4×10^5 cells/well, respectively, and were then incubated overnight to allow for cell attachment in a humidified incubator at 37ºC with 5% CO_2 and 95% RH. The following day, when the cells had attached to the plate, the media was aspirated, and the cells were washed once with 1 ml of ice-cold PBS+ and then solubilised in radioimmunoprecipitation assay (RIPA) lysis buffer supplemented with protease inhibitors. The lysates were vortexed briefly and incubated on ice for 15 min. After incubation, the lysates were

centrifuged at 16 600 ×*g* for 10 min at 4ºC. Thereafter, the supernatant was transferred into new 1.5 ml microcentrifuge tubes. Protein concentrations were determined using a BCA assay kit, following the manufacturer's protocol.

After protein extraction, prior to sample loading the 4× sample loading buffer was mixed to 1× with the protein lysates. Equal amounts of the protein lysates (10 µg) were the separated by electrophoresis using the Novex™ 10-well or 15-well gradient SDS-PAGE gels at a voltage of 120 for 90 min, using a Mini Gel Tank. Subsequently, the protein bands were transferred to polyvinylidene difluoride (PVDF) membranes on ice at a voltage of 90 for 90 min, using a Bio-Rad Wet transfer apparatus. The wet transfer is a method of transferring protein bands from the gel to the membrane, where a sandwich, which consists of the membrane and the gel, is formed and this gel-membrane sandwich is completely submerged in the transfer buffer. The sandwich was assembled in the following order. Firstly, the cassette for the assembly was placed on a clean surface and a pre-wet fibre pad was placed on the cathode (black side) side of the cassette. Pre-wetting was performed with the 1× Towbin buffer. Secondly, two pieces of pre-wet thin blotting papers were placed on top of the fibre pad. Thirdly, the gel was inverted and placed on top of the filter papers. Fourthly, the PVDF membrane, which was first activated by soaking in 100% methanol for at least 1 min, was placed on top of the gel. Next, two pre-wet filter papers were placed on top of the membrane. Finally, a second pre-wet fibre pad was placed on top of the blotting papers, and the cassette was closed and immersed in the gel transfer tank containing the 1× Towbin buffer. After transfer, the membranes were transferred put into 50 ml falcon tubes containing 5% (w/v) blocking solution 1, in order to block for non-specific binding. After blocking, the membranes were washed three times with 0.2% (v/v) Tween® PBS at 5 min intervals with gentle agitation on a roller mixer. Afterwards, immunoblotting analysis was performed for β-arrestin 1 and β-arrestin 2 using the primary antibodies, Anti-β1-Arrestin mAb (1: 1 000), or Goat pAb to Beta Arrestin2 (1:1 000), respectively, diluted in 2.5% (w/v) BSA in 0.2% (v/v) Tween® PBS. The membranes were incubated with these primary antibodies overnight at 4ºC, on a roller mixer. After overnight incubation, the membranes were washed three times with 0.2% (v/v) Tween® PBS, at 5 min intervals with gentle agitation on a roller mixer. This was followed by a 1 hr incubation with the secondary antibodies, Goat Anti-Mouse HRP conjugate (1:10 000) and or Alexa Fluor® 555 Donkey anti-goat IgG (1:5 000), diluted

in the 5% (w/v) blocking solution 1. Thereafter, the membranes were washed three times with 0.2% (v/v) Tween® PBS at 10 min intervals at room temperature with gentle agitation on the roller mixer. Subsequently, the β-arrestin 1 membrane was incubated with the Clarity™ Western ECL chemiluminescent reagents, as per manufacturer's instructions, Clarity Western ECL substrate and Clarity ECL luminol in a 1:1 ratio, for at least 1 min before image acquisition. Thereafter, chemiluminescence detection was performed (30 s exposure time) using a ChemiDoc system. The chemiluminescence channel was adjusted manually to 30 sec so that a proper image can be acquired. Whereas for the β-arrestin 2 membrane, image acquisition was performed by fluorescence because a fluorescently tagged secondary antibody was used, using the ChemiDoc system. Bands were detected by doing a manual exposure on the Alexa Fluor 546 channel for a 10 secs exposure time.

β-tubulin was used as a loading internal control.

Therefore, in order to detect the level of β-tubulin in the protein lysates, after image acquisition the membranes were washed once with 0.2% (v/v) Tween® PBS containing 0.02% (v/v) sodium azide, in order to remove the previous secondary antibody for 10 min at room temperature on the roller mixer. Thereafter, the membranes were incubated with Monoclonal Anti-β-tubulin antibody (1:1 000) and were incubated overnight at 4ºC on the roller mixer. After overnight incubation, the membranes were washed three times at 5 min intervals with 0.2% (v/v) Tween® PBS and then incubated at room temperature for 1 hr with horseradish peroxidase-conjugated goat anti-mouse secondary antibody (1:10 000). After the secondary antibody incubation, the membranes were washed three times with 0.2% (v/v) Tween® PBS at 10 min intervals with gentle agitation on the roller mixer. Thereafter, chemiluminescence detection was performed using the ChemiDoc system, following the method described above. All immunoblots were quantified by densitometric analysis by normalising the β-arrestin 1 and β-arrestin 2 bands to β-tubulin in the same lanes, using the Image Lab 6.0 software (Bio-Rad). The experiment was repeated three times independently.

2.3. Western blotting to assess phospho-activation of ERK1/2 and Akt

2.3.1. Materials used for Western blotting to assess phospho-activation ERK1/2 and Akt

Table 2.6: List of antibodies used for ERK and Akt signalling assay.

Name of antibody	**Catalogue number**	**Dilution and Concentration**	**Manufacturer**
P-p44/42 MAPK (T202/Y204) rabbit Ab	9101S	1:750	Cell signalling Technology, Danvers, Massachusetts, USA.
p44/42 MAPK (ERK1/2) Rabbit Ab	9102S	1:750	Cell Signalling technology, Danvers, Massachusetts, USA.
p-Akt (S473) Rabbit Ab	9271S	1:750	Cell Signalling, Danvers, Massachusetts, USA.
Akt Rabbit Ab	9272S	1:750	Cell Signalling Technology, Danvers, Massachusetts, USA.
Monoclonal Anti-β-tubulin antibody produced in mouse	T5293-2 mL	1:10 000	Sigma-Aldrich, St. Louis, Missouri, USA.
Goat Anti-Rabbit HRP conjugate	1705046	1:10 000	Bio-Rad, Hercules, California, USA.

Goat Anti-Mouse HRP conjugate	1705047	1:10 000	Bio-Rad, Hercules, California, USA.

Table 2.7: List of ligands and inhibitors used in this study.

Name	**Catalogue number**	**Stock concentration**	**Manufacturer**
Kisspeptin-10 (KP-10): Tyr-Asn-Trp-Asn-Ser-Phe-Gly-Leu-Arg-Phe-NH_2	205403	1 mM in 100% DMSO	GL Biochem (Shanghai) Ltd, Shanghai, China.
Barbadin	2774	100 mM in 100% DMSO	Axon Medchem, Groningen, Netherlands.

2.3.2. Methods for assessing activation of phospho-ERK1/2 and phospho-Akt signalling pathways

The activation of the ERK1/2 and Akt signalling pathways in response to stimulation with KP-10 was assessed in the BT-20 and MDA-MB-231 cells by measurement of phosphorylated ERK1/2 and Akt, respectively, via Western blotting. Therefore, BT-20 and MDA-MB-231 cells were seeded in 12-well cell culture plates at densities of 1.5 $\times 10^5$/well and 1.2 $\times 10^5$/well, respectively, in a final volume of 1 mL per well and were allowed to attach by incubating overnight in a humidified incubator at 37ºC with 5% CO_2 and 95% RH 37ºC in a humidified incubator. The following day the cells were starved for at least 4 hr in serum-free media (SFM) before stimulation with 100 nM KP-10 diluted in SFM for 5, 10, 30, 45, and 60 min. Unstimulated (0 min) and vehicle (0.1 % (v/v) DMSO in SFM) controls were included. The cells were treated with the vehicle control for only 60 min. For measurement of the effects of β-arrestin inhibition assay, after the 4 hrs starvation, the BT-20 cells were pre-treated for 30 min with 0.1 µM, 0.5 µM, 1 µM, 2 µM, and 3 µM of Barbadin (a β-arrestin inhibitor [140]), diluted in SFM, and then stimulated for 60 min with 100 nM KP-10. After stimulation, the cells were solubilized with RIPA lysis buffer, protein concentration of the lysates measured using

BCA assay protein quantification kit, following the manufacturer's protocol and samples diluted with 4 × sample loading buffer, following the method described above (see Section 2.2.2).

Prior to sample loading, the 4× loading buffer was mixed to 1× with the protein lysates and denatured by boiling at 95ºC for 5 min. Equal amounts (10 µg for BT-20 ERK1/2 assay or 15 µg for MDA-MB-231 ERK1/2 assay and BT-20 and MDA-MB-231 Akt assay) of the lysates were separated by electrophoresis, and using the Novex™ 10 well or 15 well gradient SDS-PAGE gels and transferred onto PVDF membranes, following the method described above (see Section 2.2.2). After transfer, the membranes were transferred to 50 ml falcon tubes containing 5% (w/v) blocking solution 2, in order to block for non-specific binding. Thereafter, the membranes were washed three times at 5 min intervals using 0.2% (v/v) Tween® PBS on a roller mixer. Phosphorylated ERK1/2 and phosphorylated Akt were detected by immunoblotting with the following primary antibodies, rabbit polyclonal anti-phospho-p44/42 MAPK (1:750), or rabbit polyclonal anti-phospho-Akt (1:750) primary antibodies, respectively. The primary antibody incubation was performed overnight at 4ºC. After the primary antibody incubation, the membranes were washed three times with 0.2% (v/v) Tween® PBS at 5 min interval on a roller mixer. Subsequently, the membranes were incubated with the horseradish conjugated secondary antibody, goat Anti-Rabbit HRP conjugate (1:10 000), for 1 hr at room temperature. Followed by three washes at 10 min interval. The bands were detected by chemiluminescence following the chemiluminescence protocol as described above (see Section 2.2.2).

After acquiring the images for the phosphorylated proteins, the membranes were stripped (as described above, Section 2.2.2) and re-probed with total ERK1/2 or total-Akt antibodies, respectively, and incubated overnight at 4ºC. After overnight incubation at 4ºC, the membranes were washed, three times at 5 min interval and incubated at room temperature for 1 hr with secondary antibody (horseradish peroxidase conjugated goat anti-rabbit) (1:10 000). After the secondary antibody incubation, the membranes were washed again, and bands detected by chemiluminescence as described above (see Section 2.2.2). β-tubulin was used as an internal control. Therefore, after acquiring the images for total ERK1/2 and total-Akt, the membranes were once again stripped and were re-probed with for 10 min using 0.2% Tween®

(v/v) PBS containing 0.02% sodium azide. Thereafter, the membranes were re-probed with β-tubulin antibody using the method described above (see Section 2.2.2).

All immunoblots were quantified by densitometry using the Image Lab 6.0 software (Bio-Rad). The phosphorylated ERK1/2, phosphorylated Akt, and total-ERK1/2 and total Akt bands were normalised to β-tubulin in the same lanes. Each KP-10 treatment or different concentrations of bardadin or barbadin + KP-10 were then divided by their respective normalised total ERK1/2 or total Akt signals and then finally divided by the DMSO vehicle control, in order to plot a graph of relative ERK1/2 and Akt phosphorylation over time or relative ERK1/2 over the different concentrations of Barbadin. The experiment was repeated at least three times independently.

2.4. Calcium mobilization assay using Fluo-3 am

2.4.1. Materials used for calcium signalling assay

Table 2.8: Materials used for calcium signalling that available commercially.

Name	**Catalogue number**	**Manufacturer**
Fluo-3-AM, 1 mM stock dissolved in 100% DMSO, diluted to 2.5 uM in HBSS-BSA-probenecid buffer + Pluronic® F-127	F1241	Thermo Fisher Scientific, Waltham, Massachusetts, USA.
8-well µ-slide ibidi plate	80826	Ibidi, Grafelfing, Germany.
Ionomycin, Calcium salt, 5 mM stock dissolved in 100% DMSO, diluted to 1 µM in HBSS-BSA-probenecid buffer	124222	Thermo Fisher Scientific, Waltham, Massachusetts, USA.
Kisspeptin-10 (KP-10), 1 mM stock dissolved in 100% DMSO, then diluted	205403	GL Biochem (Shanghai) Ltd, Shanghai, China.

to 100 nM in HBSS-BSA-probenecid		
Albumin, Bovine (BSA)	0332-500G	VWR chemicals, Radnor, Pennsylvania, USA.
Pluronic® F-127	P2443-250G	Sigma-Aldrich, St. Louis, Missouri, USA.
Probenecid	P8761-25G	Sigma-Aldrich, St. Louis, Missouri, USA.

Table 2.9: List of materials used for calcium signalling assay that were prepared in house.

Name	**Composition**
10 × Hank's balanced salt solution (HBSS)	• 5.4 mM Potassium chloride (KCl) • 0.5 mM Magnesium chloride hexahydrate ($MgCl_2$-$6H_2O$) • 0.4 mM magnesium sulphate heptahydrate ($MgSO_4$-$7H_2O$) • 0.4 mM potassium dihydrogen phosphate (KH_2PO_4) • 0.34 mM disodium hydrogen phosphate heptahydrate (Na_2HPO_4-$7H_2O$) in 1000 ml of distilled water added and stored at 4ºC.
1 × HBSS	For 1000 ml final volume, 100 ml of 10× HBSS was mixed with 800 ml of distilled water. Then 1.3 mM of anhydrous $CaCl_2$, 5.5 mM of d-glucose and 4.2 mM of $NaHCO_3$ was added. And the volume was brought to 1000 mL with distilled water, pH 7.4 and it was stored at 4ºC.
1 M probenecid (an organic anion transporter inhibitor, to avoid leakage)	Dissolved 7.135 g of probenecid powder in 25 ml of 1 M NaOH and heated for about 30 min on a magnetic stirrer. Stored at -20ºC

20% Pluronic F127	Dissolved 2 g of Pluronic F-127 in 10 ml of 100% DMSO and solubilised by heating at 40ºC for 20 min. Stored at room temperature
HBSS-BSA-probenecid	45 mL of 1× HBSS + 1 mg/ml mg (w/v) of BSA + 2.5 mM Probenecid. The buffer was stored at 4°C and taken out of the pre-warmed to room-temperature before being used.
0.9 M $CaCl_2$	10 g of $CaCl_2$ in 100 ml of distilled water.

N/B: All the reagents used to make the buffers were purchased from Sigma-Aldrich.

2.4.2. Method for calcium signalling assay

The release of calcium into the cytosol, in response to KP-10 stimulation, was assessed in the BT-20 and MDA-MB-231 cells. Intracellular calcium mobilization was measured using Fluo-3-AM calcium indicator dye from Invitrogen, following the protocol described here. The BT-20 and MDA-MB-231 cells were seeded in 200 µl of complete culture media at a density of 6 × 10^4 cells/well and 5 × 10^4 cells/well, respectively, in an 8-well microslide ibidi plate with glass bottom and were incubated overnight in a humidified incubator at 37ºC with 5% CO_2 and 95% RH. The following day, when the cells had attached to the plate, 180 µl of the old media was removed and replaced with 180 µl of the dye loading solution. The cells were then incubated for 30 min in the dark at 37ºC in a humidified incubator with 5% CO_2 and 95% RH. Thereafter, the dye loading solution was removed, and the wells were washed three times with 180 µl of the HBSS-BSA-probenecid buffer. After the washing, the cells were incubated for another 30 min with HBSS-BSA-probenecid at 37ºC in a humidified incubator with 5% CO_2 and 95% RH. Calcium measurement was then detected using a laser scanning confocal microscope using the Fluo-3 channel, with an excitation wavelength of 506 nm and emission at 527 nm. The cells were viewed at a 20× objective with a numerical aperture of 0.5. The confocal scanning system was set at a fast-scanning mode and the image was created from 512 × 512 pixels every 1.5 sec with 401 frames. The size of the pinhole was set at 2.27 AU. The experiment was performed at 37ºC using the microscope's heated chamber. The microscope was switched on at least 30 min before the imaging in order to get the incubator warm.

Thereafter, the plate was put in the slide chamber of the microscope and the lid was removed and videos of the cells prior to the addition of KP-10 were taken in order to make a baseline fluorescence measurement. After about 20 secs, 100 nM KP-10 was added, followed by 1 µM ionomycin + with 0.9 M $CaCl_2$ (positive control) after at least 300 secs. The fluorescence was quantified using the Bio-format plugin in ImageJ software. The analysis steps were as follows; Bio-format importer > select file > analyse > set measurement > select mean grey value only > analyse > tools > ROI manager > add > select background (sections with no cells) > 20 cells per frame were selected for analysis using the oval shape (the video was fast tracked to the time were Ionomycin was added since that was when the cells were more visible) > multi measure > measure all slices and save Excel sheet generated. Three background or blank controls (which where regions with no cells) were also included in the analysis. The average of these background controls was then subtracted from the signals and raw data for each cell at the different time points was blank corrected by subtracting each value at each time point by the average of the three background/blank controls. Data was then calculated as a percentage maximal response by subtraction of the average baseline signals and calculating. These blank corrected values were subtracted from the average of the baseline values and then converted to percentage by dividing each value by the signals as a percentage of the highest signal obtained in response to Ionomycin value and multiplying by 100. The experiment was repeated three times independently. The graphs were in Microsoft Excel. The maximum amplitude of calcium released was calculated by taking the average of the maximum amount of calcium released after the addition of KP-10, for the 20 cells that were selected per cell line. The time of maximum amplitude was calculated by taking the average of the time in which the maximum amplitude was attained for the 20 cells in each independent experiment per cell line.

2.5. Migration assay using the ORIS™ migration assay kit

2.5.1. Materials for migration assay

Table 2.10: List of materials used for migration assay.

Name	**Catalogue number**	**Supplier**
96-well optical btm plt polymerbase black w/Lid cell culture sterile	165305	Thermo Fisher Scientific, Waltham, Massachusetts, USA
Oris migration assay kit consisting of Oris™ cell seeding stoppers, Oris™ stopper removal tool, Oris™ detection mask	CMAU101	Platypus Technologies, Fitchburg, Wisconsin, USA.
DiI (DiIC$_{16}$(1,1'-Dihexadecyl-3,3,3',3'-Tetramethylindocarbocyanine Perchlorate))	D384	Thermo Fisher Scientific, Waltham, Massachusetts, USA
Minimum Essential Media (MEM), no glutamine, no phenol red	51200038	Thermo Fisher Scientific, Waltham, Massachusetts, USA
1.5 ml VWR microcentrifuge tubes	10025-724	VWR International, Radnor, Pennsylvania, USA
Centrifuge 5702	5702FG240241	Eppendorf, Hamburg, Germany
BMG LABTECH microplate reader and BMG LABTECH MARS data analysis software version 3.20 R2	415-2443	BMG LABTECH, Ortberg, Germany
BAPTA-AM (1,2-bis-(o-aminophenoxy)-ethane-	A1076	Sigma-Aldrich, St. Louis, Missouri, USA.

N,N,N',N'-tetra-acetic acid, tetra-acetoxymethyl ester), dissolved to 100 mM in 100% DMSO		

2.5.2. Method for migration assay

In a previous study in our lab through scratch assay it was shown that KP-10 induced migration in the MDA-MB-231 cells but not the BT-20 cells, under serum free culture conditions. Therefore, in this study the result of the scratch assay was confirmed using a more sensitive migration assay. The effect of KP-10 on cell migration was assessed in the MDA-MB-231 cell line using the Oris™ Cell Migration Assay. This migration assay uses fluorescence to quantify cell migration. In summary, fluorescently labelled cells are seeded in a the 96-well optical bottom, black-walled microplate in the presence of silicone stoppers, which serve as barriers that create cell free zones that are each about 2 mm in diameter, in the centre of the plate. These cells are then allowed to attach and spread around the stoppers. Thereafter, the stoppers are removed thereby creating a cell-free centre surrounded by a monolayer of cells. Using a luminometer the centre is measured and the increase in fluorescence is used to quantify cell migration.

MDA-MB-231 cell suspensions (achieved following resuspension of cell pellets during cell passaging, see Section 2.1.2) were diluted to 4 $\times 10^4$ cells/well in phenol red-free media and the cells were stained by mixing in a final concentration of 2.5 µM of dil with 1 ml of the cells in suspension, in a 1.5 ml microcentrifuge tube and inversion. The cells mixed with dil by inverting gently at least four times, Thereafter, they were incubated in a humidified incubator at 37ºC for 30 min, so that the dye could be incorporated in their plasma membrane. After the 30 min incubation, the cells were centrifuged at 1 000 ×*g* for 3 min. The supernatant was removed by aspirating and the pellet was resuspended in complete DMEM media. Thereafter, 4 $\times 10^4$ cells were seeded per well (100 µl per well) in a 96-well clear bottom, black-walled plate with and without the Oris™ stoppers and were incubated overnight in a humidified incubator at 37ºC with 5% CO_2 and 95% RH, to allow cell attachment. The wells without stoppers served as a positive control. The following day, the stoppers were removed using the

Oris™ stopper removal tool and the complete media in each well was aspirated and replaced with either untreated 0.1% phenol-red-free MEM media and either untreated (media-only control) or treated with 100 nM KP-10 or 0.1% DMSO in 0.1% phenol red-free MEM media. In a subsequent experiment, the cells were treated under serum-free conditions and either untreated (phenol red-free serum-free MEM media only) or treated with 100 nM KP-10, 10 µM BAPTA-AM, 100 nM KP-10 + 10 µM BAPTA-AM or vehicle (0.1% DMSO) in phenol red- free serum-free MEM media (treated). Thereafter, a detection mask was inserted into the bottom of the plates, and the plates were transferred to a fluorometer with incubator chamber which was set at 37ºC with 5% CO_2. Blanks were included, which were wells with the phenol red-free serum-free media MEM without cells. Cell migration was measured in real-time using the BMG LABTECH microplate reader. Fluorescence was measured with excitation at 544 nm and emission at 590 nm and readings were taken at 20 min intervals for 18 hrs using the bottom optics. The gain was adjusted using the positive control. After the assay, the data were analysed using the BMG LABTECH MARS data analysis software. The raw data were first corrected to subtract the blank readings. Graphs were then smoothed using the moving average function of the software and the software made a calculation of the slope of linear regression. The experiment was performed three times independently, each with four to eight technical repeats of each data point.

2.6. Cell viability assay using resazurin

2.6.1. Materials used for cell viability assay

Table 2.11: List of materials used for cell viability assay that are commercially available.

Name	**Catalogue number**	**Supplier**
Resazurin sodium powder, dissolved in DPBS to a final concentration of 0.6 mM in order to make a stock	62758138	Sigma-Aldrich, Missouri, St. Louis, USA.

solution. The stock solution was then filter sterilized using a 5 ml syringe and 0.4 µm pore membrane filter.		
96-well cell culture plate F-bottom with lid	655180	Greiner Bio-one, Kremsmunster, Austria.
Minimum Essential Media (MEM), no glutamine, no phenol red	51200038	Thermo Fisher Scientific, Waltham, Massachusetts, USA
DPBS (1 ×)	14040091	Thermo Fisher Scientific, Waltham, Massachusetts, USA
MF-Millipore™ Membrane Filter, 0.45 µm pore size	HAWP04700	Merck kGaA, Darmstadt, Germany.
10 ml syringe		Avacare Health, Kempton Park, South Africa.
BMG LABTECH microplate reader and BMG LABTECH MARS data analysis software version 3.30 R2	415-2443	BMG LABTECH, Ortberg, Germany

2.6.2. Methods for cell viability assay

The effect of KP-10 on cell viability was assessed using a resazurin assay. This assay measures the viability of cells by determining the amount of the non-fluorescent blue resazurin dye that is reduced to a pink, fluorescent resorufin through cellular metabolism. The BT-20 and MDA-MB-231 cells were seeded in a 96-well tissue culture plates at densities of 1.5×10^4 cells/well and 3×10^3 cells/well, respectively, and were incubated overnight in a humidified incubator with 5% CO2 and 95% RH at 37ºC, to allow for cell attachment. The next day, the complete media was aspirated, and the cells were either untreated (serum-free media only) or treated with 100 nM KP-10 or

vehicle (0.1% DMSO), in serum-free media, and incubated for 24, 48, 72, 96 or 120 hrs. The untreated condition contained only media in order to assess how the cells grow normally compared to when treated with KP-10, while the vehicle control was included to ensure that the DMSO, which was used to dissolve the KP-10 has no effect on cell proliferation. The media was changed every 24 hrs and each treatment was performed in quadruplicate. At the end of each time point, the phenol-red containing media was replaced with 100 µl of phenol red-free MEM media containing 20 µl of resazurin dye (final concentration of 0.15 mg/ml) and the cells were incubated in the dark at 37ºC for 4 hr. Blank controls, which contained only the phenol-red free media and resazurin dye (no cells) were included. Thereafter, the optical density of each well was measured at a wavelength of 590 nm with a BMG LABTECH microplate reader to measure cell viability indirectly. The data were analysed using BMG LABTECH MARS data analysis software. The raw data were corrected by subtraction of blank values and the average of the blank corrected values at each timepoint were used to plot a graph of time versus relative fluorescence unit. The standard error of the mean was calculated by dividing the standard deviation by the square root of the sample size. The experiment was repeated three times independently. The values obtained after blank subtraction were used to create an average and the values were used to plot a graph of time versus fluorescence intensity in Microsoft Excel.

2.7. Statistical analysis

All statistical analyses were performed using Microsoft. A paired Student's T-test was used compare the means between two groups. One-Way ANOVA followed by Dunnett's posthoc test was used to compare the means of three or more groups. Significance was defined as a value of $p \leq 0.05$.

Chapter 3 RESULTS

3.1. ERK1/2 is differentially activated in the BT-20 and MDA-MB-231 cell lines

The ERK1/2 signalling pathway is an intracellular signalling pathway that is a member of the MAPK family, which transmit extracellular signals from cell surface receptors such as RTKs and GPCRs, to intracellular targets such as transcription factors for genes that are involved in cell survival and proliferation (see Section 1.6.1). Activation of ERK1/2 by mitogens or GPCR ligands results in a biphasic ERK1/2 response, with a strong response, which peaks after 5 to 10 min, which yields a nuclear accumulation of ERK1/2, followed by a second response, which is lower but sustained and lasts for up to 2 hrs, and yields a cytoplasmic accumulation of ERK1/2.[93]

Preliminary RT-qPCR data (Appendix 6.1) suggested that the TNBC cell lines, BT-20 and MDA-MB-231 express endogenous *KISS1R* at mRNA level. However, previous study using Western blot analysis with different antibodies targeting KISS1R failed to conclusively demonstrate the presence of KISS1R in these cell lines. Therefore, to confirm that KISS1R is indeed present in these cell lines, cellular response to stimulation with Kisspeptin was examined. It was hypothesized that both cell lines would respond to KP-10 exposure by activating ERK1/2 since the *KISS1R* mRNA was present. To test this both cell lines were treated with 100 nM KP-10 for different times and ERK1/2 phosphorylation (as a downstream response to KISS1R activation) was quantified using Western blotting. The concentration of KP-10 (100 nM) was selected since it has been shown to lead to ERK1/2 activation by KISS1R in previous studies.[113,141] To assess the temporal profile of ERK1/2 pathway activation, both cell lines were stimulated with 100 nM KP-10 for 5, 10, 30, 45 or 60 min or with the vehicle control (0.1% DMSO; VC) and unstimulated control, 0 min (Figure 3.1). The cells were treated with the VC for only 60 min because previous data (see Appendix 6.2) showed that there was minimal ERK1/2 activation at 60 min.

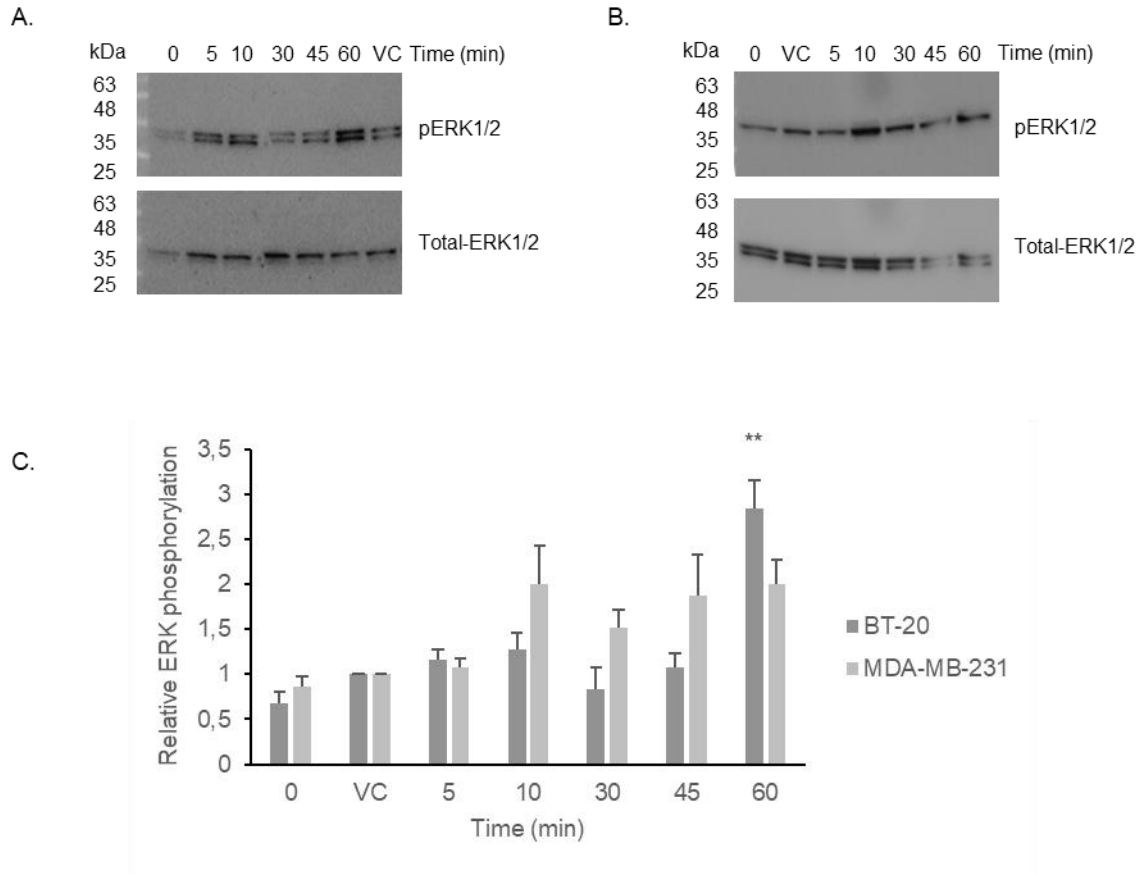


Figure 3.1: KP-10 stimulates ERK1/2 phosphorylation in BT-20 but not in MDA-MB-231 cells.

BT-20 and MDA-MB-231 cells were serum starved for 4 hrs and then treated with 100 nM KP-10 for 5, 10, 30, 45, and 60 min. Unstimulated (0 min) and vehicle treated controls, VC (0.1% DMSO) were included. Thereafter, the cells were lysed with RIPA lysis buffer and ERK1/2 phosphorylation was assessed via Western blotting, using phospho-p44/42 MAPK and p44/42 MAPK antibodies. Representative Western blot images showing ERK1/2 phosphorylation (Top panel) and total ERK1/2 expression (Bottom panel) in (A) BT-20 or and (B) MDA-MB-231 cells. Images are representative of three independent experiments (see Appendix 6.3 for individual blots from the other biological repeats and 6.7A and 6.7D Tubulin blots). The numbers on the left-hand side of the blot represent the molecular weights (kilodalton; kDa) of the BLUelf prestained protein ladder (Genedirex). (C) Densitometric analysis of relative ERK1/2 phosphorylation is depicted after treatment with 100 nM KP-10 for 0 (unstimulated control), VC (0.1% DMSO), 5, 10, 30, 45 and 60 min in (C) (dark grey) BT-20 and (D) (light grey) MDA-MB-231 cells. The graphs represent the mean ± SEM of three independent experiments. A One-Way ANOVA, followed by Dunnett's posthoc test was used to determine significant differences in ERK1/2 phosphorylation between each time point and the vehicle control (VC), **$p < 0.01$. RIPA; radioimmunoprecipitation assay, MAPK; mitogen activated protein kinase, ERK1/2; extracellular signal regulated kinase.

These results show that ERK1/2 was phosphorylated in the BT-20 cells with a significant four-fold increase only after 60 min exposure (Figure 3.1C, dark grey bars). Although, no change in ERK1/2 phosphorylation was measured at the other time points. In contrast, MDA-MB-231 cells displayed no significant ERK1/2 phosphorylation at any timepoint measured in the assay (Figure 3.1C, light grey bars).

Therefore, these data suggest that KP-10 mediates the activation of the ERK1/2 signalling pathway in the BT-20 cells, but not in the MDA-MB-231 cells. This suggests that BT-20 cells do express KISS1R protein while MDA-MB-231 cells may not express protein or the KISS1R protein in these cells does not activate a pathway leading to ERK1/2 phosphorylation. This data also suggests that in the BT-20 cells stimulated with KP-10, ERK1/2 could be activated in a β-arrestin-dependent manner and not in a G protein-dependent manner, since the robust increase in ERK1/2 phosphorylation only occurred after 60 min of stimulation.[142]

3.2 BT-20 cells activate ERK1/2 in a β-arrestin-dependent manner.

The phosphorylation of ERK1/2 in response to KP-10 stimulation on BT-20 cells, only at 60 min suggests that, in these cells, ERK1/2 could be activated in a β-arrestin-dependent manner and not in a G protein-dependent manner. Activation of the ERK1/2 signalling pathway by GPCRs can be activated through both G protein-dependent and β-arrestin-dependent pathways. The G protein-dependent ERK1/2 signalling pathway is usually transient and short-lived, lasting for 5-10 min, while the G protein-independent (β-arrestin-mediated) pathway is long-lasting, sustained and persists for more than 1 hr.[142]

As discussed earlier (Section 1.5), following activation by their ligands, GPCRs get phosphorylated by GRKs and these phosphorylated GPCRs attract β-arrestin1/2, which bind to the phosphorylated GPCRs and target them for internalization, following which they are either degraded or recycled back to the plasma membrane. In addition to inducing receptor desensitization and internalization, β-arrestin1/2 have also been shown to act as scaffold proteins for intracellular signalling pathways such as the ERK1/2 signalling pathway, and this induces a G protein-independent ERK1/2 activation.[143]

The previous data showed that the ERK1/2 response in the BT-20 cells occurred at 60 min. Thus, it was hypothesised that the ERK1/2 phosphorylation in response to KP-10 after 60 min in the BT-20 cells is β-arrestin-dependent. The expression of β-arrestin1 and 2 protein was assessed in the BT-20 and MDA-MB-231 cells through Western blotting (Figure 3.2A).

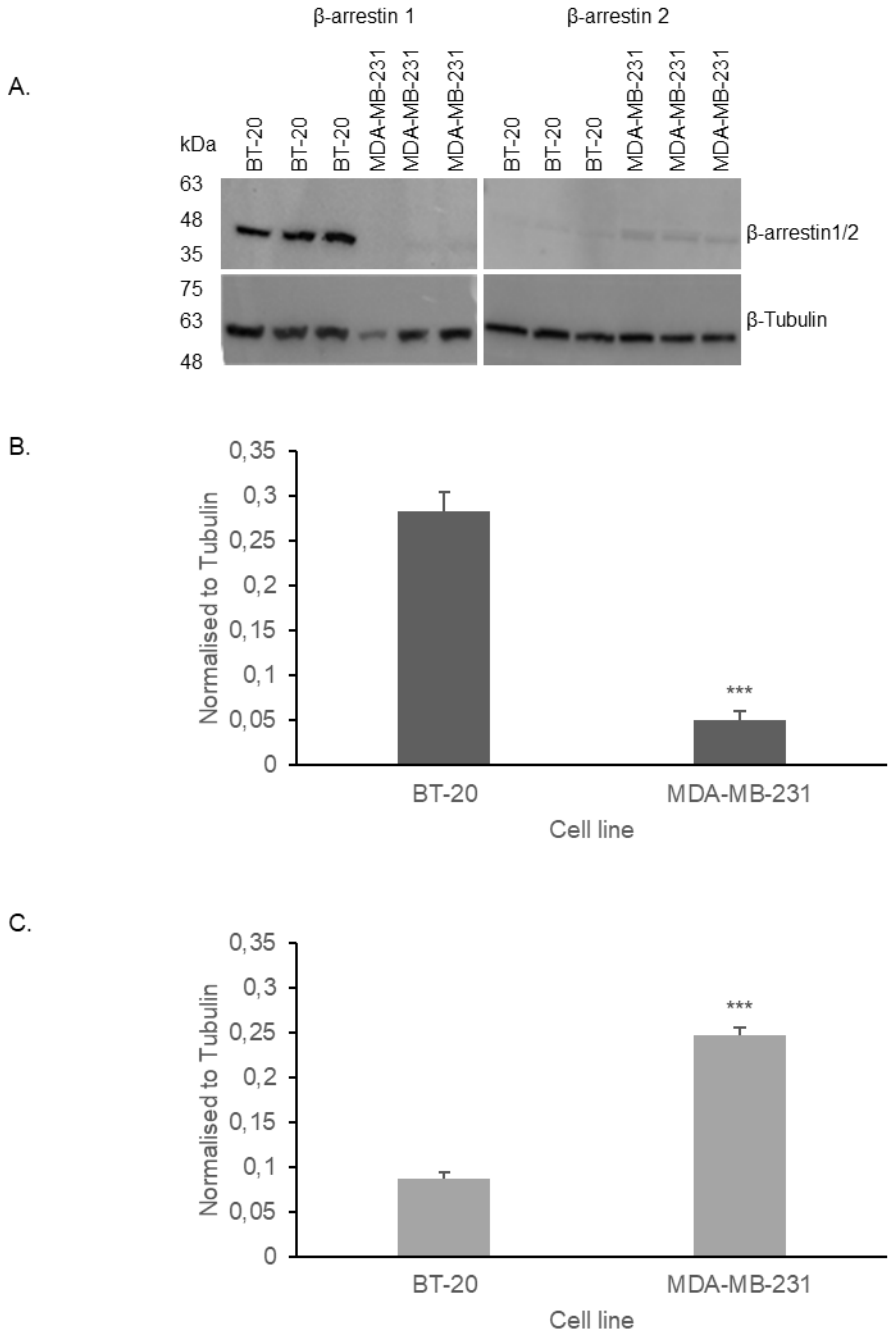


Figure 3.2: β-arrestin1 and 2 are differentially expressed in the BT-20 and MDA-MB-231 cells:

BT-20 and MDA-MB-231 cells were lysed with RIPA lysis buffer and β-arrestin1 and 2 expression was analysed through Western blotting. (A) Western blot image showing three biological repeats of the

expression of endogenous β-arrestin1 (Top panel, left blot) and β-arrestin2 (Top panel, right blot), and β-Tubulin (bottom panel) in BT-20 and MDA-MB-231. The numbers on the left-hand side of the blot represent the molecular weights (kilodalton; kDa) of the BLUelf prestained protein ladder (Genedirex). Densitometric analysis showing the expression of (B) β-arrestin1 and (C) β-arrestin2 normalised to β-tubulin, in the BT-20 and MDA-MB-231 cells. A Paired Student's t-test was performed comparing β-arrestin expression between the two cell lines, $^{***}p \leq 0.001$, n=3.

These analyses revealed that β-arrestin1 and 2 are differentially expressed in the BT-20 and MDA-MB-231 cells (Figure 3.2B and C). While BT-20 cells expressed significantly higher levels of β-arrestin1 (Figure 3.2B), MDA-MB-231 cells expressed higher levels of β-arrestin 2 (Figure 3.2C). It has to be noted that the blots appear to show that overall, there is a higher expression level of β-arrestin1 than β-arrestin2, but this could also be due to differences in the efficiency with which the antibody recognised the protein. Therefore, the differential expression levels of β-arrestin1/2 in the different cell lines could be correlated to the differences in ERK1/2 phosphorylation, since the BT-20 cells, which had an ERK1/2 response at 60 min express β-arrestin1, while the MDA-MB-231 cells, which did not have an ERK1/2 response express β-arrestin2.

Since these data showed that the BT-20 cells express endogenous β-arrestin1 at high levels, it was hypothesised that ERK1/2 phosphorylation in BT-20 following KP-10 exposure may be β-arrestin-dependent. Therefore, the ability of KP-10 to induce ERK1/2 phosphorylation β-arrestin-dependent manner was assessed using a β-arrestin inhibitor called barbadin.

To test this, the effect of the β-arrestin inhibitor, barbadin, on ERK1/2 phosphorylation was assessed in the BT-20 cells. Barbadin inhibits β-arrestin-dependent ERK1/2 phosphorylation by preventing GPCR endocytosis.[140] The BT-20 cells were pre-treated with different concentrations of barbadin for 30 min before being stimulated with 100 nM KP-10 for 60 min. Unstimulated (0 min), vehicle (0.1% DMSO) and barbadin-only treated cells were included as controls. Subsequently, ERK1/2 phosphorylation was assessed through Western blotting (Figure 3.3).

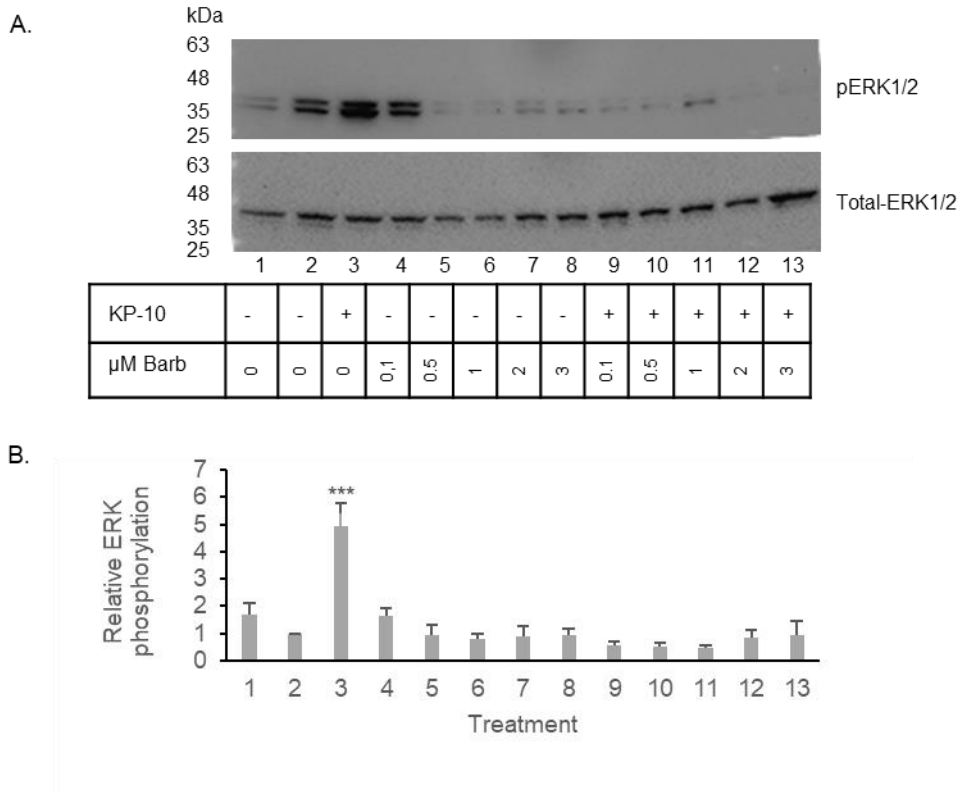


Figure 3.3: KP-10 stimulates ERK1/2 phosphorylation in a β-arrestin-dependent manner in the BT-20 cells.

BT-20 cells were serum starved for 4 h and pre-treated for 30 min with the indicated concentration of barbadin before being stimulated with 100 nM KP-10 for 60 min. Cell lysates were analysed for ERK1/2 phosphorylation by Western blotting with protein loading assessed by Tubulin expression. (A) Western blot image showing the expression of phosphorylated ERK1/2 (top panel) and total ERK1/2 (bottom panel) in BT-20 cells that were pre-treated with Barbadin and stimulated with KP-10 as indicated in the table. Images are representative of four independent experiments (see Appendix 6.4 for individual blots from the other biological repeats and Appendix 6.9A for Tubulin blot). The numbers on the left-hand side of the blot represent the molecular weights (kilodalton; kDa) of the BLUelf prestained protein ladder (Genedirex). (B) Densitometric analysis of four independent repeats of relative ERK1/2 phosphorylation after treatment depicted as fold difference over vehicle control (VC). Data are presented as mean average as error bar is depicted with a ± SEM, with *** indicating a significant difference $p<0.001$. One-Way ANOVA followed by Dunnett's posthoc test was used to compare the vehicle treated cells and the cells treated with KP-10 only or Barbadin + KP-10. VC, vehicle control (0.1% DMSO).

The results show that KP-10 stimulation increased ERK1/2 phosphorylation significantly by about 5-fold. The control cells which were pre-treated with barbadin alone did not significantly alter ERK1/2 phosphorylation. Interestingly, barbadin-treated cells stimulated with KP-10 did not present any significant increase in ERK1/2

phosphorylation. Therefore, in conclusion KP-10 activated ERK1/2 in BT-20 cells is dependent on β-arrestin.

3.3. KP-10 activates the Akt signalling pathway in BT-20 and MDA-MB-231 cells

The Akt/PKB signalling pathway has been shown to be activated in response to Kisspeptin signalling.[144] KISS1R activation can direct PI3K activity to phosphorylate PIP_2 to produce PIP_3 which in turn attracts inactive Akt in the cytosol to the plasma membrane via the PH domain of Akt. Akt signalling has been shown to play a vital role in regulating cellular processes such as cell proliferation, migration, survival and invasion (see Section 1.6.2.). Since a cell line-specific ERK1/2 response was observed after KP-10 stimulation, the presence of other signalling pathways downstream of KISS1R that may also be differentially regulated was sought. However, it was hypothesised that both cell lines would activate Akt signalling since it is a signalling pathway that is required for cell survival.

To assess Akt signalling, BT-20 and MDA-MB-231 cells were stimulated with 100 nM KP-10 for 5, 10, 30, 45 and 60 min. Unstimulated (0 min) and vehicle controls, VC (0.1% DMSO) were included. After stimulation, Akt signalling was assessed through Western blotting (Figure 3.4).

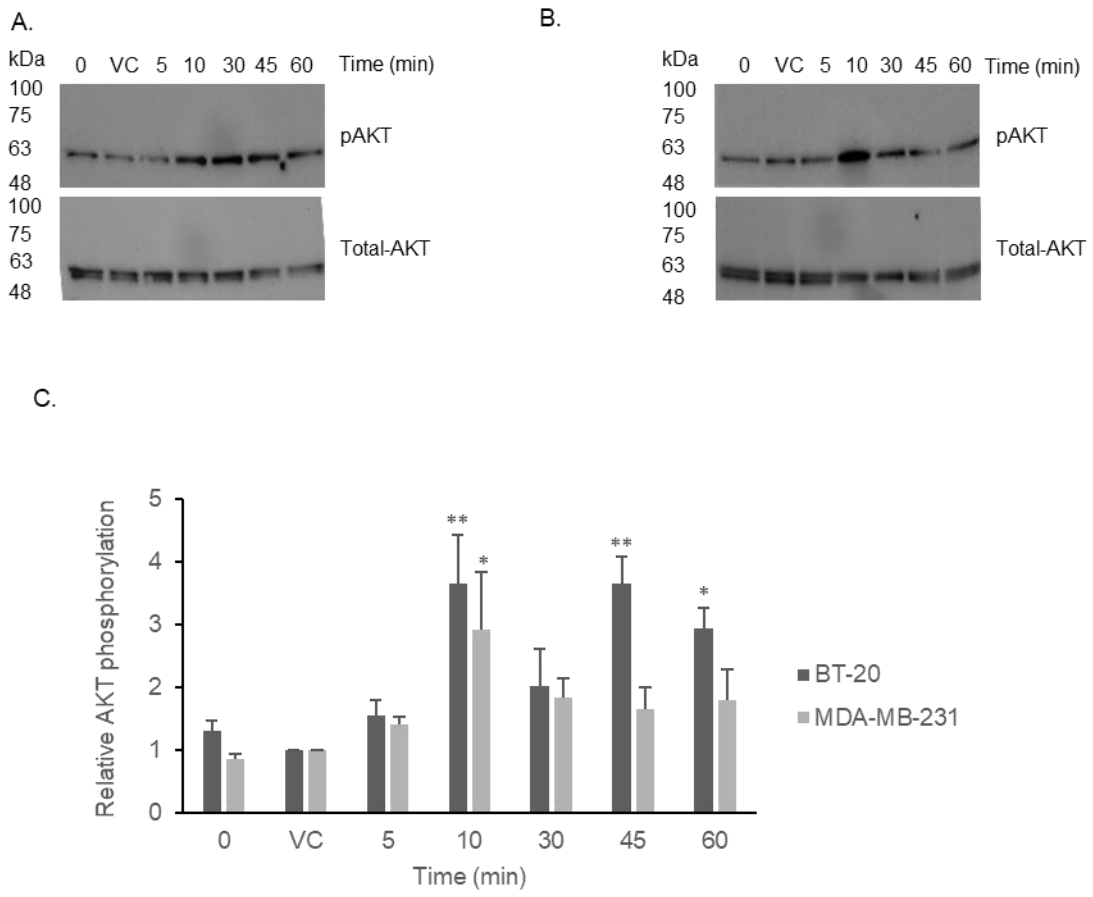


Figure 3.4: KP-10 stimulation activates Akt phosphorylation in BT-20 and MDA-MB-231 cells.

BT-20 and MDA-MB-231 cells were serum starved for 4 hrs before being treated with 100 nM KP-10 for 5, 10, 30, 45, or 60 min. Unstimulated (0 min) and vehicle (VC, 0.1% DMSO) controls were included. Cells were lysed with RIPA lysis buffer and Akt signalling was assessed through Western blotting using the phospho-Akt and Akt antibodies. Representative Western blot images showing Akt phosphorylation (Top panel) and total Akt expression (Bottom panel) in (A) BT-20 and (B) MDA-MB-231 cells. Images are representative of three independent experiments (see Appendix 6.5 for individual blots from the other biological repeats and Appendix 6.8A and 6.8C for Tubulin blots). The numbers on the left-hand side of the blot represent the molecular weights (kilodalton; kDa) of the BLUelf prestained protein ladder (Genedirex). (C) Composite graphs of three independent repeats showing average relative Akt phosphorylation in BT-20 and MDA-MB-231 cells. Data are presented as mean ± SEM, with ** indicating a significant difference $p<0.01$ and * indicating a significant difference $p<0.05$. A One-Way ANOVA followed by Dunnett's posthoc test was performed to compare the 100 nM KP-10 treatment at different timepoints to the vehicle control (VC), DMSO, dimethyl sulfoxide.

These results showed that, in the BT-20 cells, Akt phosphorylation was biphasic as there were statistically significant three-fold, three-fold and two-fold increases in Akt phosphorylation at 10, 45, and 60 min, respectively. In contrast, MDA-MB-231 cells

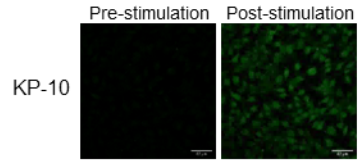
stimulated with KP-10 only induced a two-fold increase in Akt phosphorylation at 10 min.

Therefore, these data shows that KP-10 stimulates Akt signalling in both the BT-20 and MDA-MB-231 cells. This suggests that both cell lines express KISS1R protein and the KISS1R protein in these cells activate a pathway leading to Akt phosphorylation.

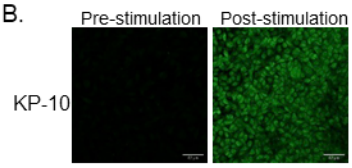
3.4. KP-10 stimulation results in calcium mobilisation in BT-20 and MDA-MB-231 cells

When Kisspeptin binds to its cognate receptor, KISS1R, it can couple through the $G\alpha_{q/11}$ pathway thereby activating PLC, which hydrolyses PIP_2 to IP_3 and DAG. IP_3 binds to the IP_3R on the endoplasmic reticulum resulting in calcium release into the cytosol (see Section 1.6.3). Since Akt and ERK1/2 signalling pathways were observed to be phosphorylated after KP-10 stimulation either in both or one of the cell lines, it was postulated a $G\alpha_{q/11}$ pathway activation might also occur, resulting in calcium mobilisation after KP-10 stimulation. The hypothesis was that stimulation of BT-20 and MDA-MB-231 cells with KP-10 will lead to intracellular calcium release. Therefore, intracellular calcium levels were assessed in both breast cancer cell lines using the calcium indicator dye, Fluo-3 AM, via confocal microscopy (Figure 3.5).

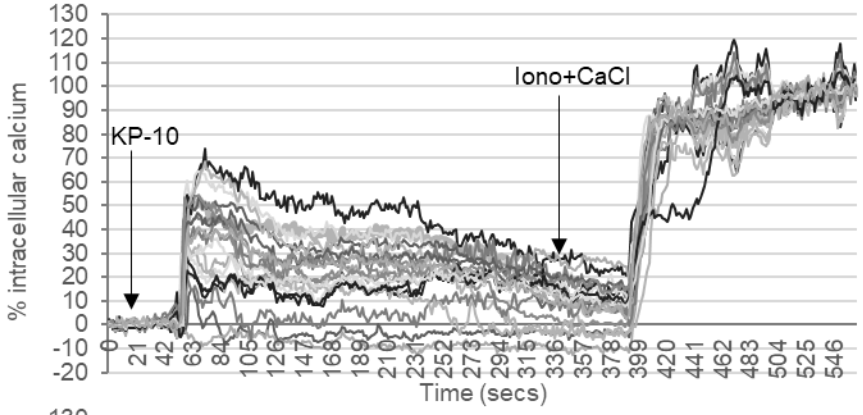
A.



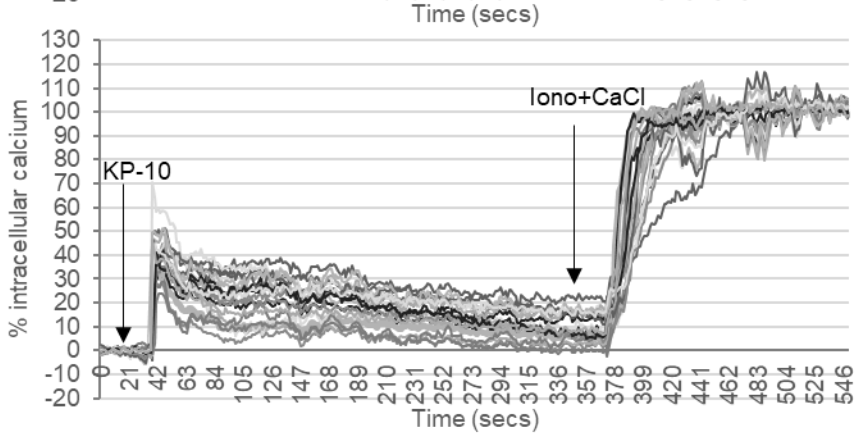
B.



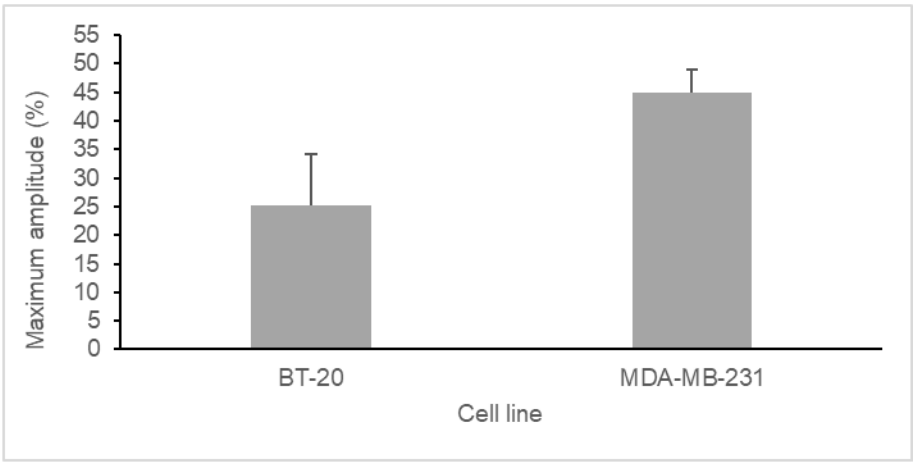
C.



D.



E.



F.

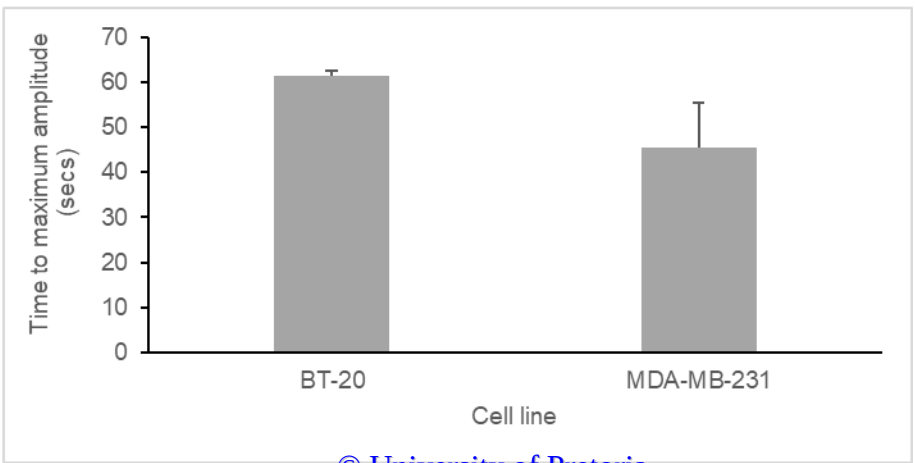


Figure 3.5: KP-10 induces calcium mobilisation in BT-20 and MDA-MB-231 cells.

BT-20 and MDA-MB-231 cells were incubated with 2 µM Fluo-3 AM for 30 min. Cells were then analysed for calcium release by confocal imaging at 200× magnification using a 20× objective and 506 nm excitation and 527 nm emission. Images were taken every 1.5 sec with at least 20 sec imaging to acquire a baseline before stimulation with 100 nM KP-10. Cells were stimulated with 100 nM KP-10 and imaged for a further 350 sec. To determine maximum fluorescence, cells were exposed to 1 µM ionomycin + 0.9 µM $CaCl_2$. Fluorescence images of BT-20 (A) and MDA-MB-231 (B) before stimulation (left panel) and after stimulation with 100 nM KP-10 (right panel). Line traces of individual cells within a representative experiment showing percentage intracellular calcium mobilisation over time of twenty cells of the BT-20 (C) and MDA-MB-231 (D) cell lines before and after stimulation with KP-10 and after ionomycin + $CaCl_2$ treatment. Graphs are representative of three independent experiments (see Appendix 6.6 for individual graphs from the other biological repeats) (E) Mean ± SEM from three independent experiments showing the KP-10 response as a percentage of the maximum amplitude (ionomycin + $CaCl_2$) response in the BT-20 and MDA-MB-231 cells from three independent experiments. A paired student's t-test was performed to compare the maximum KP-10 response amplitude in the BT-20 and MDA-MB-231 cells, $p > 0.05$ (no significant difference). (F) Mean ± SEM from three independent experiments showing the time of maximum KP-10 response amplitude in the BT-20 and MDA-MB-231 cells. A paired Student's t-test was performed to compare the time to maximum KP-10 response between the two cell lines, $p > 0.05$ (no significant difference).

Interestingly, calcium mobilisation occurred after KP-10 stimulation in both cell lines to a similar extent, with ~25% (in BT-20) and ~45% (in MDA-MB-231) (Figure 3.5E) of the total fluorophore loaded being bound. Mobilisation occurred rapidly, with similar time to maximum amplitude after KP-10 exposure reached after ~61 secs in (BT-20) and ~45 secs in (MDA-MB-231) (Figure 3.5F).

Therefore, these data shows that KP-10 stimulates calcium mobilisation in BT-20 and MDA-MB-231 cells.

Thus, the data presented thus far, shows that stimulation with KP-10 in BT-20 cells activates the ERK1/2, Akt, and calcium signalling pathways. Whereas in the MDA-MB-231 cells, KP-10 stimulates Akt and calcium signalling. It does suggest that both cell lines do express KISS1R protein but there are cell context-dependent differences mediating differential KP-10 responses.

3.5 KP-10 stimulates migration in the MDA-MB-231 cells in a calcium-dependent manner

Cell migration is one of the prerequisites for metastasis to occur.[145] Kisspeptin was originally identified as an inhibitor of metastasis.[132] Since it was observed that there was KP-10-dependent signalling in both cell lines the effect of the activation of these signalling pathways on cell migration was assessed. However, previously performed experiments showed that BT-20 cells are not migratory even with stimulation by KP-10 or other strong migration inducers. Thus, the effect of KP-10 on migration was only assessed in MDA-MB-231 cells. MDA-MB-231 migration was analysed using the Oris™ migration assay, which creates cell-free zones in 96-well plates. Following stimulation, the coverage of these zones can be monitored. Migration was measured under low-serum (0.1% FBS-containing media) conditions, since previous data showed that normal serum conditions (10% FBS-containing media) induced rapid migration which would overrule any effects of KP-10 (Figure 3.6).

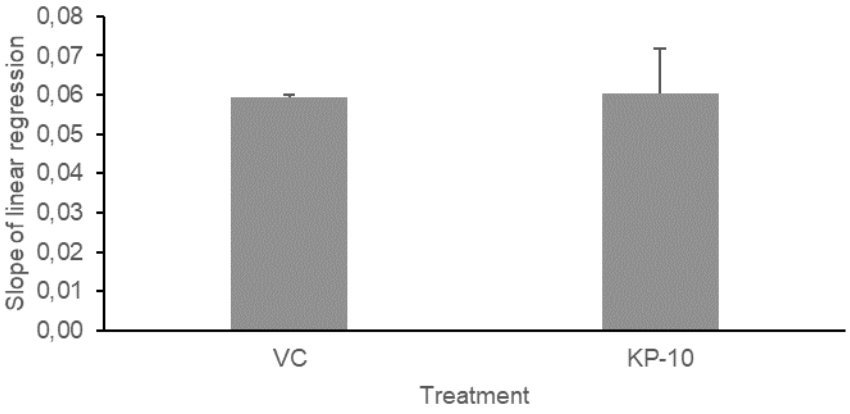


Figure 3.6: KP-10 exposure has no effect on the migration of MDA-MB-231 cells under low-serum culture conditions.

MDA-MB-231 cells were stained with 2.5 uM DiI and seeded in a 96-well black walled clear bottom plate and allowed to attach by incubation overnight. The following day, complete media was replaced with 0.1% serum media and exposed to 100 nM KP-10 or vehicle control (0.1% DMSO). Migration was measured in real- time over 18 hrs in a BMG Labtech Omega with 37ºC and 5% CO_2. Three independent repeats were performed, each with at least four technical repeats per treatment condition. The graph depicts the average slope of the linear regression from each curve, mean +/- SEM. A paired student's

t-test was performed to compare the VC to the KP-10 treated cells. $p > 0.05$. VC; vehicle control, DMSO; dimethyl sulfoxide, relative migration was measured as a slope of linear regression.

The migration data shows that KP-10 exposure in the presence of low serum had no significant effect in the migration rate of MDA-MB-231 cells. Therefore, the experiment was repeated under serum free conditions.

Since KP-10 exposure led to calcium mobilisation in MDA-MB-231 cells (Figure 3.5D), it was hypothesized that the effect of KP-10 on cell migration may be dependent on calcium mobilisation. To determine if this is the case, cells were pre-treated with the calcium-chelating compound BAPTA-AM, before migration rates were assessed in response to KP-10 stimulation. Cells were loaded with dil dye, serum-starved and treated with 100 nM KP-10, 10 µM BAPTA-AM, or 10 µM BAPTA-AM + 100 nM KP-10 for 18 hrs. Vehicle (0.1% DMSO) control was included (Figure 3.7).

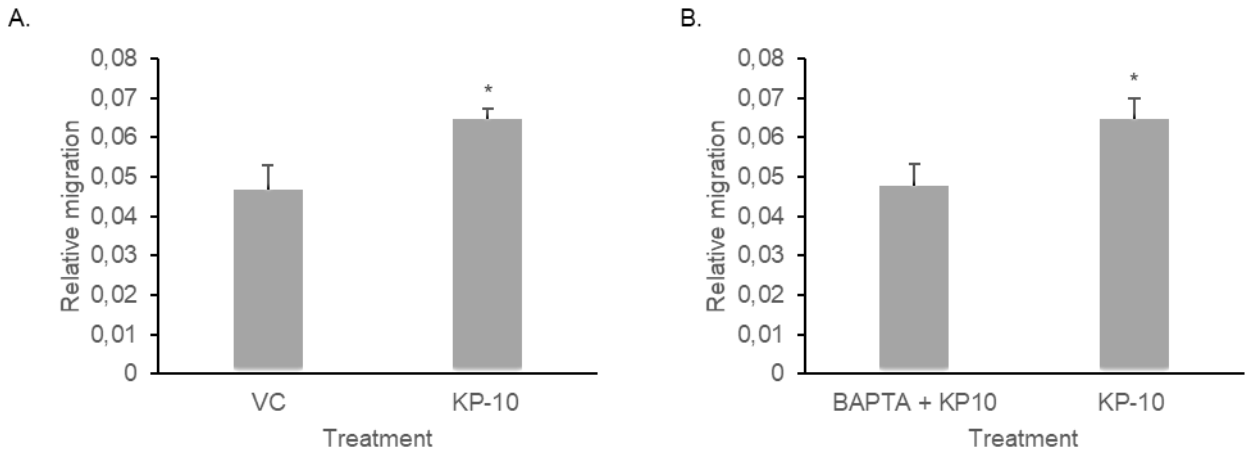


Figure 3.7: KP-10 increases migration in a calcium-dependent manner in MDA-MB-231 cells under serum-free culture conditions.

MDA-MB-231 cells were stained with dil and seeded in a 96-well black walled clear bottom plate and allowed to attach overnight. The following day, complete media was replaced with serum-free media and cells were treated with either (A) 0.1% DMSO (VC) or 100 nM KP-10 and (B) 10 µM BAPTA-AM + 100 nM KP-10, or 100 nM KP-10. Migration was measured in real-time over 18 hrs in a BMG Labtech Omega at 37ºC with 5% CO_2. Three independent repeats were performed, each with at least four technical repeats per treatment condition. The graph depicts the average slope of the linear regression from each curve, mean +/- SEM. A paired Student's t-test was performed comparing the VC to the KP-10, and BAPTA-AM + KP-10 to the KP-10 treated cells. *$p \leq 0.05$. VC, vehicle control (0.1% DMSO), DMSO, dimethyl sulfoxide, relative migration was measured as a slope of linear regression.

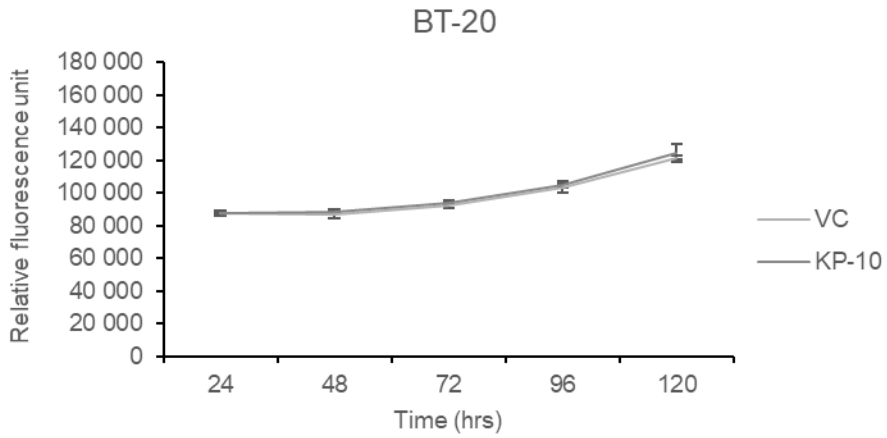
The data (Figure 3.7A) shows that compared to the vehicle control KP-10 exposure increased migration by approximately 30% in MDA-MB-231 cells in the absence of serum. However, co-treatment with the calcium inhibitor BAPTA-AM negated this increase with the migration rate of these cells being similar to the vehicle control (Figure 3.7B).

Together, these data suggest that KP-10 and KISS1R can induce cell migration in a calcium-dependent manner.

3.6. KP-10 does not stimulate BT-20 or MDA-MB-231 cell proliferation

Previous studies have suggested that Kisspeptin and KISS1R may influence cell proliferation.[146] ERK1/2 signalling and Akt signalling are both known to be involved in the regulation of cell proliferation.[147] Therefore, the effect of KP-10 on cell proliferation in the two cell lines was determined. It was hypothesised that KP-10 would influence proliferation in both cell lines since it stimulates different growth-related signalling pathways. The effect of KP-10 on cell proliferation was assessed in the BT-20 and MDA-MB-231 cells using a resazurin assay, for a periods of 24, 48, 72, 96 and 120 hrs. Cells were cultured during this assay under serum-free conditions to enable the visualisation of smaller effects on proliferation which would normally be overshadowed by the pro-proliferative effects of serum on cells. The resazurin assay measured reductive capacity of cells as a proxy for metabolically active cell numbers which is commonly used as a measure of cell proliferation (Figure 3.8).

A.



B.

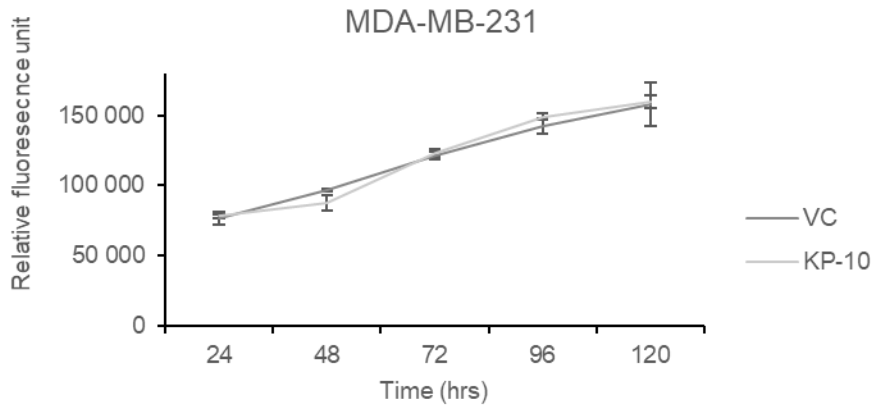


Figure 3.8: KP-10 exposure does not significantly affect cell proliferation of BT-20 and MDA-MB-231 cells.

BT-20 and or MDA-MB-231 cells were seeded in 96-well plates and incubated overnight. The following day, the complete media was replaced with serum-free media containing 100 nM KP-10 or vehicle control (0.1% DMSO), and plates were incubated for 24, 48, 72, 96, and 120 hrs after which phenol-red free media containing 0.56 mg/ml resazurin was added. Fluorescence was measured at a wavelength of 590 nm. Composite graphs of three independent repeats, mean ± SEM, showing growth curve of (A) BT-20 and (B) MDA-MB-231 cells at 24, 48, 72, 96 and 120 hrs. A paired Student's t-test was performed comparing the VC to the KP-10 treated cells at each timepoint. No statistically significant effect was measured with $p > 0.05$. VC; vehicle control, DMSO; dimethyl sulfoxide.

KP-10 stimulation of either BT-20 (Figure 3.8A) or MDA-MB-231 (Figure 3.8B) cells did not affect the growth curve over 120 hrs, with similar curves being obtained with DMSO-treated cells.

Therefore, even though KP-10 does affect cell signalling, no change in cell proliferation was observe in any of the cell lines as a result of KP-10 stimulation.

Chapter 4 DISCUSSION

Kisspeptin is a neuropeptide encoded by the *KISS1* gene that was first identified as it acts as a suppressor of metastasis in melanoma.[114] Subsequent studies showed that Kisspeptin and its cognate receptor, KISS1R, suppress metastasis in lung,[148] colorectal,[127] prostate,[130] and ovarian,[131] cancers. In fact, *KISS1* and *KISS1R* expression have been shown to be lower in many metastatic tumours when compared to the primary tumour or normal cells. [127,148,149] However, controversy exists regarding the role of Kisspeptin in breast cancer. Blake *et al*. 2016, showed that KISS1 and KISS1R expression were increased in tumour tissue of TNBC patients compared to neighbouring normal breast tissue.[138] In the metastatic TNBC cell line, MDA-MB-231, overexpression of the *KISS1* gene and stimulation with Kisspeptin increased invasiveness and migration, which are prerequisites for metastasis. [135,150,151] These data are all in contrast to the believed anti-metastatic role of this receptor. Moreover, it is now clear that this pro-metastatic effect occurs only in the absence of ERα. In fact, even inhibition of ER^+ breast cancer cells through tamoxifen treatment increased Kisspeptin and KISS1R expression.[152]

This study aimed to determine the signalling pathways that are activated in response to KP-10 stimulation in two TNBC cell lines expressing endogenous *KISS1R* mRNA and how these signalling pathways affect breast cancer biology. It was hypothesised that in such cells different pathways will be activated downstream of KISS1R and these will activate different physiological outputs. Previous studies in our lab provided some data that the metastatic TNBC cell line MDA-MB-231 migrated faster after stimulation with Kisspeptin under serum free culture conditions while the non-metastatic TNBC cell line BT-20 did not respond.

Two TNBC cell lines with very different origins, migratory ability and metastatic potential were selected to represent two divergent cell lines within the ER^- subtype. Assuming that the non-metastatic BT-20 line would be devoid of *KISS1R* expression, this study began by assessing the expression of *KISS1R* mRNA in both cell lines through RT-qPCR. Interestingly, the data showed that *KISS1R* mRNA was present in both cell lines at relatively similar levels (Appendix 6.1). This confirms the data from Blake *et al*. 2013 that showed that MDA-MB-231 cells express endogenous *KISS1R,* and this is the first study to show that endogenous *KISS1R* is expressed in BT-20 cells. This result further suggests that metastatic potential may not necessarily

correlate with *KISS1R* expression in TNBC cells, however a larger panel of different cell lines would be required to confirm this.

Zajac *et al* 2011, showed that MMP-9 expression, which is related to the metastatic potential of ER$^-$ breast cancer cells, is activated by KISS1R through an ERK1/2-dependent pathway.[151] Since *KISS1R* was expressed in BT-20 and MDA-MB-231 cells, the effect of KP-10 stimulation on ERK1/2 activation was assessed in both cell lines. This also served to assess if functional KISS1R protein was present in these cells, since Western blot analysis for KISS1R protein expression was inconclusive. The data showed that KP-10 stimulation led to ERK1/2 phosphorylation in BT-20 cells, but not in MDA-MB-231 cells, at 60 minutes after stimulation (Figure 3.1). This late response contrasts with other studies, which showed that KP-10 induced ERK1/2 activation at an early time point, in mouse embryonic fibroblasts (MEFs) and MDA-MB231 cells [113,141] and suggest that the ERK1/2 activation might be dependent on β-arrestin action. However, one previous study showed that ERK1/2 remains activated for up to 60 minutes after stimulation in COS-7 cells (African green monkey fibroblast-like cell line) exogenously expressing R386P, which is a variant of *KISS1R*.[153] The *KISS1R* sequence gene in both cell lines studied here was sequenced previously and this mutation was not found in both cell lines. Thus, this mutation does not seem to be essential for the later ERK1/2 signalling response.

To confirm if ERK1/2 activation in BT-20 cells was indeed dependent on β-arrestin activity, KP-10-induced ERK1/2 phosphorylation was assessed in the presence of the potent β-arrestin inhibitor, barbadin. Barbadin treatment completely inhibited ERK1/2 phosphorylation in response to KP-10 (Figure 3.2) suggesting that KP-10 activates ERK1/2 in a β-arrestin dependent manner in BT-20 cells. A previous study showed that in MEFs Kisspeptin activated an early ERK1/2 at 5 and 10 min in both a β-arrestin-2 and $G\alpha_q$ specific manner while β-arrestin-1 was found to inhibit ERK1/2 activation.[113] Therefore, β-arrestin expression was assessed in both cell lines and the data showed that BT-20 cells expressed high levels of β-arrestin-1, while β-arrestin-2 was measured in MDA-MB-231 cells, suggesting that the presence of β-arrestin-1 could be mediating the ERK1/2 response in BT-20 cells. Since this protein seemed to be absent in MDA-MB-231 cells, this could also explain the divergent signalling response to KP-10 in the two cell lines. This data is in direct contrast to the study by Szereszewski *et al*. 2010, who showed that KP-10 activated ERK1/2 in both a β-arrestin-2 and $G\alpha_q$-

specific manner while β-arrestin-1 was found to inhibit ERK1/2 activation.[113] However, it must be noted that in that study they used mouse embryonic fibroblasts (MEFs) exogenously expressing KISS1R. They further used MEFs from β-arrestin knockout mice to determine the role of the different isoforms of β-arrestin. As with many studies done, it appears that the overexpression of KISS1R may lead to alternative outcomes in signalling, as opposed to the signalling in response to endogenously expressed KISS1R which was studied here. That β-arrestin-2 is involved in KISS1R-mediated ERK1/2 phosphorylation was further highlighted by a study from Pampillo *et al*. 2009,[141] who showed that 100 nM KP-10 induces ERK1/2 activation in MDA-MB-231 cells at 10 minutes which is lost in cells expressing shRNA targeting β-arrestin-2.

To further confirm that the ERK1/2 activation observed in BT-20 cells is β-arrestin-1 dependent, in future, shRNA could be used to diminish β-arrestin-1 expression after which ERK1/2 activation upon KP-10 stimulation can be assessed. It is also possible that the reason there was no early ERK1/2 response in the BT-20 cells was due to the low level of β-arrestin-2 present in the BT-20 cells. Similarly, MDA-MB-231 cells overexpressing exogenous β-arrestin-1 could be generated and the ERK1/2 response to KP-10 assessed. While data suggest that a difference in β-arrestin1/2 expression may be responsible for the difference in KP-10 dependent ERK1/2 phosphorylation, other differences in the cell signalling apparatus could also activate this process. For instance, the ERK1/2 signalling pathway includes a number of proteins such as Ras, Raf, and MEK that could be altered.[111] It is possible that any of these proteins are mutated to be non-functional or deleted in the MDA-MB-231 cells. Further analysis of each of these mediators for their expression and functionality would need to be performed to conclusively identify the step in the pathway leading to ERK1/2 phosphorylation that differs between the two cell lines. A strong ERK1/2 response was observed in the unstimulated cells in the MDA-MB-231 cells (Figure 3.1B, 0 min). This could be another reason why there was no ERK1/2 response after KP-10 stimulation at any of the time points. It could be that prior to KP-10 stimulation, the ERK1/2 signalling pathway was already active. Hence adding KP-10 desensitized KISS1R. A previous study in our laboratory showed that the ERK1/2 signalling pathway is activated in both cell lines in response to serum. Therefore, the lack of ERK1/2 activation in the MDA-MB-231 cells has to do with the Kisspeptin/KISS1R signalling pathway. Suffice to say that this data shows that with the same ligand and receptor

present, and in the same breast cancer subtype, different signalling outputs can be found.

Similarly, to ERK1/2 activation, the Akt/PKB pathway can be activated downstream of Kisspeptin stimulation via KISS1R as has been shown in thyroid cancer cells expressing exogenous KISS1R.[144] Moreover, in clear cell renal carcinoma the long noncoding RNA, TP73-AS1, was shown to regulate *KISS1* expression which in turn was responsible for Akt phosphorylation.[154] The data showed that Akt signalling is activated in BT-20 cells in a biphasic manner, at an early timepoint, at ten minutes and at late time points (45 and 60 min), but in MDA-MB-231 cells, there was only an early response at ten minutes after stimulation with KP-10 (Figure 3.3). The Akt activation in the BT-20 and MDA-MB-231 cells, which occurred at ten minutes is similar to KISS1R overexpressing thyroid cancer cells where Akt phosphorylation appeared between five and ten minutes after 500 nM KP-10 stimulation.[144] The data shows that Akt activation does take place after Kisspeptin stimulation. Whether this activation is dependent on the same mechanism as ERK1/2 activation, which would explain the similar divergence in response between the two cell lines tested, remains unclear. It is possible that the increased β-arrestin1 expression in the BT-20 cells was responsible for the late Akt response. This can be confirmed by using RNA interference to decipher the effect of β-arrestin1 on the late Akt response in the BT-20 cells.

A previous study showed that in addition to ERK1/2 activation, $G\alpha_{q/11}$ activation with subsequent activation of PLC is a common response to Kisspeptin stimulation.[113] Activation of $G\alpha_{q/11}$ by Kisspeptin has been shown to lead to RhoA activation in several cancer types,[155] including breast cancer,[156] mediating diminished metastatic potential. Previous studies have shown that Kisspeptin exposure also induces calcium mobilisation in CHO cells transfected with *KISS1R*.[157] Downstream of the GPCR, the G protein complex $G\alpha_{q/11}$ activates PLC, which cleaves PIP_2 to IP_3 and DAG. DAG in turn activates PKC while IP_3 passes through the cytoplasm and binds to its receptor, IP_3R, on the endoplasmic reticulum resulting in the release of calcium from the endoplasmic reticulum to the cytoplasm.[157] Calcium mobilisation was assessed in both cell lines after KP-10 exposure using a calcium probe in living cells. The data showed that KP-10 stimulation causes a rapid, temporary release of calcium in both the BT-20 and MDA-MB-231 cell lines (Figure 3.4). This data indicates that there is functional

KISS1R protein present that can activate IP_3. Thus, the differences between the two cell lines observed, with regards to ERK1/2 and Akt pathway activation, are predicted to be unrelated to $G\alpha_{q/11}$ activation. Rather it may be specific to β-arrestin-dependent or non-GTPase dependent pathways functional in the BT-20, but not the MDA-MB-231 cell line. Further investigation using the PLC inhibitor, U-73122,[158] and the $G\alpha_{q/11}$ inhibitor, YM-254890[159] would allow the verification that there are indeed separate pathways regulating Akt and ERK1/2, and calcium mobilisation, and whether these are differentially active in the two cell lines.

While many studies suggest that Kisspeptin induces invasion and migration, one previous study found that Kisspeptin stimulation in MDA-MB-231 cells resulted in apoptosis.[160] To test if this was the case, and if there is a difference in response between the two cell lines under investigation in this study, cell proliferation was assessed in response to KP-10. The results (Figure 3.8) show that KP-10 stimulation does not have any mitogenic or apoptotic effects on BT-20 or MDA-MB-231 cells. This data correlates with a study by Ziegler *et al*. 2013,[161] where KP-10 did not have an effect on the proliferation of MDA-MB-231 cells but did inhibit proliferation when KISS1R was exogenously overexpressed in this cell line.[161] The lack of effect of KP-10 on BT-20 cell proliferation was not such a surprise since it is usually G protein dependent or early phase ERK1/2 activation that regulates transcription factor expression related to cell proliferation.[84] In contrast, ERK1/2 activated through β-arrestin remains in the cytosol where it interacts with the endosomes and plays a role in processes such as translation, mitosis, and crosstalk with other signalling pathways.[68,162]

Previous studies have shown that Kisspeptin and KISS1R exert their anti-metastatic effects in lung,[148] ovarian,[131] melanoma,[114,163] and prostate,[130] cancers through the inhibition of cell migration and invasion. In contrast, it is thought that, in ER^- breast cancers, this stimulation results in increased invasion and migration.[164] Previous studies in our lab showed that while MDA-MB-231 cells are migratory, the BT-20 cells are completely non-migratory even under strong chemotactic stimulation. Therefore, the effect of KP-10 exposure on MDA-MB-231 cell migration was assessed using an Oris™ migration assay kit. The effect of KP-10 exposure on cell migration in the presence of low serum or no serum was assessed. The result (Figure 3.7A) shows that KP-10 stimulates cell migration but only in the absence of serum. Further analysis

shows that in the presence of 0.1% serum (normal medium contains 10% serum), the positive effect on migration by KP-10 is lost (Figure 3.6). Cvetkovic *et al*. 2013,[150] showed that Kisspeptin increased migration in the non-tumour, migratory MCF10A cell line overexpressing exogenous KISS1R in the presence of 5% serum.[150] Therefore, the data in this study confirms that KP-10 also increases migration in certain breast cancer cell lines expressing the endogenous receptor. The loss of the positive effect on cell migration in the presence of 0.1% serum could be due to the presence of other compositions of the serum that could be competing with the KP-10 for binding to receptor. Therefore, this suggests that Kisspeptin/KISS1R on its own may not really be a strong regulator of migration. Future studies modulating the expression of KISS1R in the MDA-MB-231 cell line by reducing or knocking out KISS1R and analysing its effect on cell migration may show that there is such a receptor expression level-dependent response. In fact, it is important to point out that migration was only analysed after exposure with 100 nM Kisspeptin. It is possible that higher concentrations could induce a better response and even a response in the presence of serum. Interestingly, the pro-migratory effect of Kisspeptin on MDA-MB-231 cells was lost when the cells were pre-incubated with cell membrane permeant calcium chelator, BAPTA-AM (Figure 3.7B).[165] These data confirm that the Kisspeptin-induced calcium mobilisation may be responsible for the effect on cell migration. A study by Agle *et al*. 2010 [108] showed that calcium signalling influenced cell migration in small intestinal cells. Moreover, calcium mobilization has been shown to regulate actin rearrangement, cell migration, and integrin activation. It influences cell migration by regulating the polarization of migrating cells and stabilizing their leading edges.[166]

In conclusion, the purpose of this study was to determine the signalling pathway that are activated in response to Kisspeptin in two similar but non-identical breast cancer cell lines and to understand the contrasting data that has been published regarding the role of Kisspeptin in breast cancer. The data suggest that Kisspeptin activates different signalling pathways leading to divergent physiological outputs in two TNBC cell lines. In the non-migratory BT-20 cell line, ERK1/2, Akt and calcium signalling pathways are activated by Kisspeptin while in the migratory MDA-MB-231 cell line only Akt and calcium signalling pathways are activated. The activation of these signalling pathways by Kisspeptin did not affect cell proliferation, and cell migration was increased in a calcium-dependent manner in only MDA-MB-231 cell line. Thus, this

study shows that endogenous KISS1R is available for signalling in these cell lines and that cellular context i.e., possible differential expression of pathway components may be responsible for the divergent signalling outcomes. This study goes some way to explain how it is possible to generate contrasting results of Kisspeptin exposure in breast cancer.

Limitations and suggestions

While this study does provide evidence for the existence of cell-specific differences that could go towards explaining the contrasting findings related to Kisspeptin and breast cancer, this work has only focused on two cell lines. To expand this study and obtain definitive proof that such divergent signalling may exist one could make use of primary cancer tissue grown as organoids to assess signalling competency responses after Kisspeptin exposure. The data shows a strong correlation between β-arrestin-1 expression and ERK signalling downstream of KISS1R. While the data shows that KISS1R mediated ERK signalling is indeed β-arrestin-1 dependent in BT-20 cells, we have not shown that the lack of β-arrestin-1 in MDA-MB-231 cells is the reason for the lack of ERK activation after Kisspeptin exposure. To show that this is the source of divergence among these cell lines, β-arrestin-1 could be overexpressed in the MDA-MB-231 cells. Should Kisspeptin exposure now elicit an ERK response, we will have proof that it is indeed the expression of β-arrestin-1 that mediates signalling specificity between these two cell lines. Furthermore, β-arrestin-1 expression can be assessed in patient material and a correlation drawn between Kisspeptin response and its expression.

While this study has shown that Kisspeptin stimulates Akt and ERK activation and calcium mobilisation in the BT-20 cell line, there is no data indicating what the physiological output of such activation is. Firstly, transcriptomic analysis can be performed to determine if transcriptional regulation results from this activation and if so, what genes are involved. Secondly, to determine if ERK remains cytoplasmic after activation in the BT-20 cells, subcellular localisation of active ERK1/2 can be performed.

Chapter 5 REFERENCES

1. Ciaramella V, Corte CMD, Ciardiello F, Morgillo F. Kisspeptin and Cancer: Molecular interaction, Biological Functions, and Future Perspectives. Front. Endocrinol. 2018;9(115):1–5.

2. Faguet GB. A brief history of cancer: Age-old milestones underlying our current knowledge database. Int. J. Cancer. 2015; 136:2022–36.

3. Kunjumoideen K. History of cancer. 2005; 154:19–31.

4. Lukong KE. Understanding breast cancer – The long and winding road. BBA Clin. 2017;7:64–77.

5. Kocarnik JM, Compton K, Dean FE, Fu W, Gaw BL, Harvey JD, *et al*. Cancer incidence, mortality, years of life lost, years lived with disability, and disability-adjusted life years for 29 cancer groups from 2010 to 2019 a systematic analysis for the global burden of disease study 2019. JAMA Oncol. 2022;8(3):420–44.

6. Sudhakar A. History of cancer, ancient and modern treatment methods. J Cancer Sci. Ther. 2009;1(2):1–4.

7. Stewart BW, Wild, C. World Cancer Report 2014. Int. Agency Res. Cancer. 2014; 7:1–630.

8. Sung H, Ferlay J, Siegel RL, Laversanne M, Soerjomataram I, Jemal A, *et al*. Global cancer statistics 2020: GLOBOCAN estimates of incidence and mortality worldwide for 36 cancers in 185 countries. CA Cancer J Clin. 2021;71(3):209–49.

9. Siegel RL, Miller KD, Fuchs HE, Jemal A. Cancer statistics, 2022. CA Cancer J Clin. 2022; 72(1):7–33.

10. Wang H, Naghavi M, Allen C, Barber RM, Carter A, Casey DC, *et al*. Global, regional, and national life expectancy, all-cause mortality, and cause-specific mortality for 249 causes of death, 1980–2015: a systematic analysis for the Global Burden of Disease Study 2015. Lancet. 2016; 388: 1459–544.

11. Cooper G. The development and causes of Cancer. 2000. 2nd ed. Sunderland: Sinauer Associates.

12. Sarkar S, Horn G, Moulton K, Oza A, Byler S, Kokolus S, *et al*. Epigenetics in Cancer development and progression. Int. J. Mol. Sci. 2013; 14: 21087–113.

13. Vogelstein B, Kinzler KW. Cancer genes and the pathways they control. Nat. Med. 2004; 10(8):789–99.

14. Chen H, Liu H, Qing G. Targeting oncogenic Myc as a strategy for cancer treatment. Signal Transduct. Target Ther. 2018; 3:5.

15. Perera RM, Bardeesy N. On oncogenes and tumour suppressor genes in the mammary gland. Cold Spring Harb. Perspect Biol. 2012;4: a013466.

16. National Cancer Institute. What is cancer? 2015. [Internet]. Available from: https://www.cancer.gov/about-cancer/understanding/what-is-cancer?fbclid=/wARO Accessed: 23/05/2020

17. Feng Y, Spezia M, Huang S, Yuan, C, Zeng, Z, Zhang, L, *et al*. Breast cancer development and progression: Risk factors, cancer stem cells, signaling pathways, genomics, and molecular pathogenesis. Genes & Diseases. 2018; 5:77–106.

18. Sinha T. Tumors: Benign and Malignant. Canc Therapy & Oncol Int J. 2018;10(3):1–3.

19. Moses C, Garcia-bloj B, Harvey AR, Blancafort P. Hallmarks of cancer: The CRISPR generation. Eur. J. Cancer. 2018; 93:10–18.

20. Hanahan, D, Weinberg, RA. The Hallmarks of Cancer. Cell. 2000; 100:57–70.

21. Hanahan D, Weinberg, RA. Biological hallmarks of cancer. 9th ed. Robert C, Bast Jr, Croce, CM, Hait, WN, Hong, WK, Kufe, DW, Piccart-Gebhart, M, Pollock, RE, Weichselbaum, RR, Wang H, Holland, JF. editors. Holland-Frei cancer Medicine. John Wiley & Sons Inc.; 2017. 1–10 p.

22. Polyak K. Heterogeneity in breast cancer. J. Clin. Invest. 2011; 121(10):3786–8.

23. Nguyen QH, Pervolarakis N, Blake K, Ma D, Davis RT, James N, *et al*. Profiling human breast epithelial cells using single cell RNA sequencing identifies cell diversity. Nat. Commun. 2018; 9(2028).

24. Koren S, Bentires-Alj M. Breast Tumor Heterogeneity: Source of fitness, hurdle for therapy. Mol. Cell. 2015; 60(4):537–46.

25. Redig AJ, McAllister SS. Breast cancer as a systemic disease: a view of metastasis. J. Intern. Med. 2013; 274:113–26.

26. Mørch LS, Skovlund CW, Hannaford PC, Iversen L, Fielding S, Lidegaard Ø. Contemporary hormonal contraception and the risk of breast cancer. N. Engl. J. Med. 2017;377(23):2228–39.

27. Campa CM, Menéndez JM, González CA, González A, García VÁ, Cos S. What is known about melatonin, chemotherapy and altered gene expression in breast cancer. Oncol. Lett. 2017;13: 2003–14.

28. Rayter Z. History of breast cancer therapy. Cambridge University Press.

29. Yersal O, Barutca S. Biological subtypes of breast cancer: Prognostic and therapeutic implications. World J. Clin. Oncol. 2014; 5(3):412–24.

30. Aiad HA, Samaka RM, Asaad NY, Kandil MA, Shehata MA, Miligy IM. Relationship of CK8/18 expression pattern to breast cancer immunohistochemical subtyping in Egyptian patients. ecancer. 2014;8(404).

31. Noonan MM, Dragan M, Mehta MM, Hess DA, Brackstone M, Tuck AB, *et al*. The matrix protein Fibulin-3 promotes KISS1R induced triple negative breast cancer cell invasion. Oncotarget. 2018;9(53):30034–52.

32. Jin X, Mu P. Targeting Breast Cancer Metastasis. Breast Cancer: Basic and Clinical Research. 2015;9(S1):23–34.

33. Dias K, Dvorkin-Gheva A, Hallett RM, Wu Y, Hassell J, Pond GR, *et al*. Claudin-low breast cancer; clinical & pathological characteristics. PLoS ONE. 2017;12(1).

34. Fougner C, Bergholtz H, Norum JH, Sørlie T. Re-definition of claudin-low as a breast cancer phenotype. Nat. Commun. 2020;11(1).

35. Eeden, RV, Rapoport B. Triple-negative breast cancer – the past, present and future: recent and emerging trends in immunotherapy. Breast Cancer Manag. 2016; 5(1):1–5.

36. Guan X. Cancer metastases: challenges and opportunities. Acta Pharmaceutica Sinica B. 2015;5(5):402–18.

37. Seyfried, TN, Huysentruyt L. On the origin of cancer. Crit. Rev. Oncol. 2013;18(1–2):43–73.

38. Su Z, Yang Z, Xu Y, Chen Y, Yu Q. Apoptosis, autophagy, necroptosis, and cancer metastasis. Mol. Cancer. 2015;14(1):1–14.

39. Drukteinis JS, Mooney BP, Flowers CI, Gatenby RA. Beyond mammography: new frontiers in breast cancer screening. Am. J. Med. 2013;126(6):472–9.

40. Bio-render.com. Tumour metastasis Layout (2) [Internet]. 2022. Available from: https://app.biorender.com/biorender-templates/t-61742cab928fc800ad4743af-tumor-metastasis-layout-2. Accessed: 03/06/2022.

41. Geiger TR, Peeper DS. Metastasis mechanisms. Biochim. Biophys. Acta. 2009; 1796(2):293–308.

42. Novikov NM, Zolotaryova SY, Gautreau AM, Denisov EV. Mutational drivers of cancer cell migration and invasion. Br. J. Cancer. 2021; 124: 102–14.

43. McSherry EA, Donatello S, Hopkins AM, McDonnell S. Molecular basis of invasion in breast cancer. Cell. Mol Life Sci. 2007;64(24):3201–18.

44. Li DM, Feng YM. Signaling mechanism of cell adhesion molecules in breast cancer metastasis: Potential therapeutic targets. Breast Cancer Res. Treat. 2011;128(1):7–21.

45. Berx G, Cleton-Jansen AM, Nollet F, de Leeuw WJ, van de Vijver M, Cornelisse C, *et al*. E-cadherin is a tumour/invasion suppressor gene mutated in human lobular breast cancers. EMBO. 1995;14(24):6107–15.

46. Yilmaz M, Christofori G. Mechanisms of Motility in Metastasizing Cells. Mol. Cancer Res. 2010;8(5):629–42.

47. Kalluri R, Weinberg RA. The basics of epithelial-mesenchymal transition. J. Clin. Invest. 2009;119(6):1420–8.

48. Lamouille S, Xu J, Derynck R. Molecular mechanisms of epithelial-mesenchymal transition. Nat. Rev. Mol. Cell Biol. 2014; 15;178–96.

49. Eccles SA, Welch DR. Metastasis: recent discoveries and novel treatment strategies. Lancet. 2007;369(9574):1742–57.

50. Hamidi H, Ivaska J. Every step of the way: integrins in cancer progression and metastasis. Nat. Rev. Cancer. 2018; 18:533–48.

51. Krakhmal NV, Zavyalova MV, Denisov EV, Vtorushin SV, Perelmuter VM. Cancer invasion: patterns and mechanisms. Acta Naturae. 2015;7(2):17–28.

52. Chiang SP, Cabrera RM, Segall JE. Tumor cell intravasation. Am. J. Physiol Cell Physiol. 2016; 311:1–14.

53. Fares J, Fares MY, Khachfe HH, Salhab HA, Fares Y. Molecular principles of metastasis: a hallmark of cancer revisited. Signal Transduct. Target Ther. 2020; 5:28.

54. Strell C, Entschladen F. Extravasation of leukocytes in comparison to tumor cells. Cell Commun. Signal. 2008; 6:1–13.

55. Paget S. The Distribution of Secondary Growths in Cancer of the Breast. Lancet. 1889;133(3421):571–3.

56. de Castro Junior G, Puglisi F, de Azambuja E, el Saghir NS, Awada A. Angiogenesis and cancer: A cross-talk between basic science and clinical trials (the “do ut des” paradigm). Crit. Rev. Oncol. Hematol. 2006;59(1):40–50.

57. Usman S, Khawer M, Ra S, Naz Z, Saleem K. The current status of anti-GPCR drugs against different cancers. J. Pharm. Anal. 2020; 10:517-521.

58. Desale SE, Chidambaram H, Chinnathambi S. G-protein coupled receptor, PI3K and Rho signaling pathways regulate the cascades of Tau and amyloid-β in Alzheimer’s disease. Molecular Biomedicine. 2021;2(17):1–18.

59. Hanlon CD, Andrew DJ. Outside-in signaling - a brief review of GPCR signaling with a focus on the Drosophila GPCR family. J. Cell Sci. 2015;128(19):3533–42.

60. Mohan ML, Vasudevan NT, Gupta MK, Elizabeth E, Prasad SVN, Foundation CC. G-Protein Coupled Receptor Resensitization – Appreciating the balancing act of receptor function. Curr. Mol. Pharmacol. 2015.

61. Liccardo F, Luini A, Martino R Di. Endomembrane-based signaling by GPCRs and G-Proteins. Cells. 2022;11(3):528.

62. Gurevich VV, Gurevich EV. GPCR signaling regulation: The role of GRKs and arrestins. Front. Pharmacol. 2019; 10:125.

63. Odoemelam CS, Percival B, Wallis H, Chang MW, Ahmad Z, Scholey D, et al. G-Protein coupled receptors: Structure and function in drug discovery. RSC Adv. 2020; 10(60):36337–48.

64. Mekahli D, Bultynck G, Parys JB, de Smedt H, Missiaen L. Endoplasmic-reticulum calcium depletion and disease. Cold Spring Harb. Perspect. Biol. 2011. 1–30.

65. Kamato D, Thach L, Bernard R, Chan V, Zheng W, Kaur H, et al. Structure, Function, Pharmacology, and Therapeutic Potential of the G Protein, Gα/q,11. Front. Cardiovasc. Med. 2015; 2:14.

66. Wang J, Gareri C, Rockman HA. G-protein-coupled receptors in heart disease. Circ. Res. 2018; 716–35.

67. Bio-render.com. GPCR effector pathway [Internet]. 2022. Available from:https://app.biorender.com/illustrations/624aae3c48f642e3606caebd. Accessed: 03/06/2022.

68. DeFea K. β-arrestins and heterotrimeric G-proteins: Collaborators and competitors in signal transduction. Brit. J. Pharmacol. 2008; 153:298–309.

69. Zhao P, Metcalf M, Bunnett NW. Biased signaling of protease-activated receptors. Front. Endocrinol. 2014. 5: 67.

70. Zhao L, He X, Jiang H, Cheng X. Computational characterization of transducer recognition of β2 adrenergic receptor. Biochem. Biophys. Res. Commun. 2022; 12(592):67–73.

71. Xiang Y, Yao X, Chen K, Wang X, Zhou J, Gong W, *et al*. The G-protein coupled chemoattractant receptor FPR2 promotes malignant phenotype of human colon cancer cells. Am J Cancer Res. 2016; 6(11): 2599-2610.

72. Su L-D, Peng J-M, Ge Y-B. Formyl peptide receptor 2 mediated chemotherapeutics drug resistance in colon cancer cells. Eur. Rev. med. Pharmacol. Sci. 2018; 22:95-100.

73. Weng J, Wang J, Cai Y, Stafford LJ, Mitchell D, Ittmann M, *et al*. Increased expression of prostate-specific G-protein-coupled receptor in human prostate intraepithelial neoplasia and prostate cancers. Int. J. Cancer. 2005; 113:811–18.

74. Feigin ME, Xue B, Hammell MC, Muthuswamy SK. G-protein-coupled receptor GPR161 is overexpressed in breast cancer and is a promoter of cell proliferation and invasion. Proc Natl Acad Sci. 2014. 111(11):4191–6.

75. Lappano R, Jacquot Y, Maggiolini M. GPCR modulation in breast cancer. Int. J. Mol. Sci. 2018; 19: 3840.

76. Pérez-Gómez E, Andradas C, Flores JM, Quintanilla M, Paramio JM, Guzmán M, *et al*. The orphan receptor GPR55 drives skin carcinogenesis and is upregulated in human squamous cell carcinomas. Oncogene. 2013; 32(20):2534–42.

77. Guo Y, Pan W, Liu S, Shen Z, Xu Y, Hu L. ERK/MAPK signalling pathway and tumorigenesis (Review). Exp. Ther. Med. 2020; 1997–2007.

78. Li L, Zhao GD, Shi Z, Qi LL, Zhou LY, Fu ZX. The Ras/Raf/MEK/ERK signaling pathway and its role in the occurrence and development of HCC (Review). Oncol. Lett. 2016; 12(5):3045–50.

79. Roberts PJ, Der CJ. Targeting the Raf-MEK-ERK mitogen-activated protein kinase cascade for the treatment of cancer. Oncogene. 2007;26(22):3291–310.

80. Cao Z, Liao Q, Su M, Huang K, Jin J, Cao D. AKT and ERK dual inhibitors: The way forward? Cancer Lett. 2019; 459:30–40.

81. Chen K, Zhang Y, Qian L, Wang P. Emerging strategies to target RAS signaling in human cancer therapy. J. Hematol. Oncol. 2021;14(1):1–23.

82. Bos JL. Ras Oncogenes in Human Cancer: A Review. Cancer Res. 1989;49(17):4682–9.

83. Prior IA, Hood FE, Hartley JL. The frequency of ras mutations in cancer. Cancer Res. 2020; 80(14):2669–974.

84. Mebratu Y, Tesfaigzi Y. How ERK1/2 activation controls cell proliferation and cell death: Is subcellular localization the answer? Cell Cycle. 2009; 8(8):1168–75.

85. Desideri E, Cavallo AL, Baccarini M. Alike but Different: RAF paralogs and their signaling outputs. Cell. 2015; 161: 967–70.

86. Migliaccio N, Sanges C, Ruggiero I, Martucci NM, Rippa E, Arcari P, *et al*. Raf kinases in signal transduction and interaction with translation machinery. Biomol. Concepts. 2013; 4(4):391–9.

87. Matallanas D, Birtwistle M, Romano D, Zebisch A, Rauch J, von Kriegsheim A, *et al*. Raf family kinases: old dogs have learned new tricks. Genes & Cancer. 2011; 2(3):232–60.

88. McCubrey JA, Steelman LS, Chappell WH, Abrams SL, Wong EWT, Chang F, *et al*. Roles of the Raf/MEK/ERK pathway in cell growth, malignant transformation, and drug resistance. Biochim. Biophysica Acta. 2007;1773(8):1263–84.

89. Shaul YD, Seger R. The MEK/ERK cascade: From signaling specificity to diverse functions. Biochimica. Biophys. Acta. 2007; 1773:1213–26.

90. Zhou L, Tan X, Kamohara H, Wang W, Wang B, Liu J, *et al*. MEK1 and MEK2 isoforms regulate distinct functions in pancreatic cancer cells. Oncol. Reports. 2010; 24:251–5.

91. Lawrence MC, Jivan A, Shao C, Duan L, Goad D, Zaganjor E, *et al*. The roles of MAPKs in disease. Cell Res. 2008; 18:436–42.

92. Flores K, Yadav SS, Katz AA, Seger R. The nuclear translocation of mitogen-activated protein kinases: Molecular mechanisms and use as novel therapeutic target. Neuroendocrinol. 2019; 108:121–31.

93. Sipieter F, Cappe B, Leray A, de Schutter E, Bridelance J, Hulpiau P, *et al*. Characteristic ERK1/2 signaling dynamics distinguishes necroptosis from apoptosis. iScience. 2021; 24(103074).

94. New DC, Wu K, Kwok AWS, Wong YH. G protein-coupled receptor-induced Akt activity in cellular proliferation and apoptosis. FEBS J. 2007; 274(23):6025–36.

95. Nitulescu GM, van de Venter M, Nitulescu G, Ungurianu A, Juzenas P, Peng Q, *et al*. The Akt pathway in oncology therapy and beyond (Review). Int. J. Oncol. 2018; 53(6):2319–31.

96. Riggio M, Perrone MC, Polo ML, Rodriguez MJ, May M, Abba M, *et al*. AKT1 and AKT2 isoforms play distinct roles during breast cancer progression through the regulation of specific downstream proteins. Sci. Rep. 2017; 7:44244.

97. Hinz N, Jücker M. Distinct functions of AKT isoforms in breast cancer: a comprehensive review. Cell Commun. Signal. 2019; 17:154.

98. Chin YR, Yoshida T, Marusyk A, Beck AH, Polyak K, Toker A. Targeting Akt3 signaling in triple-negative breast cancer. Cancer Res. 2014; 74(3):964–73.

99. Vogt PK, Hart JR, Gymnopoulos M, Jiang H, Kang S, Bader AG, *et al*. Phosphatidylinositol 3-Kinase (PI3K): the Oncoprotein. Curr. Topic Microbiol. Immunol. 2011; 347:79–104.

100. Pratt SJ, Hernández-Ochoa E, Martin SS. Calcium signaling: breast cancer's approach to manipulation of cellular circuitry. Biophys. Rev. 2020; 12:1343–59.

101. Wu L, Lian W, Zhao L. Calcium signaling in cancer progression and therapy. FEBS J. 2021; 288: 6187–205.

102. Kania E, Roest G, Vervliet T, Parys JB, Bultynck G. IP3 receptor-mediated calcium signaling and its role in autophagy in cancer. Front. Oncol. 2017; 7: 140.

103. Bootman MD. Calcium signaling. Cold Spring Harb. Perspect. Biol. 2012; 4: a011171.

104. Romero-Garcia S, Prado-Garcia H. Mitochondrial calcium: Transport and modulation of cellular processes in homeostasis and cancer (Review). Int. J. Oncol. 2019; 54: 1155–67.

105. Roderick HL, Cook SJ. Ca2+ signalling checkpoints in cancer: Remodelling Ca 2^{+} for cancer cell proliferation and survival. Nat. Rev. Cancer. 2008; 8:361–75.

106. Lanner JT, Georgiou DK, Joshi AD, Hamilton SL. Ryanodine receptors: structure, expression, molecular details, and function in calcium release. Cold Spring Harb. Perspect. biol. 2010; 2: a003996.

107. Evans JH, Falke JJ. Ca2+ influx is an essential component of the positive-feedback loop that maintains leading-edge structure and activity in macrophages. Proc. Natl. Acad. Sci. 2007; 104:16176-81.

108. Agle KA, Vongsa RA, Dwinell MB. Calcium mobilization triggered by the chemokine CXCL12 regulates migration in wounded intestinal epithelial monolayers. J. Biol. Chem. 2010; 285(21):16066–75.

109. Leung CS, Yeung TL, Yip KP, Pradeep S, Balasubramanian L, Liu J, Wong KK, *et al*. Calcium dependent FAK/CREB/TNNC1 signaling mediates the effect of stromal MFAP5 on ovarian cancer metastatic potential. Nat. Commun. 2014; 5:5092.

110. van Gastel J, Hendrickx JO, Leysen H, Santos-Otte P, Luttrell LM, Martin B, *et al*. β-Arrestin based receptor signaling paradigms: potential therapeutic targets for complex age-related disorders. Front. Pharmacol. 2018; 9: 1369.

111. Buscà R, Pouysségur J, Lenormand P. ERK1 and ERK2 map kinases: Specific roles or functional redundancy? Front. Cell Dev. Biol. 2016; 4:53.

112. Luttrell LM, Ferguson SSG, Daaka Y, Miller WE, Maudsley S, Della Rocca SC, *et al*. β-Arrestin-dependent formation of β2 adrenergic receptor-Src protein kinase complexes. Sci. 1999; 283:655-661.

113. Szereszewski JM, Pampillo M, Ahow MR, Offermanns S, Babwah AV. GPR54 Regulates ERK1 / 2 Activity and Hypothalamic Gene Expression in a Gα q/11 and β-Arrestin-Dependent Manner. PLoS ONE. 2010;5(9): e12964.

114. Lee J, Miele ME, Hicks DJ, Karen K, Trent J, Weissman B, *et al*. KiSS-1, a novel human malignant melanoma. J. Nat. Cancer Inst. 1996; 88:1731–7.

115. Clarke H, Dhillo WS, Jayasena CN. Comprehensive Review on Kisspeptin and its role in reproductive disorders. Endocrinol. Metab. 2015; 30:124–41.

116. Kotani M, Detheux M, Vandenbogaerde A, Communi D, Vanderwinden JM, le Poul E, *et al*. The metastasis suppressor gene KiSS-1 encodes kisspeptins, the natural ligands of the orphan G Protein-coupled receptor GPR54. J. Biol. Chem. 2001; 276:34631–6.

117. Roseweir AK, Millar RP. The role of kisspeptin in the control of gonadotrophin secretion. Hum. Reprod. Update. 2009; 15(2):203–12.

118. Pheng V, Uenoyama Y, Homma T, Inamoto Y, Takase K, Yoshizawa-Kumagaye K, *et al*. Potencies of centrally or peripherally injected full-length kisspeptin or its C-terminal decapeptide on LH release in intact male rats. J. Reprod. Dev. 2009; 55(4):378–82.

119. Trevisan CM, Montagna E, De Oliveira R, Christofolini DM, Barbosa CP, Crandall KA, *et al*. Kisspeptin/GPR54 System: What Do We Know about Its Role in Human Reproduction? Cell. Physiol. Biochem. 2018; 49:1259–76.

120. Murphy KG. Kisspeptins: Regulators of Metastasis and the Hypothalamic-Pituitary-Gonadal Axis. J. Neuroendocrinol. 2005; 17:519–25.

121. Lee DK, Nguyen T, Neill GPO, Cheng R, Liu Y, Howard AD, *et al*. Discovery of a receptor related to the galanin receptors. FEBS Lett. 1999; 446:103–7.

122. Kotani M, Detheux M, Vandenbogaerde A, Communi D, Vanderwinden JM, Le Poul E, *et al*. The metastasis suppressor gene KiSS-1 encodes Kisspeptins, the natural ligands of the orphan G Protein-coupled Receptor GPR54. J. Biol. Chem. 2001; 276:34631–6.

123. Hori A, Honda S, Asada M, Ohtaki T, Oda K, Watanabe T, *et al*. Metastin suppresses the motility and growth of CHO cells transfected with its receptor. Biochem. Biophys. Res. Commun. 2001; 286:958–63.

124. Muir AI, Chamberlain L, Elshourbagy NA, Michalovich D, Moore DJ, Calamari A, *et al*. AXOR12, a novel human G Protein-coupled receptor, activated by the peptide KiSS-1. J. Biol. Chem. 2001; 276:28969–75.

125. De Tassigny X, Fagg LA, Dixon JPC, Day K, Leitch HG, Hendrick AG, *et al*. Hypogonadotropic hypogonadism in mice lacking a functional Kiss1 gene. Proc. Natl. Acad. Sci. 2007; 104(25):10714-19.

126. Seminara SB, Messager S, Chatzidaki EE, Thresher RR, Acierno JS, Shagoury JK, *et al*. The GPR54 gene as a regulator of puberty from the reproductive endocrine unit. N Engl J Med. 2003; 349: 1614-27.

127. Ji K, Ye L, Ruge F, Hargest R, Mason MD, *et al*. Implication of metastasis suppressor gene, Kiss-1 and its receptor Kiss-1R in colorectal cancer. BMC Cancer. 2014; 14:723.

128. Sanchez-carbayo M, Capodieci P, Cordon-cardo C. Tumor suppressor role of KiSS-1 in bladder cancer loss of KiSS-1 expression is associated with ladder cancer. Am. J. Path. 2003; 162(2):609–17.

129. Ikeguchi M, Hirooka Y, Kaibara N. Quantitative reverse transcriptase polymerase chain reaction analysis for KiSS-1 and orphan G-protein-coupled receptor (hOT7T175) gene expression in hepatocellular carcinoma. J. Cancer Res. Clin. Oncol. 2003; 129:531–5.

130. Wang H, Jones J, Turner T, He QP, Hardy S, Grizzle WE, *et al*. Clinical and biological significance of KISS1 expression in prostate cancer. Am. J. Path. 2012; 180:1170–8.

131. Jiang Y, Berk M, Singh LS, Tan H, Yin L, Powell CT, *et al*. KiSS1 suppresses metastasis in human ovarian cancer via inhibition of protein kinase C alpha. Clin. Ex. Metastasis. 2005; 22:369–76.

132. Masui T, Doi R, Mori T, Toyoda E, Koizumi M, Kami K, *et al*. Metastin and its variant forms suppress migration of pancreatic cancer cells. Biochem. Biophys. Res. Commun. 2004; 315:85–92.

133. Makri A, Pissimissis N, Lembessis P, Polychronakos C, Koutsilieris M. The kisspeptin (KiSS-1)/GPR54 system in cancer biology. Cancer Treat. Rev. 2008;34(8):682–92.

134. Stark AM, Tongers K, Maass N, Mehdorn HM, Held-Feindt J. Reduced metastasis-suppressor gene mRNA-expression in breast cancer brain metastases. J. Cancer Res. Clin. Oncol. 2005; 131:191–8.

135. Martin TA, Watkins G, Jiang WG. KiSS-1 expression in human breast cancer. Clin. Ex. Metastasis. 2005; 22:503–11.

136. Cvetković D, Babwah AV, Bhattacharya M. Kisspeptin/KISS1R System in Breast Cancer. J Cancer. 2013; 4:653–61.

137. Goertzen CG, Dragan M, Turley E, Babwah AV, Bhattacharya M. KISS1R signaling promotes invadopodia formation in human breast cancer cell via β-arrestin2/ERK. Cell. Signal. 2016; 28:165–76.

138. Blake A, Dragan M, Tirona RG, Hardy DB, Brackstone M, Tuck AB, *et al*. G protein-coupled KISS1 receptor is overexpressed in triple negative breast cancer and promotes drug resistance. Sci. Reports. 2017; 7:46525.

139. Dragan M, Nguyen M, Guzman S, Goertzen C, Brackstone M, Dhillo WS, *et al*. G protein-coupled kisspeptin receptor induces metabolic reprograming and tumorigenesis in estrogen receptor-negative breast cancer. Cell Death & Disease. 2020; 11:106.

140. Beautrait A, Paradis JS, Zimmerman B, Giubilaro J, Nikolajev L, Armando S, *et al*. A new inhibitor of the β-arrestin/AP2 endocytic complex reveals interplay between GPCR internalization and signalling. Nat. Commun. 2017; 8:15054.

141. Pampillo M, Camuso N, Taylor JE, Szereszewski JM, Ahow MR, Zajac M, *et al*. Regulation of GPR54 Signaling by GRK2 and β-Arrestin. Mol. Endocrinol. 2009; 23:0000-0000.

142. Eishingdrelo H, Sun W, Li H, Wang L, Eishingdrelo A, Dai S, *et al*. ERK and β-arrestin interaction: A converging point of signaling pathways for multiple types of cell surface receptors. J. Biomol. Screen. 2015; 20:341–9.

143. Davis RJ, Cassier E, Gallay N, Bourquard T, Claeysen S, Bockaert JL, *et al*. Phosphorylation of β-arrestin2 at Thr 383 by MEK underlies β-arrestin-dependent activation of Erk1/2 by GPCRs. eLife. 2017; 6: e23777.

144. Stathatos N, Bourdeau I, Espinosa AV, Saji M, Vasko VV, Burman KD, *et al*. KiSS-1/G protein-coupled receptor 54 metastasis suppressor pathway increases myocyte-enriched calcineurin interacting protein 1 expression and chronically inhibits calcineurin activity. J. Clin. Endocrinol. Met. 2005; 90:5432–40.

145. Kramer N, Walzl A, Unger C, Rosner M, Krupitza G, Hengstschläger M, *et al*. In vitro cell migration and invasion assays. J. Vis. Exp. 2014; 88: e51046.

146. Stafford LJ, Xia C, Ma W, Cai Y, Liu M. Advances in brief identification and characterization of mouse metastasis-suppressor KiSS1 and its G-Protein coupled receptor. Cancer Res. 2002; 62:5399–404.

147. Gharibi B, Ghuman MS, Hughes FJ. Akt- and Erk-mediated regulation of proliferation and differentiation during PDGFRβ-induced MSC self-renewal. J. Cell. Mol. Med. 2012; 16:2789–801.

148. Sun Y, Xu S. Expression of KISS1 and KISS1R (GPR54) may be used as favorable prognostic markers for patients with non-small cell lung cancer. 2013;521–30.

149. Xoxakos I, Petraki C, Msauel P, Armakolas A, Grigorakis A, Stefanakis S, *et al*. Expression of Kisspeptin (KISS1) and its receptor GPR54 (KISS1R) in prostate cancer. Anticancer Res. 2020; 40:709–18.

150. Cvetković D, Dragan M, Leith SJ, Mir ZM, Leong HS, Pampillo M, *et al*. KISS1R induces invasiveness of estrogen receptor-negative human mammary epithelial and breast cancer cells. Endocrinol. 2013; 154:1999–2014.

151. Zajac M, Law J, Cvetkovic DD, Pampillo M, Mccoll L, Pape C, *et al*. GPR54 (KISS1R) transactivates EGFR to promote breast cancer cell invasiveness. PLos One. 2011; 6(6): e21599.

152. Marot D, Bieche I, Aumas C, Kuttenn F, Lidereau R, Roux N. High tumoral levels of Kiss1 and G-protein- coupled receptor 54 expression are correlated with poor prognosis of estrogen receptor-positive breast tumors. Endocrine-Related Cancer. 2007; 14:691–702.

153. Teles MG, Bianco SDC, Brito VN, Tratbach EB, Kuohung W, Seminara SB, *et al*. A GPR54-activating mutation in a patient with central precocious puberty. N. Eng. J. Med. 2008; 358:709–15.

154. Liu G, Zhao X, Zhou J, Cheng X, Ye Z, Ji Z. LncRNA TP73-AS1 promotes cell proliferation and inhibits cell apoptosis in clear cell renal cell carcinoma through repressing KISS1 expression and inactivation of PI3K/Akt/mTOR signaling pathway. Cell. Physio. Biochem. 2018; 48:371–84.

155. Navenot JM, Fujii N, Peiper SC. Activation of Rho and Rho-associated kinase by GPR54 and KiSS1 metastasis suppressor gene product induces changes of cell morphology and contributes to apoptosis. Mol. Pharmacol. 2009; 75:1300–6.

156. Cho SG, Li D, Stafford LJ, Luo J, Rodriguez-Villanueva M, Wang Y, *et al*. KiSS1 suppresses TNFα-induced breast cancer cell invasion via an inhibition of RhoA-mediated NF-κB activation. J. Cell. Biochem. 2009; 107:1139–49.

157. Min L, Soltis K, Reis ACS, Xu S, Kuohung W, Jain M, *et al*. Dynamic kisspeptin receptor trafficking modulates kisspeptin-mediated calcium signaling. Mol. Endocrinol. 2014; 28:16–27.

158. Bae YS, Lee TG, Park JC, Hur JH, Kim Y, Heo K, *et al*. Identification of a compound that directly stimulates phospholipase C activity. Mol. Pharmacol. 2003; 63:1043–50.

159. Uemura T, Kawasaki T, Taniguchi M, Moritani Y, Hayashi K, Saito T, *et al*. Biological properties of a specific Gαq/11 inhibitor, YM-254890, on platelet functions and thrombus formation under high-shear stress. Br. J. Pharmacol. 2006; 148:61–9.

160. Song GQ, Zhao Y. Kisspeptin-10 inhibits the migration of breast cancer cells by regulating epithelial-mesenchymal transition. Oncol. Reports. 2015; 33:669–74.

161. Ziegler E, Olbrich T, Emons G, Gründker C. Antiproliferative effects of kisspeptin-10 depend on artificial GPR54 (KISS1R) expression levels. Oncol. Reports. 2013; 29:549–54.

162. Tohgo A, Pierce KL, Choy EW, Lefkowitz RJ, Luttrell LM. β-arrestin scaffolding of the ERK cascade enhances cytosolic ERK activity but inhibits ERK-mediated transcription following angiotensin AT1a receptor stimulation. J. Biol. Chem. 2002; 277:9429–36.

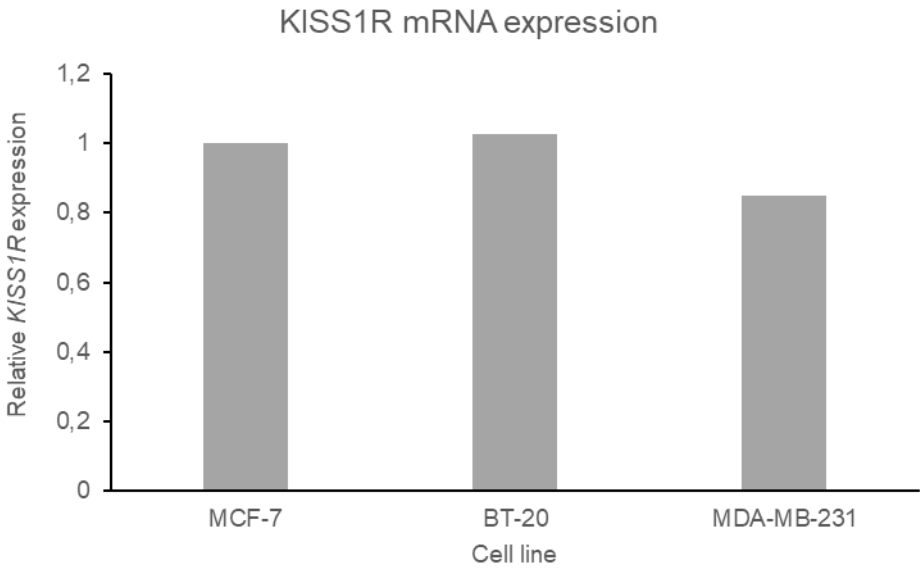
163. Goldberg SF, Miele ME, Hatta N, Takata M, Paquette-Straub C, *et al*. Melanoma Metastasis Suppression by Chromosome 6. Cancer Res. 2003; 63:432–40.

164. Guzman S, Brackstone M, Wondisford F, Babwah AV, Bhattacharya M. KISS1/KISS1R and Breast Cancer: Metastasis Promoter. Semin. Reprod. Med. 2019; 37:197–206.

165. Wang N, Hao HS, Li CY, Zhao YH, Wang HY, Yan CL, *et al*. Calcium ion regulation by BAPTA-AM and ruthenium red improved the fertilisation capacity and developmental ability of vitrified bovine oocytes. Sci. Reports 2017; 7:10652.

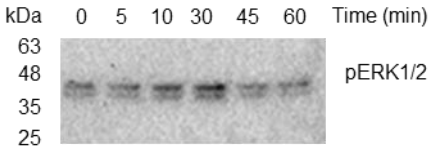
166. Evans JH, Falke JJ. Ca2+ influx is an essential component of the positive-feedback loop that maintains leading-edge structure and activity in macrophages. Proc. Natl. Acad. Sci. 2007; 104:16176–81.

Chapter 6 APPENDICES



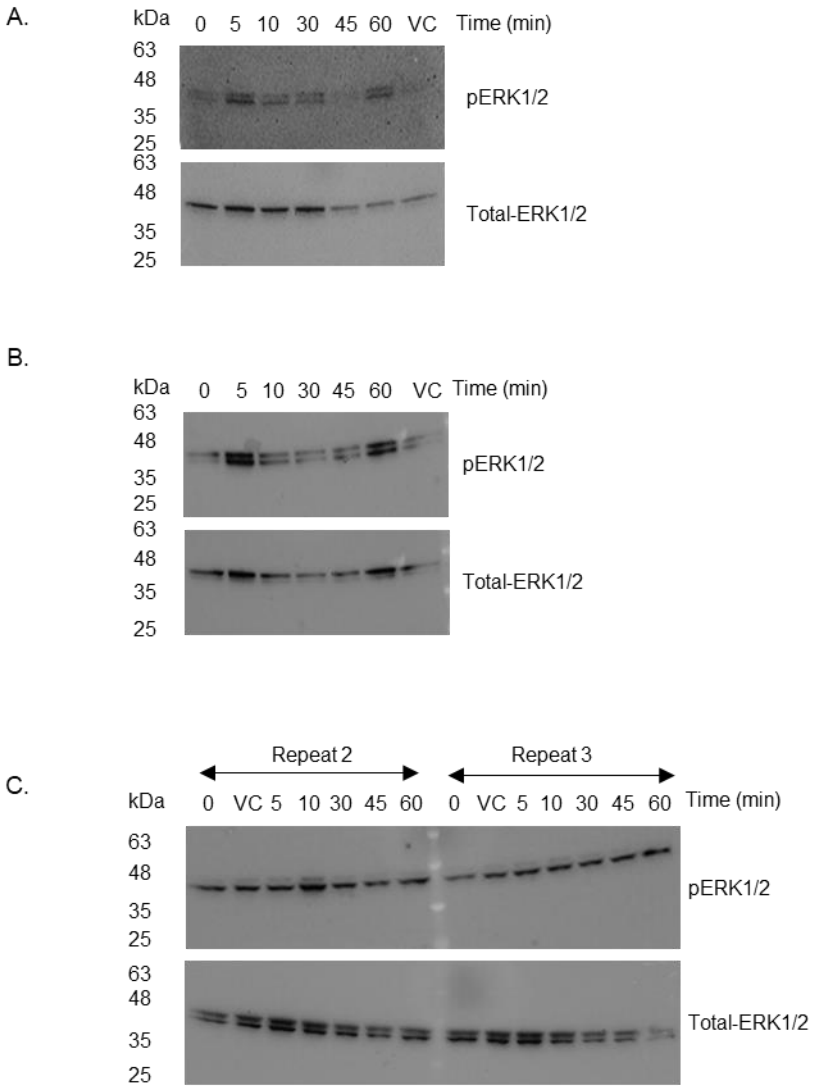
Appendix 6.1: KISS1R mRNA is expressed at relatively similar levels in BT-20 and MDA-MB-231 cells:

Total RNA was extracted from MCF-7, BT-20 and MDA-MB-231 cell lines and then the expression of KISS1R mRNA was assessed through RT-qPCR. The relative expression of *KISS1R* was analysed using the housekeeping gene, *β-actin*, as an internal control for qPCR normalisation. The bars represent the means of two technical repeats.



Appendix 6.2: BT-20 cells stimulated with vehicle control only at different time points.

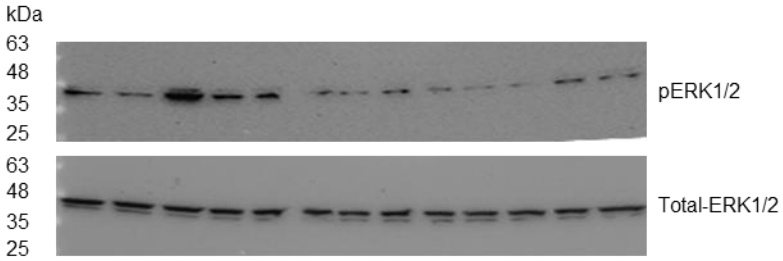
BT-20 cells were serum starved for 4 hrs and then treated with VC (0.2% propylene glycol) for 5, 10, 30, 45, and 60 min, unstimulated control (0 min) was included. Thereafter, the cells were lysed with RIPA lysis buffer and ERK1/2 phosphorylation was assessed via Western blotting, using phospho-p44/42 MAPK antibody.



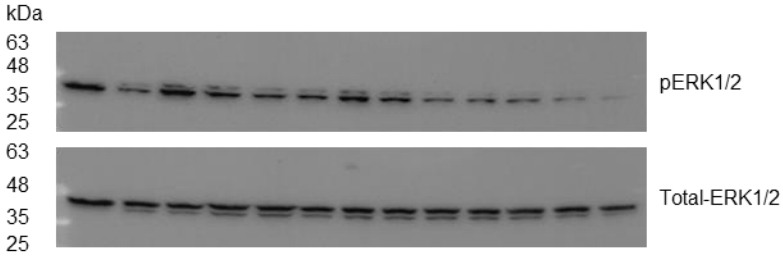
Appendix 6.3: Remaining biological repeats for measurement of ERK1/2 signalling phosphorylation in BT-20 (A and B) and MDA-MB-231 cells (C) displayed in Figure 3.1:

BT-20 and MDA-MB-231 cells were serum starved for 4 hrs and then treated with 100 nM KP-10 for 5, 10, 30, 45, and 60. Unstimulated (0 min) and vehicle treated controls, VC (0.1% DMSO) were included. Thereafter, the cells were lysed with RIPA lysis buffer and ERK1/2 phosphorylation was assessed via Western blotting, using phospho-p44/42 MAPK and p44/42 MAPK antibodies. Western blot images showing ERK1/2 phosphorylation (Top panel) and total ERK1/2 expression (Bottom panel) in (A and B) BT-20 or and (C) MDA-MB-231 cells.

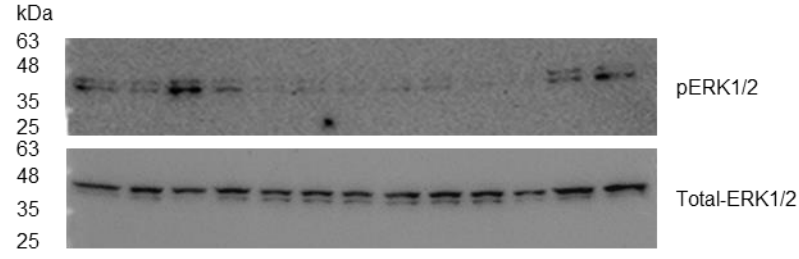
A.



B.



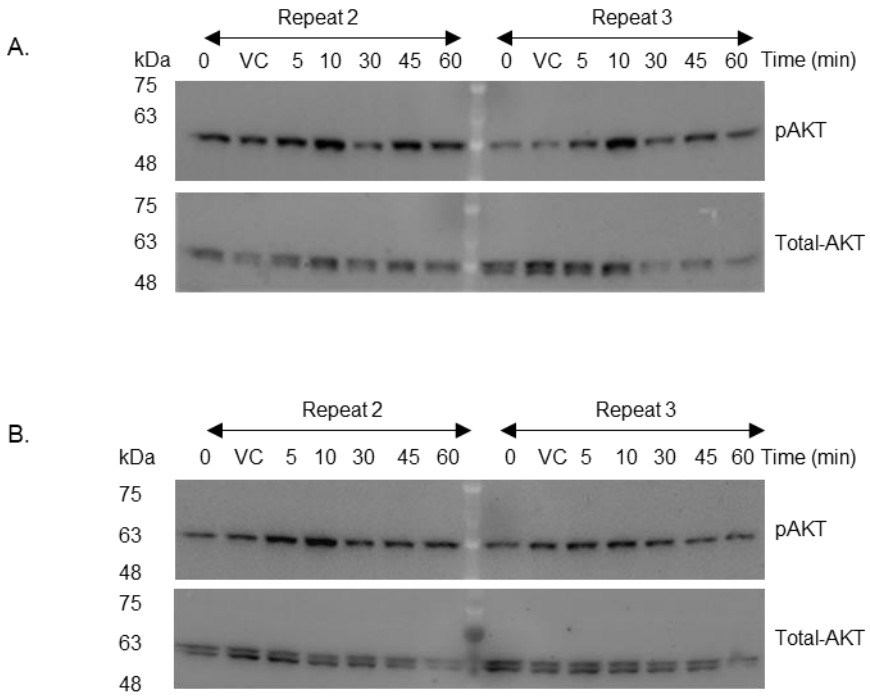
C.



	1	2	3	4	5	6	7	8	9	10	11	12	13
KP-10	-	-	+	-	-	-	-	-	+	+	+	+	+
µM Barb	0	0	0	0,1	0.5	1	2	3	0.1	0.5	1	2	3

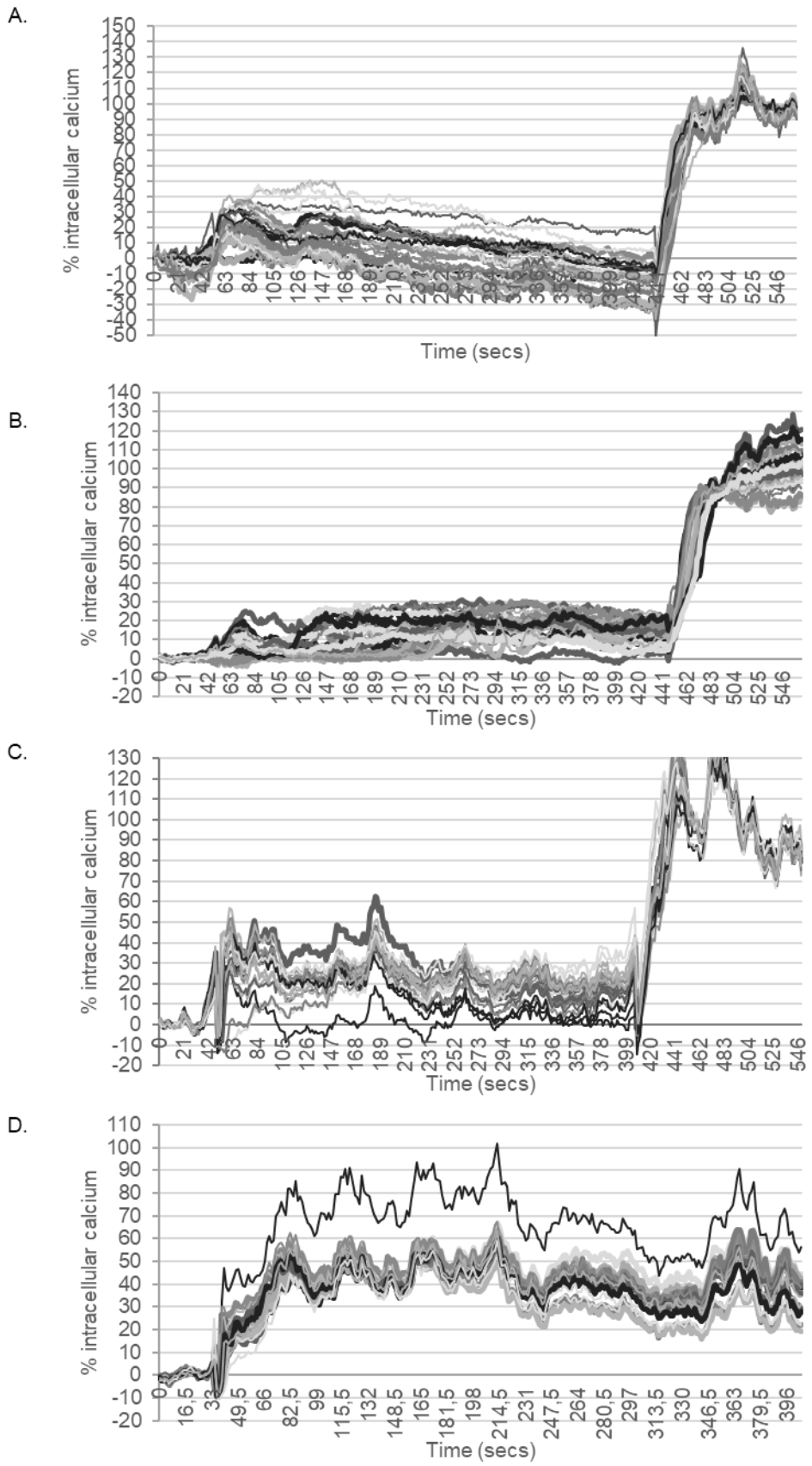
Appendix 6.4: Remaining biological repeats for barbadin assay measuring β-arrestin-dependence of ERK1/2/ phosphorylation in BT-20 cells, displayed in Figure 3.2:

BT-20 cells were serum starved for 4 h and pre-treated for 30 min with the indicated concentration of Barbadin before being stimulated with 100 nM KP-10 for 60 min. Cell lysates were analysed for ERK1/2 phosphorylation by Western blotting with protein loading assessed by Tubulin expression (see Appendix 6.9 for Tubulin blots). (A, B and C) Western blot image showing the expression of phosphorylated ERK1/2 (top panel) and total ERK1/2 (bottom panel) in BT-20 cells that were pre-treated with Barbadin and stimulated with KP-10 as indicated in the table.

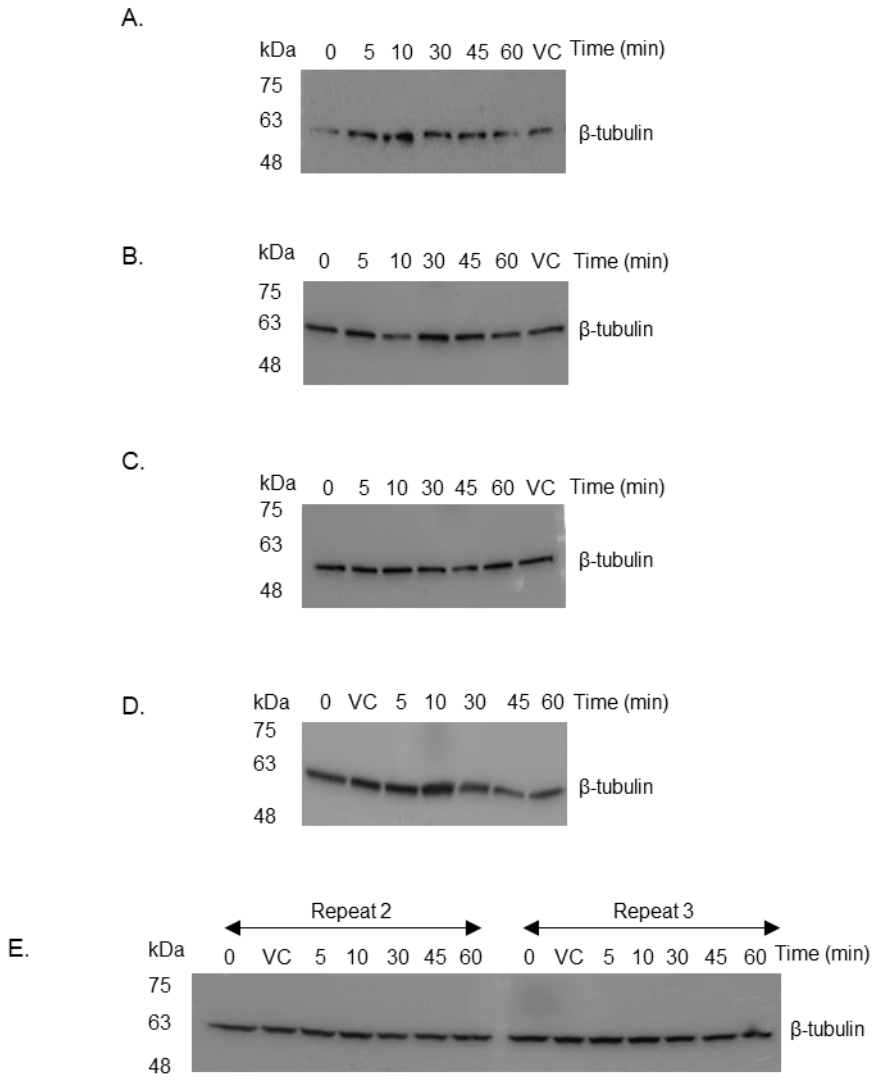


Appendix 6.5: Remaining biological repeats for measurement of Akt phosphorylation signalling in (A) BT-20 and (B) MDA-MB-231 cells, displayed in Figure 3.3:

BT-20 and MDA-MB-231 cells were serum starved for 4 hrs before being treated with 100 nM KP-10 for 5, 10, 30, 45, or 60 min. Unstimulated (0 min) and vehicle (VC, 0.1% DMSO) controls were included. Cells were lysed with RIPA lysis buffer and Akt signalling was assessed through Western blotting using the phospho-Akt and Akt antibodies. Western blot images showing Akt phosphorylation (Top panel) and total Akt expression (Bottom panel) in (A) BT-20 and (B) MDA-MB-231 cells.

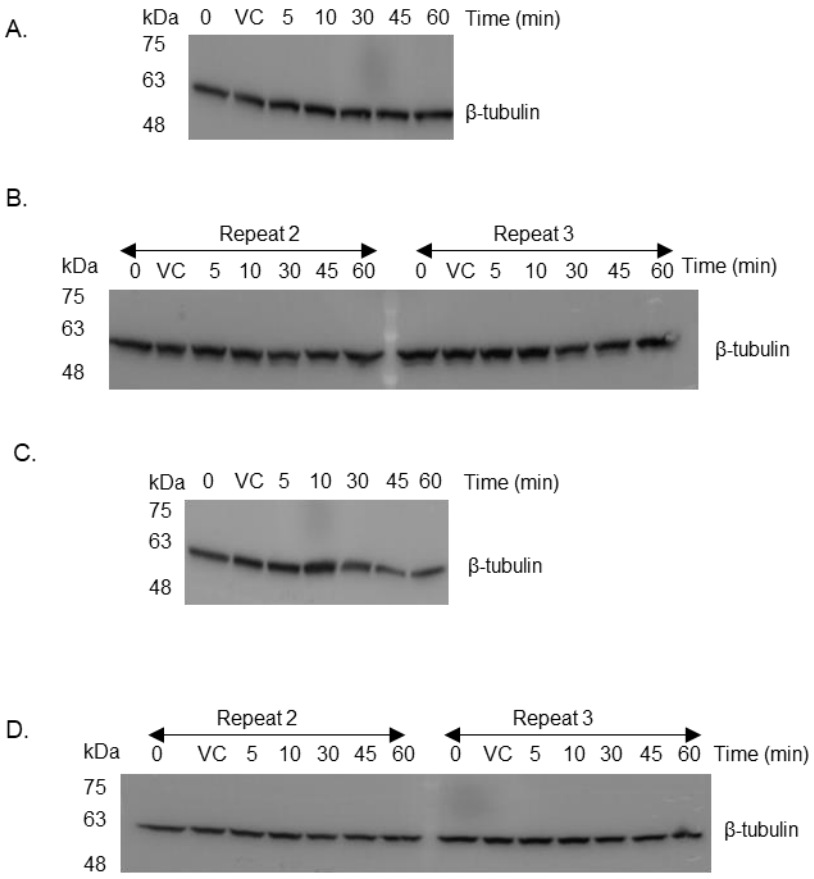


Appendix 6.6: Remaining biological repeats for calcium signalling in BT-20 (A and B) and MDA-MB-231(C and D) cells, displayed in Figure 3.4.



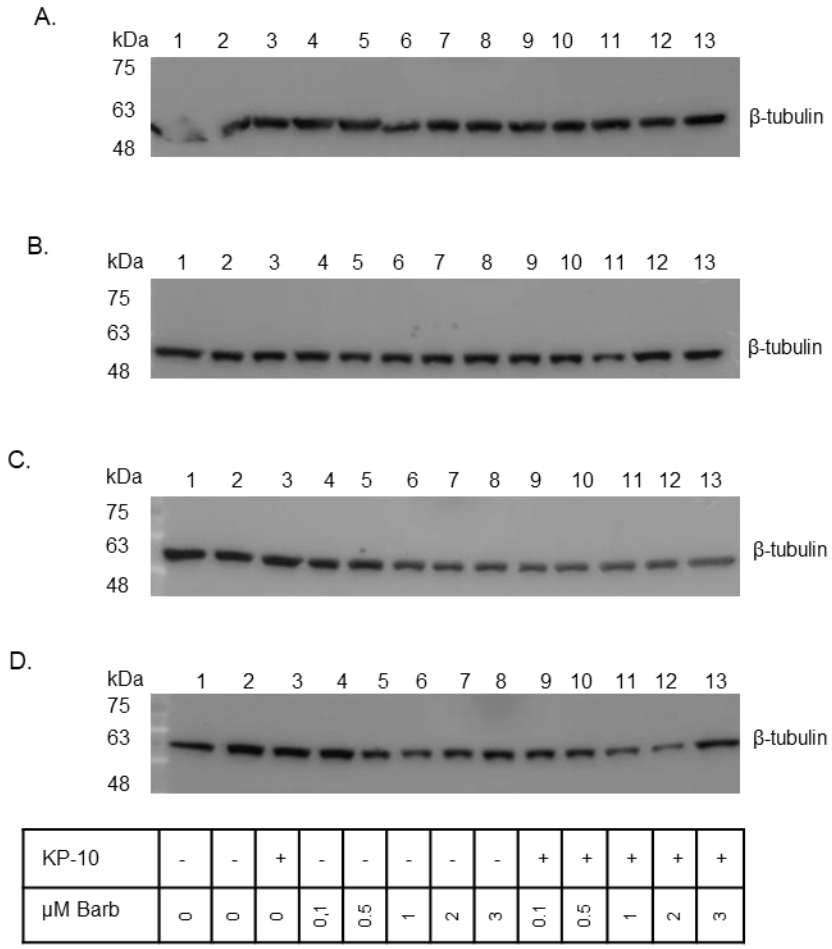
Appendix 6.7: β-tubulin blots for BT-20 (A, B, and C) and MDA-MB-231 (D and E) ERK1/2 assays repeat 1, 2 and 3.

BT-20 and MDA-MB-231 cells were serum starved for 4 hrs and then treated with 100 nM KP-10 for 5, 10, 30, 45, and 60. Unstimulated (0 min) and vehicle treated controls, VC (0.1% DMSO) were included. Thereafter, the cells were lysed with RIPA lysis buffer and β-tubulin expression was assessed via Western blotting, using β-tubulin antibody. Western blot images showing β-tubulin in (A, B, C) BT-20 repeat 1, 2 and 3, respectively and (D and E) MDA-MB-231 cells repeat 1, 2, and 3, respectively.



Appendix 6.8: Tubulin blots for BT-20 (A and B) and MDA-MB-231 (C and D) Akt assays repeat 1, 2 and 3.

BT-20 and MDA-MB-231 cells were serum starved for 4 hrs and then treated with 100 nM KP-10 for 5, 10, 30, 45, and 60. Unstimulated (0 min) and vehicle treated controls, VC (0.1% DMSO) were included. Thereafter, the cells were lysed with RIPA lysis buffer and β-tubulin expression was assessed via Western blotting, using β-tubulin antibody. Western blot images showing β-tubulin in (A and B) BT-20 repeat 1, 2 and 3, respectively and (C and D) MDA-MB-231 cells repeat 1, 2, and 3, respectively.



Appendix 6.9: Tubulin blots for BT-20 Barbadin assay repeat 1 (A), repeat 2 (B), repeat 3 (C), and repeat 4 (D).

BT-20 cells were serum starved for 4 h and pre-treated for 30 min with the indicated concentration of Barbadin before being stimulated with 100 nM KP-10 for 60 min. Thereafter, the cells were lysed with RIPA lysis buffer and β-tubulin expression was assessed via Western blotting, using β-tubulin antibody. Western blot images showing β-tubulin for BT-20 (A) repeat 1, (B) repeat 2, (C) repeat 3, and (D) repeat 4.

Thank you for your order!

Dear Miss. Udochi Felicia Azubuike,

Thank you for placing your order through Copyright Clearance Center's RightsLink® service.

Order Summary

Licensee:	Miss. Udochi Felicia Azubuike
Order Date:	Apr 13, 2022
Order Number:	5287061484566
Publication:	CA: Cancer Journal for Clinicians
Title:	Global Cancer Statistics 2020: GLOBOCAN Estimates of Incidence and Mortality Worldwide for 36 Cancers in 185 Countries
Type of Use:	Dissertation/Thesis
Order Total:	0.00 USD

View or print complete details of your order and the publisher's terms and conditions.

Sincerely,

Copyright Clearance Center

Tel: +1-855-239-3415 / +1-978-646-2777
customercare@copyright.com
https://myaccount.copyright.com

Appendix 6.10: Permission confirmation for "The global distribution of cancer the incidence and mortality for the 10 most common cancers in 2020" (Figure 1.1).

LETTER OF STATISTICAL CLEARANCE **Date: 26 / 05 / 2021**

LETTER OF CLEARANCE FROM THE BIOSTATISTICIAN

This letter is to confirm that, Ms Udochi Felicia Azubuike from UP **discussed with me the study titled:** An investigation of the Kisspeptin/KISS1R signalling node and its role in breast cancer biology**.**

I hereby confirm that I am aware of the project and also undertake to assist, if possible, with the Statistical analysis of the data generated from the project.

The analytical tool(s) that will be used is(are) The data from this two-factor experimental design, cell line at two levels (MDA-MB-231 and BT-20) and concentration at eight level (0, 100 fM, 1 pM, 10 pM, 100 pM, 1 nM, 10 nM and 100 nM), will be analysed in an appropriate analysis of variance (ANOVA)/regression analysis. The two-way ANOVA with factors cell line and concentration, including interaction, will have 32 residual degrees of freedom, which exceeds the norm of 30 and hence sample size is adequate.

Data summary, by cell line and concentration, will report descriptive statistics, including but may not be restricted to, mean, standard deviation and 95% confidence intervals. Data will be analysed in a two-way ANOVA or its regression equivalent. For the latter the margins command in Stata should be able to facilitate comparisons of interest. This analysis will hold for the activity assays including western blot of phosphorylation of proteins and second messenger accumulation assays, signalling pathways including MAPK, Akt/PI3K, and the β-arrestin pathways will be assessed for modulation by KISS1R activation. Testing will be at the 0.05 level of significance **to achieve the objective(s) of the study.**

Signature 

PJ Becker (Tel: 012-319-2203**)**

Research Office, Faculty of Health Sciences

LETTER OF APPROVAL BY PHD COMMITTEE

UNIVERSITEIT VAN PRETORIA
UNIVERSITY OF PRETORIA
YUNIBESITHI YA PRETORIA

Faculty of Health Sciences

19 August 2021

Dr I van den Bout
Department of Physiology
Faculty of Health Sciences

Dear Dr van den Bout

Student: F Azubuike (PhD Physiology)
Title: The Kisspeptin signalling pathway and its role in breast cancer biology

The above-mentioned student's protocol has been approved by the PhD committee. We wish the student all the best with their studies.

Kind regards,

Prof. Martin Brand
Chair: PhD Committee

Email: martin.brand@up.ac.za
Tel +27 (0)12 354 2097

Department of Surgery
Room 71107, Level 7, Bridge E, Steve Biko Academic Hospital
Steve Biko Academic Hospital, Private Bag X169
Pretoria, 0001, South Africa

Fakulteit Gesondheidswetenskappe
Lefapha la Disaense tša Maphelo

ETHICS APPROVAL LETTER 1

UNIVERSITEIT VAN PRETORIA
UNIVERSITY OF PRETORIA
YUNIBESITHI YA PRETORIA

Faculty of Health Sciences

Institution: The Research Ethics Committee, Faculty Health Sciences, University of Pretoria complies with ICH-GCP guidelines and has US Federal wide Assurance.

- FWA 00002567, Approved dd 22 May 2002 and Expires 03/20/2022.
- IORG #: IORG0001762 OMB No. 0990-0279 Approved for use through February 28, 2022 and Expires: 03/04/2023.

Faculty of Health Sciences **Research Ethics Committee**

16 September 2021

Approval Certificate
Amendment

Dear Dr JI van den Bout

Ethics Reference No.: 271/2019
Title: The kisspeptin signalling pathway and its role in breast cancer biology.

The **Amendment** as supported by documents received between 2021-08-23 and 2021-09-15 for your research, was approved by the Faculty of Health Sciences Research Ethics Committee on 2021-09-15 as resolved by its quorate meeting.

Please note the following about your ethics approval:

- Please remember to use your protocol number (271/2019) on any documents or correspondence with the Research Ethics Committee regarding your research.
- Please note that the Research Ethics Committee may ask further questions, seek additional information, require further modification, monitor the conduct of your research, or suspend or withdraw ethics approval.

Ethics approval is subject to the following:

- The ethics approval is conditional on the research being conducted as stipulated by the details of all documents submitted to the Committee. In the event that a further need arises to change who the investigators are, the methods or any other aspect, such changes must be submitted as an Amendment for approval by the Committee.

We wish you the best with your research.

Yours sincerely

On behalf of the FHS REC, Dr R Sommers
MBChB, MMed (Int), MPharmMed, PhD
***Deputy Chairperson** of the Faculty of Health Sciences Research Ethics Committee, University of Pretoria*

The Faculty of Health Sciences Research Ethics Committee complies with the SA National Act 61 of 2003 as it pertains to health research and the United States Code of Federal Regulations Title 45 and 46. This committee abides by the ethical norms and principles for research, established by the Declaration of Helsinki, the South African Medical Research Council Guidelines as well as the Guidelines for Ethical Research: Principles Structures and Processes, Second Edition 2015 (Department of Health).

ETHICS APPROVAL LETTER 2

UNIVERSITEIT VAN PRETORIA
UNIVERSITY OF PRETORIA
YUNIBESITHI YA PRETORIA

Faculty of Health Sciences

Institution: The Research Ethics Committee, Faculty Health Sciences, University of Pretoria complies with ICH-GCP guidelines and has US Federal wide Assurance.
- FWA 00002567, Approved dd 22 May 2002 and Expires 03/20/2022.
- IORG #: IORG0001762 OMB No. 0990-0279 Approved for use through February 28, 2022 and Expires: 03/04/2023.

Faculty of Health Sciences **Research Ethics Committee**

17 March 2022

Approval Certificate
Annual Renewal

Dear Dr JI van den Bout,

Ethics Reference No.: 271/2019 – Line 4
Title: The kisspeptin signalling pathway and its role in breast cancer biology.

The **Annual Renewal** as supported by documents received between 2022-03-10 and 2022-03-16 for your research, was approved by the Faculty of Health Sciences Research Ethics Committee on 2022-03-16 as resolved by its quorate meeting.

Please note the following about your ethics approval:
- Renewal of ethics approval is valid for 1 year, subsequent annual renewal will become due on 2023-03-17.
- Please remember to use your protocol number (271/2019) on any documents or correspondence with the Research Ethics Committee regarding your research.
- Please note that the Research Ethics Committee may ask further questions, seek additional information, require further modification, monitor the conduct of your research, or suspend or withdraw ethics approval.

Ethics approval is subject to the following:
- The ethics approval is conditional on the research being conducted as stipulated by the details of all documents submitted to the Committee. In the event that a further need arises to change who the investigators are, the methods or any other aspect, such changes must be submitted as an Amendment for approval by the Committee.

We wish you the best with your research.

Yours sincerely

On behalf of the FHS REC, Dr R Sommers
MBChB, MMed (Int), MPharmMed, PhD
***Deputy Chairperson** of the Faculty of Health Sciences Research Ethics Committee, University of Pretoria*

The Faculty of Health Sciences Research Ethics Committee complies with the SA National Act 61 of 2003 as it pertains to health research and the United States Code of Federal Regulations Title 45 and 46. This committee abides by the ethical norms and principles for research, established by the Declaration of Helsinki, the South African Medical Research Council Guidelines as well as the Guidelines for Ethical Research: Principles Structures and Processes, Second Edition 2015 (Department of Health)

RESEARCH OUTPUT

Oral presentation at the International Union of Physiological Sciences (IUPS) conference in May 2022.

Dear Udochi Felicia Azubuike

Greetings from Beijing!

On behalf of the Local Organizing Committee of the 39th Congress of the International Union of Physiological Sciences (IUPS2022), we are pleased to inform you that your abstract 'The Kisspeptin signalling pathway and its role in breast cancer biology.' has been accepted as an ORAL to present in Young Physiologist Symposium 3.

According to the program of the congress, your symposium is scheduled as below:

[Date, Time, and Duration]

- Beijing Time (UTC +8): 15:50-17:50, Monday, May 9th, 2022.
- Each Young Physiologist Symposium will be divided into two sessions of 1 hour each, one session with 6 speakers and each speaker will be allocated 8 minutes, and a total of 12 minutes at the end of a session for Group Discussion/Q & A time.
- This may be ungodly hours for some of you to present. Please note that talks can be prerecorded. However, it would be much appreciated if you are available for live discussion/Q & A time in the last 12 minutes of each session in this time slot.

Oral presentation at South African Society of Biochemistry and Molecular Biology (SASBMB) conference in January 2022.

13 January 2022

Dear Udochi Felicia Azubuike,

We are pleased to inform you that your submission entitled “The Kisspeptin signalling pathway and its role in breast cancer biology.”, Submission ID: 6, has been accepted as an **ORAL PRESENTATION** at the SASBMB 2022 Congress.

Below, please find your provisional presentation details. Please note that these may still change. The programme will be published on the website asap. Please check back regularly for updates and changes.

Presentation Date: Tuesday, 25 January
Presentation Time: 11:40 - 11:55
Session Name: Understanding and Treating Cancer II
Full Session Time: 10:40 - 12:25
Session Joining Time: 10:20:00 AM

Oral presentation at the Physiological Society of Southern Africa (PSSA) conference in September 2021. The Wyndham award was received for the presentation.

Dear Ms Udochi Felicia Azubuike

Thank you for having shown interest in the joint Virtual AAPS-PSSA 2021 Congress (12 – 15 September 2021) and for your scientific participation and contribution to the field.

We are pleased to inform you that your abstract entitled *"An investigation of the Kisspeptin signaling pathway and its role in breast cancer biology."* was peer reviewed by a scientific committee and has been accepted for **ORAL** presentation.

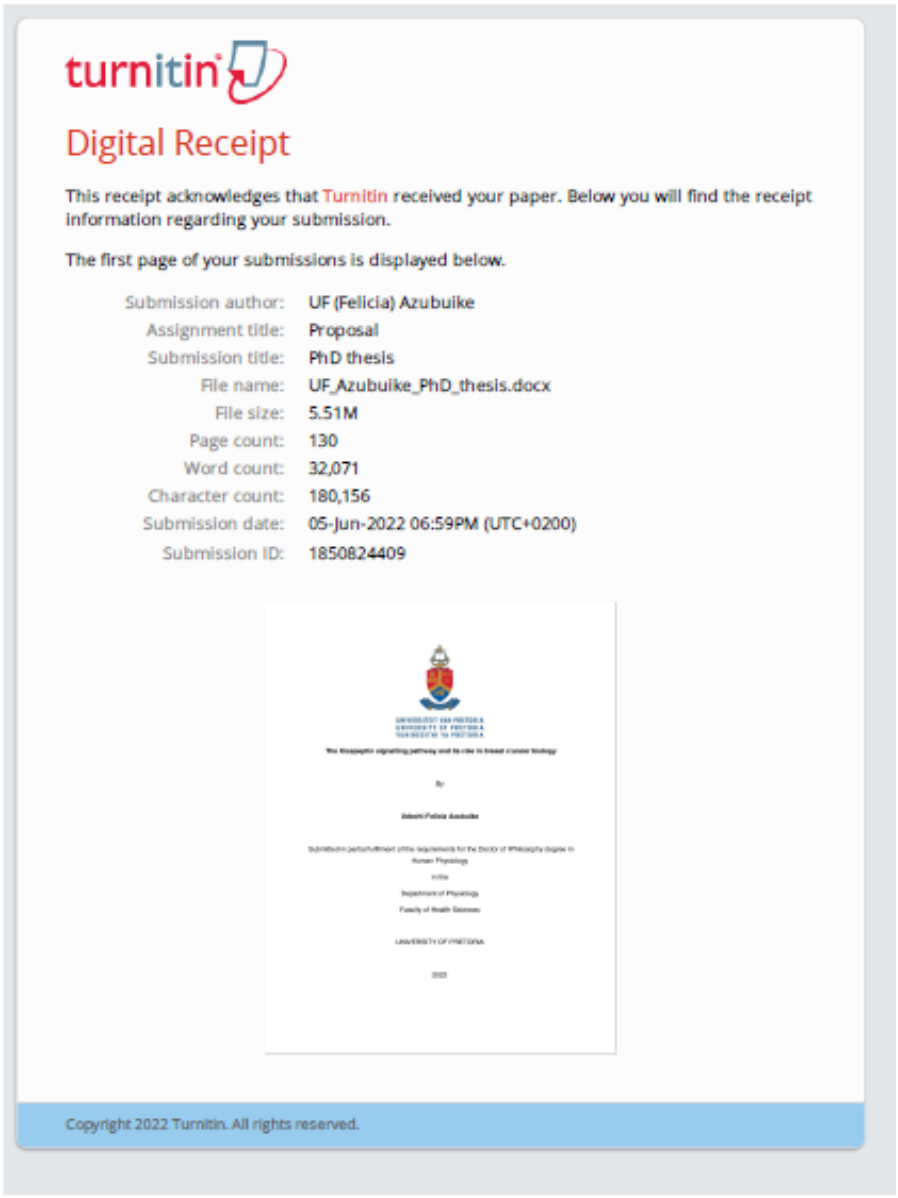
COMPETITION ENTRY:

If you have submitted an abstract for consideration for the Wyndham Prize Competition, will you kindly **CONFIRM** this via email.

For entrants in the Wyndham competition, there is a need for verification of eligibility of the entrants, (see rules at: https://physiolsoc.org.za/wyndham-prize-competition/)

The Wyndham award is an award given to the young scientist with the best oral presentation at the PSSA annual conference.

TURNITIN RECEIPT



turnitin

Digital Receipt

This receipt acknowledges that Turnitin received your paper. Below you will find the receipt information regarding your submission.

The first page of your submissions is displayed below.

Submission author:	UF (Felicia) Azubuike
Assignment title:	Proposal
Submission title:	PhD thesis
File name:	UF_Azubuike_PhD_thesis.docx
File size:	5.51M
Page count:	130
Word count:	32,071
Character count:	180,156
Submission date:	05-Jun-2022 06:59PM (UTC+0200)
Submission ID:	1850824409