Functional studies of kisspeptin analogues and the human kisspeptin receptor.

Thesis submitted in accordance with the requirements of the University of Liverpool for the degree of Doctor in Philosophy by Xiaoyang Zhang

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Declaration

I hereby declare that the work presented within the thesis was carried out by myself during the course of my PhD and that it has not been submitted for any other degree or qualification. Where I have used the work of others, the sources of information have been detailed clearly in the presentation.

Xiaoyang Zhang

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Finally, I would like to thank my family and friends for supporting and encouraging me throughout my studies.

Abbreviations

General

ABL1	Abelson murine leukemia viral oncogene homolog 1
ARB	Angiotensin receptor blocker
BCA	Bicinchoninic acid
BSA	Bovine serum albumin
cAMP	Cyclic adenosine 3’,5’-monophosphate
CFP	Cyan fluorescent protein
CHO	Chinese hamster ovary
Co-IP	Co-immunoprecipitation
DAG	Diacylglycerol
DMEM	Dulbecco’s modified Eagle’s medium
DMSO	Dimethyl sulfoxide
DPC	Dodecylphosphocholine
ECL	Extracellular loop
EDTA	Ethylene diamine tetraacetic acid
ECM	Extracellular matrix
ERα	Estrogen receptor α
ERK1/2	Extracellular signal-regulated kinase 1/2
FAK	Focal adhesion kinase
FlAsH	Fluorophore 4’,5’-bis(1,2,3-dithioarsolan-2-yl)-fluorescein

FRET	Fluorescence resonance energy transfer
FSH	Follicle-stimulating hormone
GAP	GTPase activating protein
GEF	Guanine nucleotide exchange factors
GH	Growth hormone
GIPs	GPCR-interacting proteins
GnRH	Gonadotropin-releasing hormone
G protein	Guanine nucleotide-binding protein
GPCR	G protein-coupled receptor
GRKs	GPCR kinases
GST	Glutathione-S-transferase
H8	Helix 8
HEK293	Human embryonic kidney 293
HEPES	4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid
ICL	Intracellular loop
IQGAP1	IQ motif-containing GTPase-activating protein 1
LH	Luteinizing hormone
IHH	Idiopathic hypogonadotropic hypogonadism
IP	Inositol phosphate
IP_3	Inositol 1,4,5-trisphosphate
LB	Luria Broth
LiSS	Ligand-induced selective signalling
MAPK	Mitogen-activated protein kinase

MCF-7	Michigan Cancer Foundation-7
MEF	Mouse embryonic fibroblast
MEK1	MAPK kinase 1
MEK2	MAPK kinase 2
MMPs	Matrix metalloproteinases
MMTV	Mouse mammary tumour virus
NC-IUPHAR	International Union of Pharmacology, Committee on Receptor Nomenclature and Drug Classification
NF-κB	Nuclear factor kappa B
NMR	Nuclear magnetic resonance
NNGH	N-Isobutyl-N-(4-methoxyphenylsulfonyl)glycyl hydroxamic acid
p63RhoGEF	Rho guanine nucleotide exchange factor 25
PACAP	Pituitary adenylyl cyclase-activating polypeptide
PAGE	Polyacrylamide gel electrophoresis
PBS	Phosphate buffered saline
PCR	Polymerase chain reaction
PI3K	Phosphatidylinositide 3-kinases
PIP_2	Phosphatidylinositol 4,5-bisphosphate
PLC-β	Phospholipase C-β
POPC	1-palmitoyl-2-oleoyl-phosphatidylchline
PP2A	Protein phosphatase 2A
PVDF	Polyvinylidene difluoride
PyMT	Polyoma virus middle T antigen

SD	Standard deviation
SDF-1	stromal cell-derived factor 1
SDS	Sodium dodecyl sulfate
shRNA	Short hairpin RNA
SRF	Serum response factor
3D	Three-dimensional
TM	Transmembrane domain
YFP	Yellow fluorescent protein

Receptors

α_{1B}AR	α_{1B}-adrenergic receptor
A_{2A}AR	Adenosine A_{2A} receptor
AT_1R	Angiotensin II type 1 receptor
β_2AR	β_2-adrenergic receptor
CCR5	CC chemokine receptor 5
EGFR	Epidermal growth factor receptor
ERα	Estrogen receptor α
GALR1	Galanin receptor 1
H_2R	Histamine H_2 receptor
NTR1	Neurotensin receptor type 1
PAR1	Protease-activated receptor 1
μ-OR	μ-opioid receptor

20 amino acids

Amino Acid	Three-letter	One-letter	Amino Acid	Three-letter	One-letter
Alanine	Ala	A	Leucine	Leu	L
Arginine	Arg	R	Lysine	Lys	K
Asparagine	Asn	N	Methionine	Met	M
Aspartic Acid	Asp	D	Phenylalanine	Phe	F
Cysteine	Cys	C	Proline	Pro	P
Glutamine	Gln	Q	Serine	Ser	S
Glutamic Acid	Glu	E	Threonine	Thr	T
Glycine	Gly	G	Tryptophan	Trp	W
Histidine	His	H	Tyrosine	Tyr	Y
Isoleucine	Ile	I	Valine	Val	V

Abstract

Kisspeptins and their cognate receptor, the kisspeptin receptor, play pivotal roles in the regulation of the development of puberty and cancer metastasis. However, the signalling pathways activated by kisspeptins and the kisspeptin receptor remain largely to be demonstrated. Aims of the thesis are to investigate the biological functions of kisspeptin analogues and the human kisspeptin receptor. Since kisspeptins show low metabolic stability, it may limit the studies in the long-term signalling of kisspeptins and the kisspeptin receptor. To improve the metabolic stability of kisspeptins, in the thesis, three novel phosphinic peptides were designed and synthesised based on the amino acid sequence of KP-10, which are the last ten amino acids shared by all native and functional kisspeptins. Whether the synthetic peptides can bind to and activate the kisspeptin receptor and their ability to inhibit MMP were tested. The results showed that among synthesized peptides, compound B possesses the kisspeptin receptor-agonistic activity and could function as a selective inhibitor of MMP-2. However, the binding of the synthesized peptides to the kisspeptin receptor was undetectable using the presented methods. In addition, the Pro-rich region within the C-terminal tail of the kisspeptin receptor has been proposed to function as a SH3 binding motif to mediate the interactions of the receptor with non-G protein effectors. Therefore, the interaction of the kisspeptin receptor with two candidates, p85α or c-SRC, was elucidated by using co-IP assays and their potential biological functions were examined. Direct interactions of the human kisspeptin receptor with p85α and c-SRC were observed in human breast cells and the interactions were not affected by short-time KP-10 stimulation. Functionally, the kisspeptin receptor may directly bind to p85α or c-SRC to activate $G_{q/11}$-

independent PI3K/AKT and c-SRC-dependent ERK1/2 signalling pathways. Importantly, a weak negative crosstalk of the kisspeptin receptor with insulin receptor, which kisspeptins inhibited insulin-induced phosphorylation of AKT, was observed. Furthermore, two Cys residues, Cys^{338} and Cys^{340}, in the C-terminal tail of the kisspeptin receptor are predicted to be palmitoylation sites and, therefore, be important for the expression and function of the kisspeptin receptor. In the thesis, the roles of Cys^{338} and Cys^{340} on the receptor expression and signalling were investigated by using mutagenesis studies. The Ser substitution of Cys^{338} had little effect on receptor expression, internalization and functions. By contrast, although the mutation of Cys^{340} to Ser had little effect on expression and the downstream signalling evens measured, it did affect the internalization of the receptor. The Ser substitution of both Cys^{338} and Cys^{340} decreased the total expression of the receptor and abolished receptor signalling and internalization. The results may indicate that Cys^{340} is a primary palmitoylation site and Cys^{338} acts as an alternative palmitoylation site to rescue the signalling, at least some aspects, of the receptor, when is Cys^{340} mutated. Alternatively, Cys^{338} may be normally palmitoylated, but this has effects on signalling functions not measured here, and does not impact on receptor internalization.

Chapter 1

Introduction

1.1 Overview

Guanine nucleotide-binding (G) protein-coupled receptors (GPCRs) constitute the largest family of cell surface receptors and are encoded by approximately 800 distinct genes, which account for more than 1% of the human genome (Luttrell, 2008). GPCR family plays important roles in intercellular signalling in response to a variety of extracellular stimuli such as neurotransmitters and peptide hormones (Luttrell, 2008). Dysfunctions of GPCRs can contribute to numerous diseases from neuro diseases to cancers (Huang et al., 2017, Dorsam and Gutkind, 2007). Not surprising then, GPCRs are targets of over half of all drugs currently used in the clinic (Dorsam and Gutkind, 2007, Luttrell, 2008) and remain a main avenue for future development of drugs.

GPCRs are characterised by seven transmembrane domains (TMs), which are connected by three extracellular loops (ECL1-3) and three intracellular loops (ICL1-3). The extracellular region containing ECLs and the N-terminus, together with the extracellular ends of TMs, are responsible for the recognition and binding of ligands. The 7TMs form a structural core of a GPCR and are involved in ligand-induced signalling transduction *via* their conformational changes. The intracellular region containing ICLs and carboxyl (C)-terminus interacts with intracellular signalling transducers, such as G proteins, which mediate the amplification and diversity of the downstream signalling. Based on the similarity of amino acid sequences of TMs of GPCRs, Fredriksson and colleagues proposed a GRAFS classification system, which divided 802 known and predicted human GPCRs into five main families: Rhodopsin (R), Glutamate (G), Adhesion (A), Frizzled/taste2 (F) and Secretin (S) (Fredriksson et

al., 2003). The Rhodopsin family is the largest family and is subdivided into four subgroups: α, β, γ and δ. The GRAFS classification system is used and extended by the International Union of Pharmacology, Committee on Receptor Nomenclature and Drug Classification (NC-IUPHAR) committee, which provides guidelines for the nomenclature of GPCRs. The NC-IUPHAR committee divides human GPCRs into five main families: class A (Rhodopsin), class B (Secretin), class C (Glutamate), Frizzled and Adhesion family (Alexander et al., 2013) and this classification system is used in the thesis.

A novel GPCR cDNA was isolated from a rat brain in 1999 and the open reading frame of the cDNA encodes a 396-amino-acid protein termed GPR54 (Lee et al., 1999). Two years later, the human orthologue of GPR54, which shares 81% homology in the primary sequence with rat GPR54, was cloned from a human brain and was alternatively termed AXOR12 (Muir et al., 2001) or hOT7T175 (Ohtaki et al., 2001). The kisspeptin receptor, which is the official name for GPR54 designated by the NC-IUPHAR committee (Kirby et al., 2010), is used here. According to the GRAFS classification system, the kisspeptin receptor belongs to the γ branch of the Rhodopsin family and shares highest identity of the amino acid sequence with galanin receptor 1 (GALR1) (Fig. 1.1) (Fredriksson et al., 2003). In mammalian species, the kisspeptin receptor is encoded by a single gene known as *kissr1r* with exception of platypus (*Ornithorhynchus anatinu*s), which has two genes (*kissr1r* and *kissr4r*) that encode two subtypes of the receptor (Tena-Sempere et al., 2012). In the following sections, the characteristics of the kisspeptin receptor and its endogenous ligands, the physiological functions of the kisspeptin receptor and the kisspeptin

receptor-mediated signalling will be discussed.

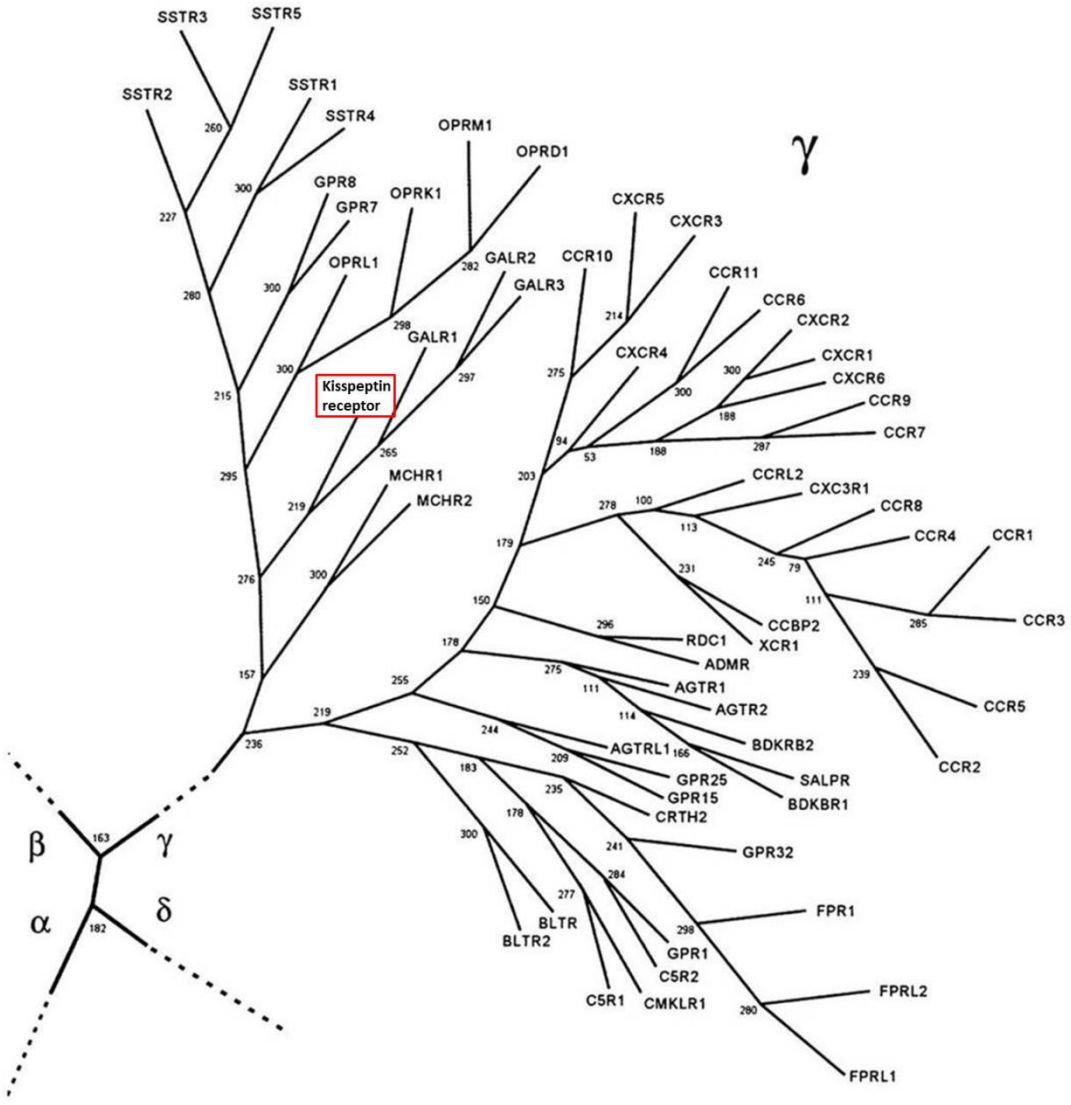


Figure 1.1 Phylogenetic relationships between GPCRs in the γ subgroup of the human Rhodopsin family. Based on the GRAFS classification system, the Rhodopsin family can be subdivided into four subgroups: α, β, γ and δ. The numbers indicate the distances between proteins, which were calculated using the maximum parsimony by using the Phylip package. The kisspeptin receptor is highlighted in a red rectangle. Figure is adapted from Fredriksson *et al*., 2003.

1.2 The kisspeptin receptor and its ligands

1.2.1 Structure-function relationships of the human kisspeptin receptor

The human kisspeptin receptor, which contains 398 amino acids, comprises an N-terminus in the extracellular region with approximate 43 amino acids, 7TMs that are

connected by ECL1-3 and ICL1-3, and a C-terminal tail in the intracellular region with approximate 70 amino acids (Fig. 1.2). The N-terminus of the human kisspeptin receptor has three Asn residues (Asn^{10}, Asn^{18} and Asn^{28}), which are located in the consensus N-linked glycosylation motif (NXS/T, where X is any amino acid except Pro) and are predicted to be N-glycosylated (Muir et al., 2001, Clements et al., 2001). Besides, the ECLs of the human kisspeptin receptor contain two Cys residues (Cys^{115} at ECL1 and Cys^{191} at ECL2), which are highly conserved and form a disulphide bridge in most GPCRs. The disulphide bridge has been reported to be important for shaping the ligand-binding pocket and stabilizing the conformation of the extracellular region in GPCRs (Venkatakrishnan et al., 2013, Zhang et al., 2015a). Therefore, the disulphide bridge formed between Cys^{115} and Cys^{191} of the human kisspeptin receptor may also exhibits similar functions.

In TMs, the human kisspeptin receptor contains several motifs, which are conserved in most class A GPCRs (Sandoval et al., 2016, Zhang et al., 2015a, Venkatakrishnan et al., 2013). It has a conserved (D/E)$R^{3.50}$(Y/W) motif [superscript denotes Ballesteros-Weinstein numbering, in which the most conserved residue among class A GPCRs in each TM is designated x.50, where x is the number of TM (1-7) located in (Ballesteros and Weinstein, 1995)] in TM3, a $CW^{6.48}XP^{6.50}$ (X is any amino acid) motif in TM6 and a $NP^{7.50}$XXY (X is any amino acid) motif in TM7. The conserved motifs may be implicated in the activation of the kisspeptin receptor, since they are classified as molecular micro-switches to regulate the activation of other GPCRs (Nygaard et al., 2009). For example, in human adenosine A_{2A} receptor (A_{2A}AR), the $Arg^{3.50}$ residue in the (D/E)$R^{3.50}$(Y/W) motif forms a salt bridge by an interaction with the Asp/$Glu^{6.30}$

residue when the receptor is inactivated (Xu et al., 2011). The salt bridge is broken when $A_{2A}AR$ is activated by a full agonist. In addition, a rotameric switch of the aromatic side chain of the $Tyr^{7.53}$ in the $NP^{7.50}XXY$ motif is observed in the full agonist-activated conformation of $A_{2A}AR$ compared with the inactive structure. Moreover, the $Trp^{6.48}$ residue in the $CW^{6.48}XP^{6.50}$ motif locates at the bottom of the ligand binding pockets of $A_{2A}AR$ and may be involved in the trigger of an outward tilt of the intracellular side of TM6 by interacting with agonists upon the activation of full agonist. In addition to the conserved motifs, the human kisspeptin receptor also contains several residues that are conserved among most class A GPCRs, including Asn^{60} in TM1, Asp^{88} in TM2, Trp^{167} in TM4 and Pro^{216} in TM5 ($Asn^{1.50}$, $Asp^{2.50}$, $Trp^{4.50}$ and $Pro^{5.50}$ according to Ballesteros-Weinstein numbering). These residues are proposed to mediate the tertiary interactions between TMs and be involved in the formation of the TM helix bundle of GPCRs (Venkatakrishnan et al., 2013).

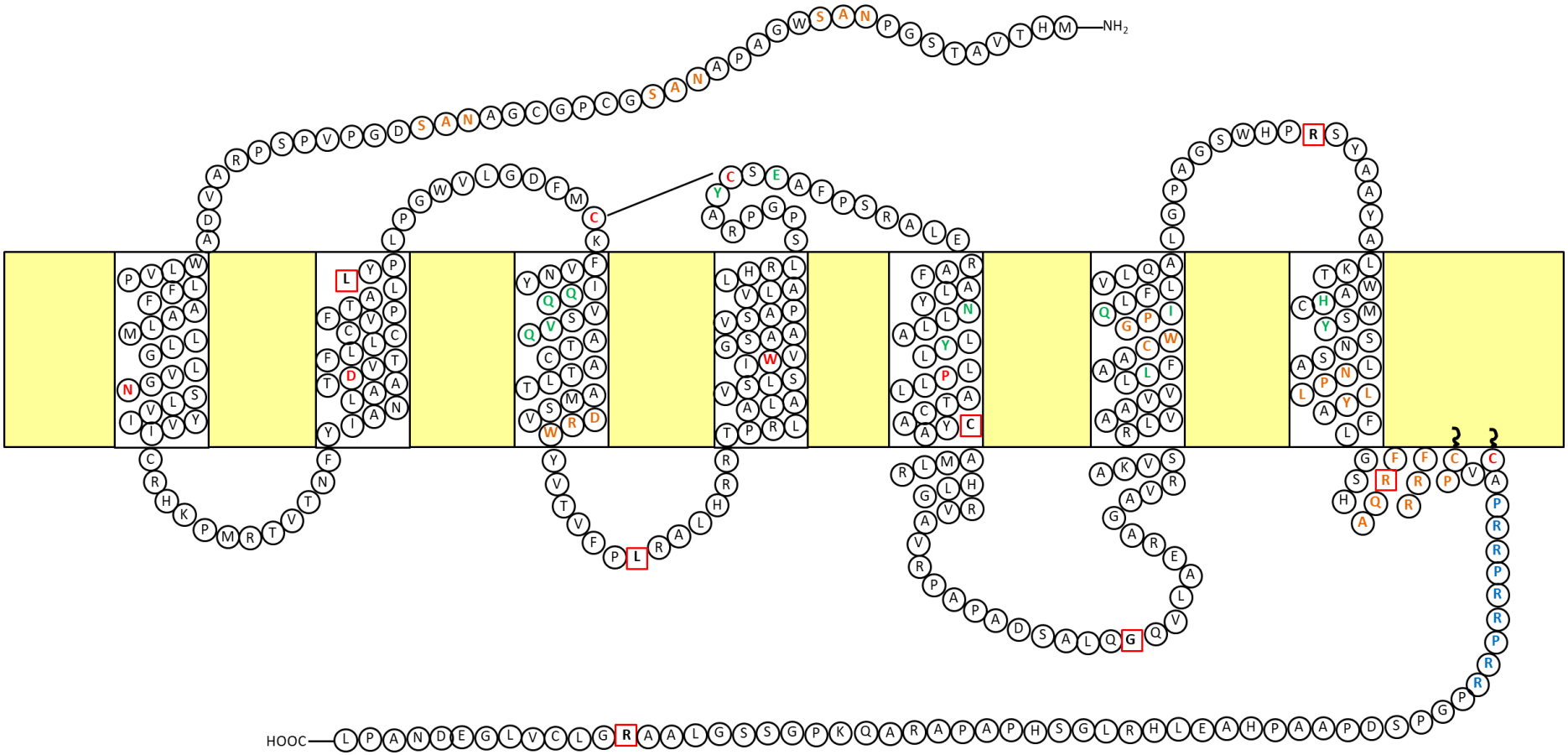


Figure 1.2 The primary structure of the human kisspeptin receptor. The consensus sequences are shown in orange and the conserved residues are shown in red. Residues predicted to be involved in the formation of ligand-binding pocket are shown in green. The residues in red square are the disease-causing mutation sites. Three Pro-Arg-Arg repeats are shown in blue. The GenBank accession number of the human kisspeptin receptor plotted here is AAK83235.1.

In the intracellular region, a short amphipathic helix, termed helix 8 (H8), is reported to be present in most crystallized class A GPCRs, such as β_2-adrenergic receptor (β_2AR) (Cherezov et al., 2007, Rasmussen et al., 2011b, Rasmussen et al., 2011a), A_{2A}AR (Jaakola et al., 2008), M_2 muscarinic acetylcholine receptor (Haga et al., 2012), dopamine D_3 receptor (Chien et al., 2010), histamine H_1 receptor (Shimamura et al., 2011) and neurotensin receptor type 1 (NTR1) (Egloff et al., 2014), with the exceptions of protease-activated receptor 1 (PAR1) (Zhang et al., 2012) and chemokine receptor type 4 (CXCR4) (Wu et al., 2010). H8 locates after the $NP^{7.50}XXY$ motif and contains a conserved sequence [F(R/K)XX(F/L)XXX(L/F), where X is any amino acid] (Fig. 1.3.). NTR1, which was previously crystalized without H8 being formed (White et al., 2012), has been recently shown to exhibit a less stable H8 in several crystalized structures compared with that of A_{2A}AR and the H8 structure may only occur under certain conditions (Egloff et al., 2014). In the same regard, PAR1, CXCR4 and the kisspeptin receptor, which have a less conserved F(R/K)XX(F/L)XXX(L/F) sequence, may possess an even less stable H8 structure that only be formed under certain scenarios. Functionally, H8 is essential for the cell surface expression of some GPCRs, such as β_2AR (Parmar et al., 2016), and can mediate the interaction of receptors with cognate G proteins (Kaye et al., 2011) and other interacting proteins, such as Rab11 (Reid et al., 2010).

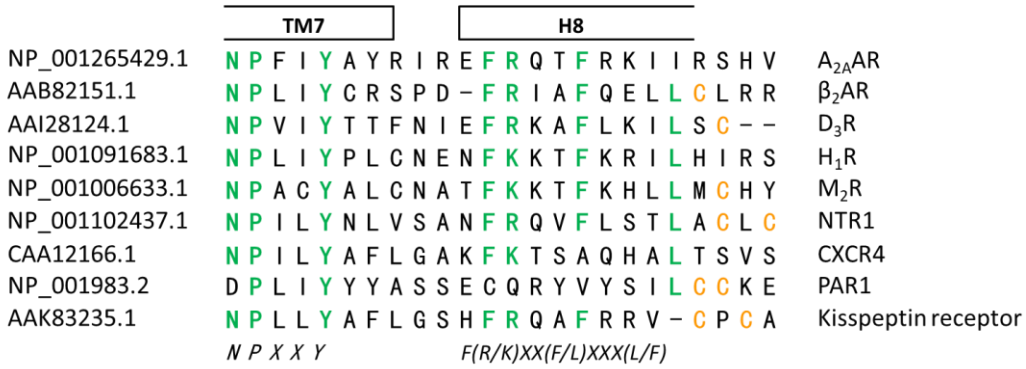


Figure 1.3 Sequence alignments among the end of TM7 and H8 region of GPCRs. The NPXXY and the F(R/K)XX(F/L)XXX(L/F) motifs are labelled in green and the putative palmitoylated Cys residues are highlighted in yellow. Accession number of GenBank or NCBI reference sequence of each receptor is given in the left. Figure is adapted from Egloff *et al*., 2014.

Pasquuier and colleagues predicted some residues of the human kisspeptin receptor that are involved in the formation of the ligand-binding pocket by the molecular modelling (Pasquier et al., 2014). They are Gln^{122}, Gln^{123}, Val^{126} and Gln^{127} within TM3, Tyr^{190}, Cys^{191} and Glu^{193} within ECL2, Asn^{208} and Tyr^{213} within TM5 ($Asn^{5.42}$ and $Tyr^{5.47}$ according to Ballesteros-Weinstein numbering), Leu^{271}, Trp^{276}, Ile^{279} and Gln^{280} within TM6 ($Leu^{6.43}$, $Trp^{6.48}$, $Ile^{6.51}$ and $Gln^{6.52}$ according to Ballesteros-Weinstein numbering) and His^{309} and Tyr^{313} within TM7 ($His^{7.39}$ and $Tyr^{7.43}$ according to Ballesteros-Weinstein numbering). In addition, some natural occurring mutations of the human kisspeptin receptor, including Leu^{102} in TM2 ($Leu^{2.64}$ according to Ballesteros-Weinstein numbering) (de Roux et al., 2003), Leu^{148} in ICL2 (Seminara et al., 2003) Cys^{223} in TM5 ($Cys^{5.57}$ according to Ballesteros-Weinstein numbering) and Arg^{297} in ECL3 (Semple et al., 2005), have been reported in the patients with idiopathic hypogonadotropic hypogonadism (IHH), which is defined by the failure to initiate puberty or display a severe delayed puberty. The mutations result in a decrease in the activity of the human kisspeptin receptor, indicating these residues

could be essential for mediating folding, trafficking and expression of the receptor and/or the activation of the receptor. Nevertheless, high-resolution structures of the kisspeptin receptor remain to be demonstrated for the understanding of the activation and activity of the receptor.

1.2.2 Post-translational modifications of the kisspeptin receptor

Several post-translational modifications, such as glycosylation (Soto and Trejo, 2010, Min et al., 2015), ubiquitination (Alonso and Friedman, 2013), phosphorylation (Butcher et al., 2012) and palmitoylation (Qanbar and Bouvier, 2003), occur on GPCRs to regulate the activities of receptors by modulating their expression, trafficking, structure and function. Upon activation by agonists, GPCRs are phosphorylated by GPCR kinases (GRKs), which specifically phosphorylate Ser and Thr residues within the ICLs and C-terminal tail of receptors. The phosphorylation of a GPCR promotes the recruitment of β-arrestins and, consequently, activates desensitization of the receptor (Ferguson et al., 1996). β-arrestins can also target GPCRs for internalization in clathrin-coated pits *via* directly interact with clathrin (Goodman et al., 1996) and the β2-adaptin subunit of the heterotetrameric complex of adaptor protein 2 (Laporte et al., 2000). Like other GPCRs, the kisspeptin receptor is able to be phosphorylated by GRKs, which is mediated by a direct interaction with GRK2 through its sequences in the ICL2 and C-terminal tail. The interaction of GRK2 with the kisspeptin receptor mediates the desensitization of the receptor, as transfection of human embryonic kidney 293 (HEK293) cells, which express exogenous the kisspeptin receptor, with GRK2 results in a right-shift of the dose-response curve of KP-10-induced turnover of inositol phosphates (IPs) (Pampillo et

al., 2009). Following the phosphorylation of kisspeptin-bound kisspeptin receptor by GRKs, β-arrestin-1 and β-arrestin-2 can be recruited to the receptor (Pampillo et al., 2009). The interaction of β-arrestins with the kisspeptin receptor is proposed to colocalize the receptor to clathrin-coated pits, which initiate the internalization of the receptor.

The statuses of other post-translational modifications on the kisspeptin receptor remain unclear. One possible post-translational modification on the human kisspeptin receptor is N-glycosylation, which attaches complex oligosaccharides to GPCRs during their journey from the endoplasmic reticulum to the Golgi apparatus (Duvernay et al., 2005, Norskov-Lauritsen and Brauner-Osborne, 2015, Zhang and Kim, 2017). The human kisspeptin receptor has three putative sites of N-glycosylation (Asn^{10}, Asn^{18} and Asn^{28}) in its N-terminus (Section 1.2.1) (Muir et al., 2001, Clements et al., 2001). They might be involved in the regulation of functions of the kisspeptin receptor, since N-glycosylation has been reported to play important and diverse roles in the regulation of activities of GPCRs, including regulations of cell surface expression, ligand binding, desensitization and endocytosis of receptors (Zhang and Kim, 2017). Another potential post-translational modification that may occur on the human kisspeptin receptor is *S*-palmitoylation, which attaches palmitate to the Cys residues within the ICLs and C-terminal tails of GPCRs *via* a reversible thioester linkage. Cys residues that locate adjacent to the end of H8 region of many GPCRs, such as β_2AR (Adachi et al., 2016), dopamine D_3 receptor (Zhang et al., 2016), PAR1 (Dowal et al., 2011) (Fig. 1.3), dopamine D_2 receptor (D_2R) (Sensoy and Weinstein, 2015) and CB_1 cannabinoid receptor (Oddi et al., 2012), are major sites of *S*-

palmitoylation. *S*-palmitoylation on these sites is thought to assist the association of H8 with the inner surface of the cell membrane (Qanbar and Bouvier, 2003, Escriba et al., 2007). Indeed, recent *in silico* analysis of CB_1 cannabinoid receptor find that palmitoylation of the Cys residue could stabilize the H8 structure (Oddi et al., 2017). In addition, *in silico* analysis of D_2R reveals that palmitoylation on the Cys residues adjacent to the end of H8 increases the depth of the insertion of H8 into plasma membrane, indicating that palmitoylation may regulate GPCR signalling by reducing the accessibility of H8 to the aqueous environment (Sensoy and Weinstein, 2015). The human kisspeptin receptor contains a putative H8 region, which ends with two Cys residues (Cys^{338} and Cys^{340}) (Fig. 1.3). Hence, Cys^{338} and Cys^{340} are highly possible to be palmitoylated. In support of the note, Cys^{340} is predicted as a potential site for *S*-palmitoylation by the NBA-Palm (Xue et al., 2006) and CSS-Palm 4.0 program (Ren et al., 2008). Functionally, since *S*-palmitoylation is a reversible modification, the turnover of *S*-palmitoylation is widely hypothesized to be involved in the signalling, trafficking and stability of GPCRs (Goddard and Watts, 2012, Norskov-Lauritsen and Brauner-Osborne, 2015, Zhang and Kim, 2017). It has been found that substitutions of palmitoylated Cys residues with Ala lead to a reduction in the surface expression of CC chemokine receptor 5 (CCR5) and decrease the half-life of the receptor (Percherancier et al., 2001). Moreover, Ala substitutions on the palmitoylation sites of μ-opioid receptor (μ-OR) result in a decrease in cholesterol association and attenuation of the signalling of the receptor *via* disrupting receptor homodimerization and G protein coupling (Zheng et al., 2012). Thus, investigations in the statuses of palmitoylation on the kisspeptin receptor may provide new understanding of its structure-functional relationships.

1.2.3 Ligands of the kisspeptin receptor

1.2.3.1 Kisspeptins

Although the closest homologue of the kisspeptin receptor is galanin receptors (Fig. 1.1), galanin is unable to bind and activate the kisspeptin receptor (Lee et al., 1999). Until 2001, three surrogate agonistic peptides of the kisspeptin receptor were firstly isolated from the human placental extracts and termed either kisspeptins (13-, 14- and 54-amino-acid peptides) (Kotani et al., 2001) or metastin (54-amino-acid peptide) (Ohtaki et al., 2001). Kisspeptins, which are used by the NC-IUPHAR nomenclature system, are encoded by *kiss1* gene (Ohtaki et al., 2001). So far, only a single *kiss1* gene is identified in eutherian mammals without any alternative splicing product (Pasquier et al., 2014). In humans, the *kiss1* gene consists of three exons and two introns and its spliced mRNA product can be translated into a 145-amino-acid precursor of kisspeptins (Fig. 1.4). The polypeptide precursor is subsequently processed to several size-varied and biologically functional kisspeptins, including 54-, 14-, 13- and 10-amino-acid peptides named KP-54, KP-14, KP-13 and KP-10.

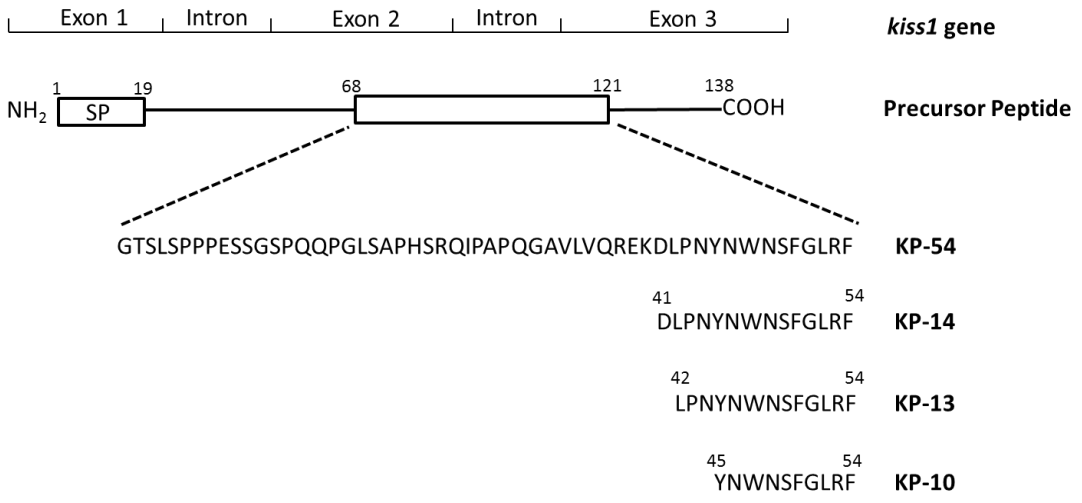


Figure 1.4 The diagrams of human *kiss1* gene and its encoded bioactive peptides. The *kiss1* gene encodes a 145-amino-acid precursor peptide. The cleavage of the precursor leads to the formation of KP-54 and the further truncation results in the generation of KP-14, KP-13 and KP-10. The protein sequences of each product are shown. SP stands for signal peptide, which is the sequence targeting the protein to the secretory pathway.

All bioactive kisspeptin isoforms (KP-54, KP-13, KP-14 and KP-10) share the last ten amino acids (45-54) at the C-terminus and possess similar potency (Ohtaki et al., 2001). The last ten amino acids are highly conserved among all vertebrate species with the exception of Trp^{47} and Ser^{49}, suggesting their important roles in the functions of kisspeptins (Pasquier et al., 2014). Indeed, systematic substitutions of each amino acid of human KP-10 with Ala (Ala scan) lead to decreased agonistic activity, with the exception of Trp^{47} and Ser^{49} (Niida et al., 2006, Orsini et al., 2007). The most dramatic decreases in the activity of KP-10 are observed in analogues with Ala substitutions of Phe^{50}, Leu^{52} and Phe^{54}. Asn^{48} and amino acids from Phe^{50} to Phe^{54} are stereochemically important, because substitutions of each residue with their D-enantiomers, which alter the conformation of the peptides, result in complete loss of the kisspeptin receptor-agonistic activity (Niida et al., 2006). In contrast, Trp^{47} and Ser^{49}, which are less conserved among vertebrates, are relatively tolerant for D-amino acid substitutions (Niida et al., 2006). In addition, substitutions of each amino acid of human KP-10 with Ala also result in reductions in the binding to the kisspeptin receptor, which positively correlate with the kisspeptin receptor-agonistic activity (Orsini et al., 2007). Moreover, the aromatic side chains of two Phe residues in the C-terminal tail (Phe^{50} and Phe^{54}) of kisspeptins are essential for the binding and activation of the kisspeptin receptor. In supports, substitutions of Phe^{50} or Phe^{54} with Tyr, Trp, cyclohexylamine and 3-pyridinylalanine, which mimic the

aromatic side chain of Phe, have little effects on receptor binding and activation (Orsini et al., 2007). By contrast, Ala substitutions of Phe^{50} or Phe^{54} result in decreases in the binding to the kisspeptin receptor and a dual Ala substitution on Phe^{50} and Phe^{54} abolished the binding and activation of the receptor.

It remains unclear that how kisspeptin binds to its receptor. Kisspeptins have been proposed to bind to the kisspeptin receptor *via* a two-step ligand transportation model, in which they initially bind to the lipid membrane and then laterally diffuse to the receptor (Lee et al., 2009). In supports, a close correlation between the binding of kisspeptins to the lipid membrane and its binding to the kisspeptin receptor is observed. For instance, human KP-10 is able to bind to 1-palmitoyl-2-oleoyl-phosphatidylchline (POPC) liposomes, which mimic the environment of the lipid membrane. The binding of human KP-10 to POPC liposomes is abolished by a substitution of Phe^{50} or Phe^{54} with Ala (Lee et al., 2009), which exhibits impaired binding ability to the kisspeptin receptor (Orsini et al., 2007). Therefore, some three-dimensional (3D) structures of kisspeptins in membrane-mimetic environments have been revealed by nuclear magnetic resonance (NMR) studies (Orsini et al., 2007, Lee et al., 2009). In a solution of sodium dodecyl sulfate (SDS) micelles, the residues from Asn^{48} to Phe^{54} within the C-terminus of human KP-13 form a helical conformation, where Phe^{50}, Phe^{54} and Arg^{53} lie on the same face of the helix (Orsini et al., 2007). The helical structure of human KP-13 is essential for its binding to the kisspeptin receptor, as single substitutions on either Gly^{51} or Leu^{52} with a helix-breaking Pro residue greatly decreases receptor binding and agonistic activity. However, instead of the helical structure, in the presence of dodecylphosphocholine (DPC) micelles, a

human KP-10 analogue with a substitution of Ser^{49} to Ala folds into several tight turn structures, encompassing the residues Trp^{47} to Phe^{54} (Lee et al., 2009). Additionally, the C-terminal region of the analogue forms a hydrophobic cluster composed of Phe^{50}, Phe^{54} and the aliphatic side-chain of Leu^{52}, which may interact with the hydrophobic carbon chains of the lipid membrane. The discrepancy between two NMR structures may be caused by different detergents used for mimicking the lipid membrane. SDS micelles provide a negatively charged surface, while DPC micelles form a neutral zwitterionic surface. To understand how the kisspeptin receptor binding with kisspeptins, the accurate 3D structures of kisspeptins remain to be solved in the further.

1.2.3.2 Synthetic ligands for the kisspeptin receptor

Kisspeptins are metabolically unstable in serum. It has been shown that more than 50% of human KP-10 can be metabolized in mouse plasma after one-minute incubation at 37^{o}C (Asami et al., 2012a). The incubation produces more than ten metabolites and the main fragments are produced by the digestion between Tyr^{45}-Asn^{46} and Arg^{53}-Phe^{54} *via* aminopeptidase and trypsin-like proteases. Moreover, kisspeptins have also been shown to be cleaved between Gly^{51}-Leu^{52} by matrix metalloproteinases (MMPs), including membrane-type 1-MMP, membrane-type 3-MMP, membrane-type 5-MMP, MMP-2 and MMP-9 (Takino et al., 2003). The cleavage leads to inactivation of all bioactive kisspeptins. Therefore, several studies have attempted to design and synthesize more potent and stable kisspeptin analogues by downsizing (Niida et al., 2006) and/or by substituting susceptible residues (Asami et al., 2012a, Asami et al., 2012b, Asami et al., 2013, Tomita et al.,

2008). For example, a substitution of Arg^{53} of KP-10, which is a target site of the digestion mediated by trypsin, with guanidino-*N* (N^{ω})-methyl Arg (Arg(Me)) enables the analogue to be resistant to trypsin-mediated hydrolysis (Asami et al., 2012a). The Arg(Me) analogue exhibits improved stability in serum and increased potency compared with KP-10. However, the peptide is still susceptible to other enzymes in mouse serum. A further study on the Arg(Me) analogue by substituting Tyr^{45}, Trp^{47} and Gly^{51} residues resulted in several more stable variants (Asami et al., 2012b). Among them, a peptide with substitutions of Tyr^{45}, Trp^{47} and Gly^{51} with D-Tyr, D-Trp, azaglycine, in which α-carbon is replaced by a nitrogen atom, and Arg(Me) respectively shows high metabolic stability with similar agonistic activity and binding affinity compared with that of KP-10.

In addition to the development of agonists of the kisspeptin receptor, several studies have focused on the design and synthesis of antagonists of the receptor to develop therapeutic drugs and assist in the elucidation of the physiological roles of kisspeptins and the kisspeptin receptor. A peptide antagonist, ac[(D)-Ala]-Asn-Trp-Asn-Gly-Phe-Gly-[(D)-Trp]-Arg-Phe-NH_2 (peptide 234), is developed by systematic substitutions of amino acids of KP-10 (Roseweir et al., 2009). The peptide potently inhibits KP-10-induced formation of IP in Chinese hamster ovary (CHO) cells, which stably express the human kisspeptin receptor, with an IC_{50} value of 7.0 nM and exhibits a similar binding affinity to the kisspeptin receptor as that of KP-10. In addition, the antagonistic activity of peptide 234 is also observed *in vivo* by directly inhibiting luteinizing hormone (LH) secretion in male mice and blocking KP-10-induced gonadotropin-releasing hormone (GnRH) neuron firing in female mice brain

slices. Besides, some small molecules, including a series of 2-acylamino-4,6-diohenylpyridines derivatives, have been reported to possess the kisspeptin receptor-antagonistic activity (Kobayashi et al., 2010a, Kobayashi et al., 2010b).

1.3 Physiological and pathophysiological roles of the kisspeptin receptor

A broader expression profile of mRNA of the kisspeptin receptor and kisspeptin has been identified in human tissues (Fig. 1.5) and high expression level is observed in human placenta, pancreas (Ohtaki et al., 2001) and pituitary (Kotani et al., 2001). The broader expression profile of the kisspeptin receptor and kisspeptin implies their potential diverse and complex physiological functions. In the following parts, functions of kisspeptins and the kisspeptin receptor in the regulation of puberty development and cancer metastasis are discussed.

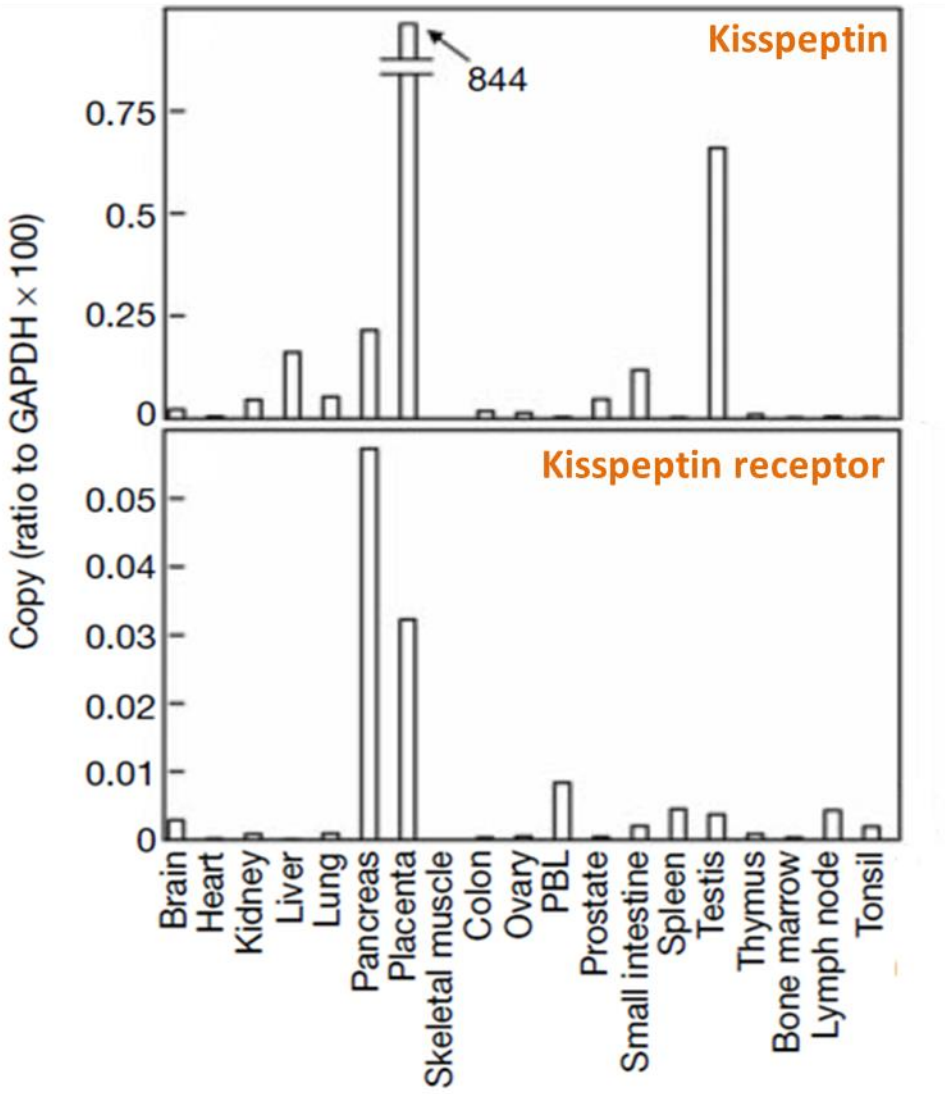


Figure 1.5 The expression profile of mRNA of kisspeptin and the kisspeptin receptor in human tissues. PBL is short for peripheral blood leukocyte. Figure is adapted from Ohtaki *et al*., 2001.

1.3.1 Roles of the kisspeptin receptor in the development of puberty

Several natural occurring mutations (Fig. 1.2) on human *kiss1r* gene are identified in the patients with IHH (Semple et al., 2005, Seminara et al., 2003, de Roux et al., 2003). Linkage analysis of the patients reveals a homozygous T-to-C transition resulting in a substitution of Leu^{148} to Ser (L148S), a heterozygous C-to-T transition leading to replacement of Arg^{331} with a premature stop codon (R331X) and a

heterozygous T-to-A transversion substituting the physiologic stop codon with Arg (X399R) (Seminara et al., 2003). Overall, these mutations result in a reduction of KP-10-induced IP response when expressed in COS-7 cells. More detailed analyses reveals that the L148S mutant impairs $G_{q/11}$-mediated signalling by decreasing the capability of the mutant receptor to catalyze the dissociation of the Gα subunit from the Gβγ complex (Wacker et al., 2008). In addition, a deletion of 155 base pairs encompassing intron 4 and exon 5 of *kiss1r* gene and a homozygous T-to-C transition replacing Leu^{102} with Pro (L102P) are identified in the patients with IHH (de Roux et al., 2003). The 155-base pair deletion of *kiss1r* gene results in a truncation of the receptor at Gly^{247} within the ICL3 and leads to a loss of function (de Roux et al., 2003), whereas the L102P mutant completely abolishes KP-10-induced turnover of IP when expressed in HEK293 cells (Tenenbaum-Rakover et al., 2007). Furthermore, two missense mutations, the substitution of Cys^{223} with Arg (C223R) and the replacement of Arg^{297} with Leu (R297L) (Semple et al., 2005), and a heterozygous insertion of one Pro-Arg-Arg repeat in the Pro-Arg-rich region of the C-terminal tail after residue 342 (Chevrier et al., 2013) are observed in the patients with IHH. The C223R mutant profoundly impairs KP-10-induced calcium flux, while the R297L mutant only has a mild effect on the function of the receptor when expressed in Flp-In^{TM}-293 cells. The insertion of a Pro-Arg-Arg causes a decrease in the maximal KP-10-stimulated accumulation of IP and phosphorylation of extracellular signal-regulated kinase 1/2 (ERK1/2), and a reduction in the cell surface expression without affecting total expression of the receptor. Besides, a gain-of-function mutation of the kisspeptin receptor, where the Arg^{386} is substituted by Pro (R386P), is observed in a patient with idiopathic central precocious puberty (Teles et al., 2008). The R386P mutant results

in prolonged receptor signalling by decreasing agonist-induced receptor desensitization. Altogether, attenuations or prolongations of the activity of the kisspeptin receptor lead to dysregulations of puberty, indicating a critical role of the kisspeptins and the kisspeptin receptor in the regulation of human reproductive axis.

Indeed, kisspeptins are able to stimulate both LH and follicle-stimulating hormone (FSH) release in a number of species, including humans (Dhillo et al., 2007, Dhillo et al., 2005), mice (Messager et al., 2005, Han et al., 2005) and rats (Irwig et al., 2004). The kisspeptin-induced release of LH is inhibited by the presence of a GnRH antagonist, acyline, suggesting that GnRH might be a mediator of kisspeptin-induced release of gonadotropin (Irwig et al., 2004). Consistently, administration of kisspeptin into the cerebrospinal fluid of sheep results in an abrupt increase in the secretion of GnRH and LH at the same time (Messager et al., 2005). Since the expression of the kisspeptin receptor is detected in GnRH neurons in both mRNA (Irwig et al., 2004, Han et al., 2005) and protein level (Messager et al., 2005), kisspeptin could activate the receptor located on GnRH neurons leading to the secretion of GnRH, and, consequently, result in the release of LH and FSH.

1.3.2 Roles of the kisspeptin receptor in the development of cancers

Since *kiss1* gene is identified as a metastasis suppresser gene (Lee et al., 1996), kisspeptins and the kisspeptin receptor have been also proposed to suppress cancer metastasis. In the supports of the note, kisspeptins and the kisspeptin receptor have been reported to suppress the metastasis of a variety of cancers, including melanoma (Ohtaki et al., 2001), breast (Tan et al., 2014, Cho et al., 2009a, Lee and

Welch, 1997), endometrial (Kang et al., 2011), thyroid (Stathatos et al., 2005), pancreatic (Masui et al., 2004), gastric (Li et al., 2012), renal (Yoshioka et al., 2008), bladder (Takeda et al., 2012) and ovarian (Jiang et al., 2005) cancers. However, mechanisms underlying the regulation of migration and invasion of cancer cells *via* kisspeptins and the kisspeptin receptor remain largely unknown. One potential mechanism for the inhibition of cancer metastasis by kisspeptins may involve the downregulation of MMPs, which play critical roles in the degradation of extracellular matrix. For example, a negative correlation between the level of expression of endogenous kisspeptin and MMP-9 is observed in hepatocellular carcinoma tissue slides, which were collected from human patients (Shengbing et al., 2009). Consistently, the transfection of BGC-823 cells, which is a gastric carcinoma cell line, with plasmids containing *kiss1* gene, leads to an increase in the expression of kisspeptin and a decrease in the expression of MMP-9 at both mRNA and protein levels (Li et al., 2012). The kisspeptin-induced downregulation of MMP-9 expression is also observed in HT-1080 cells (Yan et al., 2001), MBT-2V cells (Takeda et al., 2012) and HTC-116 cells (Chen et al., 2016) that belong to human fibrosarcoma, murine bladder and human colorectal cancer cells respectively. Further studies reveal that the downregulation of kisspeptin on the expression of MMP-9 may be mediated by nuclear factor kappa B (NF-κB). It has been demonstrated that the interaction of NF-κB with MMP-9 promoter in the nucleus is reduced in HT-1080 cells transfected with constructs containing *kiss1* gene, compared with that in untransfected cells (Yan et al., 2001). The decreased interaction, in part, results from the reduced nuclear translocation of NF-κB mediated by IκB. Similarly, a decreased nuclear translocation and increased cytoplasmic expression of NF-κB, which leads to reduced DNA-binding

activity of NF-κB, is observed in MBT-2V cells upon KP-54 stimulation (Takeda et al., 2012).

However, kisspeptins and the kisspeptin receptor have been recently demonstrated to promote invasiveness of some human breast cancer cells, including MDA-MB-231, Hs578T (Zajac et al., 2011) and SKBR3 (Cvetkovic et al., 2013). KP-10 is able to promote motility and invasion of MDA-MB-231 and Hs578T (Zajac et al., 2011) cells endogenously express the kisspeptin receptor, as well as SKBR3 cells transfected with the kisspeptin receptor (Cvetkovic et al., 2013). One common feature shared by these breast cancer cells is that they do not express endogenous estrogen receptor α (ERα). KP-10 does not enhance migration and invasion of ERα-positive breast cancer cells, including T47D (Zajac et al., 2011, Cvetkovic et al., 2013) and Michigan Cancer Foundation-7 (MCF-7) cells, which also express endogenous the kisspeptin receptor (Cvetkovic et al., 2013). Consistently, a treatment of ERα-negative MDA-MB-231 cells with kisspeptins secreted from human placental explants in the culture medium (placental kisspeptins) promotes their invasion, while a treatment of ERα-positive MCF-7 cells with placental kisspeptins does not altered their invasion (Rasoulzadeh et al., 2016). Therefore, ERα might be involved in the differential modulation of cancer metastasis by kisspeptins and the kisspeptin receptor. Indeed, KP-10 is unable to enhance invasion of the MDA-MB-231 cells, when they are transfected with ERα (Cvetkovic et al., 2013). One potential mechanism underlying kisspeptin-induced metastasis of cancer may involve the transactivation of epidermal growth factor receptor (EGFR) *via* the kisspeptin receptor. KP-10 induces the phosphorylation of EGFR in MDA-MB-231 and Hs578T (Zajac et al., 2011) cells, which endogenously

express the kisspeptin receptor, and SKBR3 cells transfected with the kisspeptin receptor (Cvetkovic et al., 2013). Inhibition of EGFR signalling by using a selective receptor tyrosine kinase inhibitor, AG-1478, abolishes KP-10-stimulated invasion of MDA-MB-231 cells (Zajac et al., 2011), suggesting that KP-10-activated the kisspeptin receptor can transactivate EGFR to promote cell invasion. However, KP-10 cannot stimulate the phosphorylation of EGFR in ERα-positive breast cancer cells, such as MCF-7 and T47D cells (Cvetkovic et al., 2013). Therefore, ERα may modulate the kisspeptin receptor signalling to EGFR and then regulate cancer metastasis. A better understanding of the mechanisms underlying the kisspeptin receptor signalling is required to evaluate its potential as a therapeutic target in the treatment of cancer metastasis.

1.4 The signalling of the kisspeptin receptor

The intracellular region of a GPCR mediates interactions of the receptor with GPCR-interacting proteins (GIPs) containing the cognate G proteins or non-G protein interacting proteins. The interactions of GPCRs with GIPs mediate a variety of intracellular responses. In the following sections, the signalling pathways activated by kisspeptins and the kisspeptin receptor are presented and are summarised in Fig. 1.6.

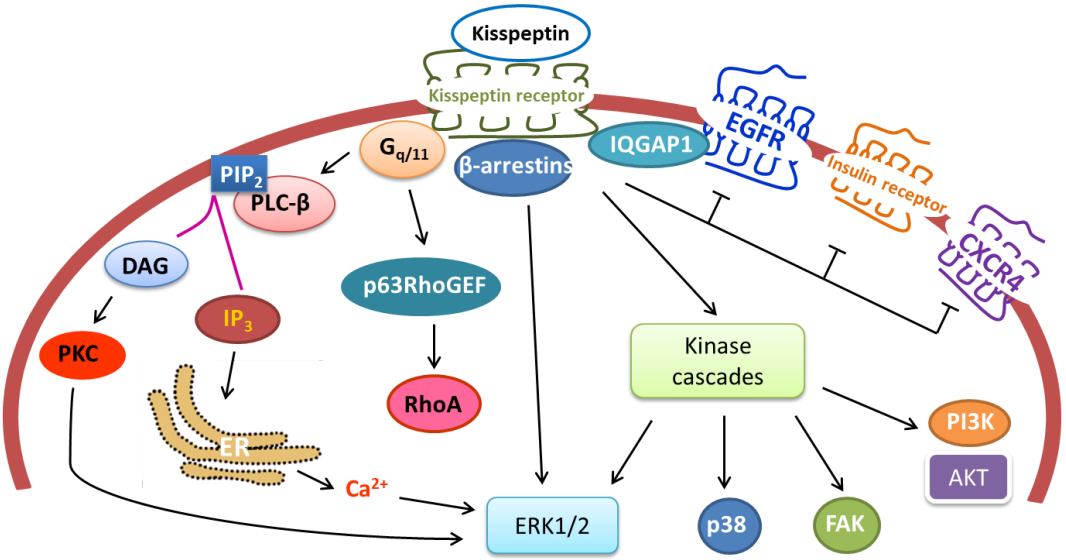


Figure 1.6 The main signalling pathways stimulated by kisspeptins and the kisspeptin receptor. The figure illustrates the signalling pathways that have been reported to be activated by the kisspeptin receptor upon the binding of kisspeptins.

1.4.1 GPCR-mediated G protein activation and G protein-dependent signalling

Canonical GPCR signalling is mediated by their cognate heterotrimeric G proteins. Heterotrimeric G proteins couple to the intracellular region of activated GPCRs and are composed of Gα, Gβ and Gγ subunits. The Gα subunit binds to GDP in the inactive status and binds to GTP, when it is activated. The Gβ and Gγ subunits always form a stable heterodimeric complex and act as a functional unit. In the basal state, the GDP-bound Gα subunit binds tightly to the Gβγ dimer. Upon the agonist-induced activation of GPCRs, conformational changes of the receptor promote the recruitment of G proteins and catalyse the exchange of GDP with GTP in the Gα subunit. The binding of GTP leads to a further conformational change of the Gα subunit and also results in the dissociation of the active Gα·GTP from the Gβγ complex and receptors. Both Gα·GTP and Gβγ dimer are able to activate downstream effector proteins and subsequently initiate diverse intracellular signalling pathways. The duration of the activated status of Gα·GTP is determined by the intrinsic GTPase

activity within the Gα subunit and is accelerated by the GTPase activating proteins (GAPs). The hydrolysis of GTP to GDP inactivates the Gα subunit and allows it to reassociate with Gβγ subunit (Fig. 1.7) (Lambert, 2008, Zhang et al., 2015b).

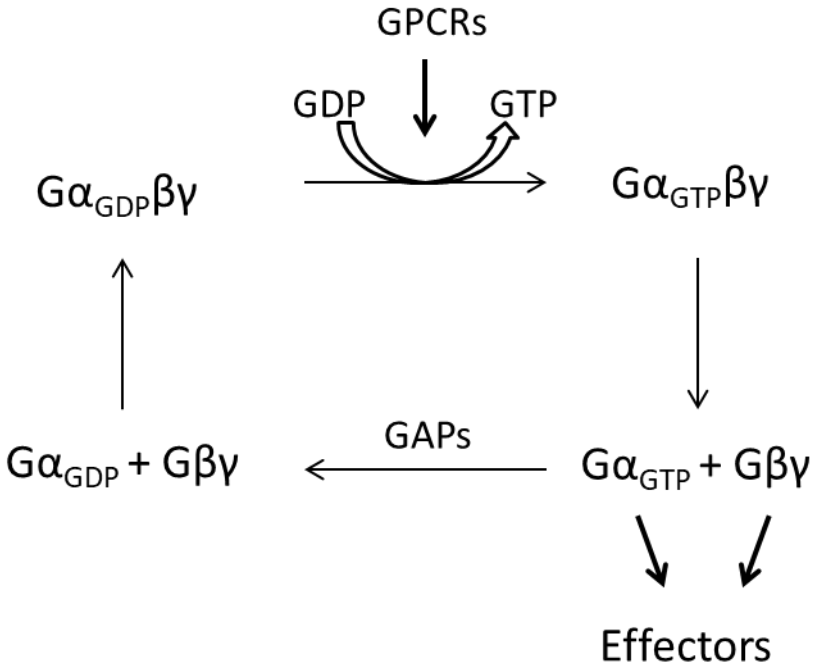


Figure 1.7 Overview of GPCR-mediated activation of G protein. In the inactivate status, the GDP-bound Gα subunit associates with the Gβγ dimer. The activation of a GPCR induces the exchange of GDP to GTP in the Gα subunit and subsequently results in a dissociation of GTP-bound Gα subunit from Gβγ. Both GTP-bound Gα and Gβγ can transduce downstream signalling *via* interaction with various effectors. The hydrolysis of GTP to GDP through the intrinsic GTPase activity of the Gα subunit with helps from GAPs terminates the Gα signalling and leads to reassociation to the Gβγ complex. Figure is adapted from Lambert, 2008.

In mammals, there are 16 different α subunits, 5 distinct β ($G\beta_{1\text{-}5}$) subunits and 12 different Gγ ($G\gamma_{1\text{-}5,\ 7\text{-}13}$) subunits (Downes and Gautam, 1999). The α subunits can be assigned into four main classes based on the similarity of their protein sequences (denoted G_x with x referring to the specific class) and each class can be further divided into specific subtypes (denoted $G\alpha_x$ with x referring to the subtypes): G_s ($G\alpha_s$

and $G\alpha_{olf}$), $G_{i/o}$ ($G\alpha_{i1\text{-}i3}$, $G\alpha_{o}$, $G\alpha_{z}$, $G\alpha_{t1\text{-}t2}$ and $G\alpha_{gust}$), $G_{q/11}$ ($G\alpha_{q}$, $G\alpha_{11}$, $G\alpha_{14}$, and $G\alpha_{15/16}$) and $G_{12/13}$ ($G\alpha_{12}$ and $G\alpha_{13}$) (Simon et al., 1991, Downes and Gautam, 1999). Following ligands binding, GPCRs couple to one or more G protein families to mediate cellular responses.

The kisspeptin receptor is a $G_{q/11}$-coupled receptor and can, therefore, activate $G_{q/11}$-dependent signalling pathways. The canonical effectors of $G_{q/11}$ family members are phospholipase C-βs (PLC-βs). The activated Gα subunit of $G_{q/11}$ binds and activates all PLC-β isoforms (PLC-$\beta_{1\text{-}4}$) (Lee et al., 1994, Runnels and Scarlata, 1999, Jiang et al., 1994, Lee et al., 1992). Activated PLC-βs then catalyse the hydrolysis of phosphatidylinositol 4,5-bisphosphate (PIP_2) to inositol 1,4,5-trisphosphate (IP_3) and diacylglycerol (DAG) (Rhee, 2001), which in turns mobilize calcium from intracellular stores and activate protein kinase C (PKC) (Berridge, 1987). *In vitro*, KP-10 can stimulate intracellular calcium mobilization and phosphatidylinositol turnover in CHO-K1 (Kotani et al., 2001), B16-BL6 mouse melanomas (Ohtaki et al., 2001) and HEK293 cells (Muir et al., 2001) transfected with the human kisspeptin receptor. The KP-10-induced calcium mobilization is unable to be altered by the treatment with pertussis toxin, which prevents the binding of $G_{i/o}$ to the receptor by ADP-ribosylation, indicating that the activation of PLC-β is mediated by $G_{q/11}$ but not $G_{i/o}$ family (Muir et al., 2001, Kotani et al., 2001). The kisspeptin receptor does not couple strongly to $G_{i/o}$ and G_s subfamilies, since KP-10 cannot elicit accumulation of cyclic adenosine 3',5'-monophosphate (cAMP), which is mediated by G_s subfamily, and inhibit the formation of cAMP, which is regulated by $G_{i/o}$ subfamily (Muir et al., 2001, Kotani et al., 2001). In physiological contexts, kisspeptins and the kisspeptin receptor

can activate the secretion of GnRH and pituitary responses *via* $G_{q/11}$/PLC- β-mediated signalling pathways. Inhibition of PLC by a PLC inhibitor (U-73122) or depletion of intracellular calcium stores by thapsigargin inhibits the KP-10-induced secretion of GnRH in rat hypothalamic explants (Castellano et al., 2006). Consistently, pretreatment of cultured hypothalamic slices from transgenic mice, which express a rat GnRH promoter-driven luciferase reporter, with a PKC inhibitor (Go-6983) inhibits kisspeptin-induced secretion of GnRH and luciferase expression (Choe et al., 2013), suggesting that $G_{q/11}$/PLC- β might mediate kisspeptin-induced secretion of GnRH by regulating the transcription of gene encoding GnRH. In addition, the inhibition of PLC by U-73122, the depletion of intracellular calcium stores by thapsigargin or the inhibition of PKC by Go-6983 can also attenuate KP-10-induced secretion of LH and GH in baboon pituitary cells (Luque et al., 2011)

In addition to $G_{q/11}$/PLC-β-mediated signalling, kisspeptins and the kisspeptin receptor can activate some other effectors of $G_{q/11}$ family. For example, KP-10 can stimulate the phosphorylation of ERK1/2 *via* $G_{q/11}$ subfamily. The KP-10-induced phosphorylation of ERK1/2 is abolished in $G_{q/11}$ knockout mouse embryonic fibroblast (MEF) cells expressing the kisspeptin receptor, which are derived from mice with $G_{q/11}$ gene deletions or disruptions (Szereszewski et al., 2010). The KP-10-induced phosphorylation of ERK1/2 is important for the secretion of hormones, since inhibition of ERK1/2 by PD-98059 abolishes the KP-10-induced secretion of GnRH in rat hypothalamic explants (Castellano et al., 2006) and release of LH in baboon pituitary cells (Luque et al., 2011). In addition, the KP-10-induced phosphorylation of ERK1/2 is also involved in the regulation of cell apoptosis, as inhibition of ERK1/2 by

using an inhibitor (U0126) increases the survival of Jurkat cells after KP-10 treatment (Navenot et al., 2009). In addition to ERK1/2, RhoA and Rho guanine nucleotide exchange factor 25 (p63RhoGEF) could also be activated by kisspeptins and the kisspeptin receptor *via* $G_{q/11}$. KP-10 activates RhoA and promotes its downstream serum response factor (SRF)-dependent transcriptional activity in HEK293 cells expressing the human kisspeptin receptor (Cho et al., 2011). The RhoA-mediated activation of SRF-mediated transcription is regulated by $G_{q/11}$/p63RhoGEF signalling pathway, since it is attenuated by transfections of HEK293 cells with a dominant negative mutant of $G\alpha_q$ or a catalytic domain-deleted mutant of p63RhoGEF. It has been reported that $G_{q/11}$ could activate RhoA *via* p63RhoGEF (Lutz et al., 2007, Lutz et al., 2005). Therefore, the kisspeptin receptor could activate RhoA through $G_{q/11}$/p63RhoGEF/RhoA signalling pathway. The $G_{q/11}$/p63RhoGEF/RhoA signalling pathway has been proposed to be involved in the regulation of metastasis of breast tumours. In primary breast tumour cells, which are isolated from mouse mammary tumour virus (MMTV)- polyoma virus middle T antigen (PyMT)/*kiss1r*$^{+/+}$ female mice, knockdown of the expression of the kisspeptin receptor by using short hairpin RNA (shRNA) decreases the formation of GTP-bound RhoA. Moreover, transfection of the primary tumour cells with dominant negative RhoA reduces the anchorage-independent growth of the cells (Cho et al., 2011). Similar results are also observed in transformed MCF-10A cells, which are human normal breast epithelial cells. Transfection of MCF-10A cells with constitutively active Ras results in the tumorigenesis and anchorage-independent growth. The Ras-induced anchorage-independent growth of MCF-10A cells is reduced by knockdown of the expression of the human kisspeptin receptor by using shRNA, inactivation of RhoA by using an

inhibitor of RhoA (Y27632) or transfecting the cells with inactive mutant of RhoA (Cho et al., 2011). Hence, in addition to the kisspeptin receptor-mediated transactivation of ERα/EGFR (Section 1.3.2), the kisspeptin receptor may be able to promote the metastasis of breast cancers *via* $G_{q/11}$/p63RhoGEF/RhoA signalling pathway.

1.4.2 G protein-independent signalling

In addition to G protein-mediated signalling pathways, increased numbers of studies show that non-G protein effectors can directly interact with GPCRs to mediate G protein-independent signalling pathways [reviewed in (Ritter and Hall, 2009)]. A variety of non-G protein effectors that can interact with GPCRs have been reported such as β-arrestins (Luttrell et al., 1999), spinophilin (Wang et al., 2004), calmodulin (Labasque et al., 2008), protein phosphatases (Evans et al., 2008) and SH2 domain-containing proteins (Bousquet et al., 2006) to diversify GPCR functions [reviewed in (Luttrell, 2008, Bockaert et al., 2010, Magalhaes et al., 2012)]. The kisspeptin receptor has been shown to interact with β-arrestins (Section 1.2.2) (Pampillo et al., 2009). Following KP-10 binding, the kisspeptin receptor can be phosphorylated by GRKs and then can recruit β-arrestin1 or β-arrestin2 to mediate the desensitization and internalization of the receptor (Pampillo et al., 2009). In addition, β-arrestins can also interact with other effectors, including mitogen-activated protein kinases (MAPKs) and SRC tyrosine kinases to phosphorylate downstream effectors (Luttrell et al., 1999), diacylglycerol kinases to induce degradation of DAG (Nelson et al., 2007), and Ral GDP dissociation stimulators to activate Ral-dependent cytoskeletal reorganization (Bhattacharya et al., 2002). Moreover, KP-10 is able to regulate the

phosphorylation of ERK1/2 through β-arrestins. Knockdown of the endogenous expression of β-arrestin2 in MDA-MB-231 cells by using shRNA suppresses the phosphorylation of ERK1/2 after the treatment with KP-10 (Pampillo et al., 2009). Consistently, a reduction in KP-10-induced phosphorylation of ERK1/2 is observed in β-arrestin1/2 double knockout MEF cells expressing the kisspeptin receptor, which are derived from mice with β-arrestin1/2 gene deletions or disruption (Szereszewski et al., 2010). However, β-arrestin1 and β-arrestin2 exhibit opposite effects on the kisspeptin receptor-dependent phosphorylation of ERK1/2. In β-arrestin1 single knockout MEF cells, the level of KP-10-induced phosphorylation of ERK1/2 is significantly higher than that of the wild type MEF cells. By contrast, KP-10-induced phosphorylation of ERK1/2 is abolished in β-arrestin2 single knockout MEF cells (Szereszewski et al., 2010). Thus, β-arrestin1 inhibits the kisspeptin receptor signalling to ERK1/2, while β-arrestin2 potentiates it.

Besides, the kisspeptin receptor has three Pro-Arg-Arg repeats within the C-terminal tail (Fig. 1.2). The Pro-rich region has been identified in the ICLs and the C-terminal tail of some GPCRs and modulates the signalling of receptors by mediating the interaction of the receptor with SH3-domain containing proteins. For example, several Pro-rich regions are identified within the ICL3 of dopamine D_4 receptor. The Pro-rich regions are able to mediate interactions of dopamine D_4 receptor with NcK, growth factor receptor bound protein 2, Abelson murine leukemia viral oncogene homolog 1 (ABL1), p85α and c-SRC (Oldenhof et al., 1998). The removal of the Pro-rich motifs abolishes dopamine D_4 receptor-mediated phosphorylation of ERK1/2 and reduces the ability of the receptor to inhibit forskolin-induced accumulation of

cAMP. Another example is P2Y purinergic receptor 2, which has two Pro-rich motifs at the C-terminal tail. Upon the activation by agonists, P2Y purinergic receptor 2 can interact with c-SRC *via* the Pro-rich motifs (Liu et al., 2004). The removal of the Pro-rich motifs inhibits the agonist-induced activation of c-SRC and proline-rich tyrosine kinase 2, and reduces the phosphorylation level of co-localized platelet-derived growth factor receptor and EGFR. The Pro-rich region within the C-terminal tail of the kisspeptin receptor has been shown to interact with protein phosphatase 2A (PP2A). A glutathione-S-transferase (GST) fusion protein containing the C-terminal tail of the kisspeptin receptor (GST-C-ter) pulls either PP2A-B or -C subunit down from the CHO cell lysates (Evans et al., 2008). The pull down of PP2A-B or -C subunit is abolished by deleting the Pro-rich region of the kisspeptin receptor (GST-C-terΔPR). The interaction between the kisspeptin receptor and PP2A may assist the subcellular localization of PP2A and, hence, promotes PP2A phosphatase activity, since the complex formed by PP2A-C subunit and GST-C-ter possesses the phosphatase activity. However, the interaction of the kisspeptin receptor with PP2A remains to be demonstrated in cellular studies.

1.4.3 Cross-talk of the kisspeptin receptor with other membrane receptors

The kisspeptin-activated kisspeptin receptor has been shown to crosstalk with other membrane receptors. For example, KP-10 stimulates the phosphorylation of EGFR in some ERα-negative breast cancer cells, such as MDA-MB-231, Hs578T (Zajac et al., 2011) and SKBR3 cells (Cvetkovic et al., 2013), to promote motility and invasion of the cells (Section 1.3.2). The kisspeptin receptor-mediated transactivation of EGFR is proposed to be mediated by IQ motif-containing GTPase-activating protein 1

(IQGAP1), which is an actin cytoskeletal binding protein and interacts with actin filaments (Cvetkovic et al., 2013). IQGAP1 has been shown to interact and be colocolized with both EGFR (McNulty et al., 2011) and the kisspeptin receptor (Cvetkovic et al., 2013). In addition, the kisspeptin receptor can also directly interact with EGFR and it is promoted by the treatment with KP-10 (Zajac et al., 2011). Therefore, the kisspeptin receptor could form a complex with IQGAP1 and EGFR. It has been shown that depletion of the expression of IQGAP1 by using shRNA in MDA-MB-231 cells abolishes KP-10-induced phosphorylation of EGFR. Since IQGAP1 can act as a scaffold to bind to ERK2 (Roy et al., 2004), B-Raf (Ren et al., 2007), MAPK kinase 1 and 2 (MEK1 and MEK2) (Roy et al., 2005) and modulates their functions, the kisspeptin receptor might recruit and activate the signalling cascade of MAPK *via* IQGAP1 to phosphorylate EGFR. Functionally, the KP-10-induced transactivation of EGFR might be involved in the regulation of activity of MMP-9 in ERα-negative breast cancer cells. Inhibition of EGFR by using an inhibitor (AG-1468) inhibits KP-10-stimulated activity of MMP-9 in MDA-MB-231 cells (Zajac et al., 2011).

On the other hand, the kisspeptin-induced activation of the kisspeptin receptor can downregulate the activities of CXCR4 (Navenot et al., 2005), insulin receptor and EGFR (Navenot et al., 2009) by suppressing their signalling. A prior treatment of CHO cells, which are co-transfected with the kisspeptin receptor and CXCR4, with KP-10 disables the stromal cell-derived factor 1 (SDF-1, also known as CXCL12)-induced calcium mobilization and phosphorylation of AKT (Navenot et al., 2005). Similarly, pretreatment of HEK293 cells, which are transfected with the kisspeptin receptor, with KP-10 inhibits EGF and insulin-induced phosphorylation of AKT (Navenot et al.,

2009). The precise mechanisms underlying the negative crosstalk of the kisspeptin receptor with CXCR4, EGFR and insulin receptor remain to be investigated. The different signalling of kisspeptins and the kisspeptin receptor to EGFR might be due to the distinct cell types used.

1.4.4 Other signalling pathways

Kisspeptins and the kisspeptin receptor are also able to mediate some kinase cascades, in which the precise signalling pathways remain to be elucidated. For example, KP-54 induces the phosphorylation of focal adhesion kinase (FAK) and paxillin, which play important roles in the formation of focal adhesions, in B16-BL6 mouse melanoma cells transfected with the kisspeptin receptor (Ohtaki et al., 2001). In addition, kisspeptins can also elicit the phosphorylation of p38 (Castellano et al., 2006) and AKT (Luque et al., 2011). The signalling of kisspeptins and the kisspeptin receptor to p38 and AKT is involved in the regulation of secretion of neuroendocrine. In fact, inhibition of phosphatidylinositide 3-kinases (PI3K)/AKT by using wortmannin abolishes the KP-10-induced release of LH in baboon pituitary cells (Luque et al., 2011), while the treatment of rat hypothalamic explants with p38 inhibitor (SB-203580) prevents KP-10-induced GnRH secretion (Castellano et al., 2006). However, the signalling to p38 is dependent on the cellular context studied, especially in cancer cell lines. For example, KP-10 triggers the phosphorylation of p38 in CHO cells expressing the human or rat kisspeptin receptor (Kotani et al., 2001), but not in MDA-MB-435S cells transfected with the kisspeptin receptor, which are derived from breast cancer cells, (Becker et al., 2005) and in ARO cells, which are thyroid cancer cells and express the endogenous kisspeptin receptor (Ringel et al., 2002).

Consistently, studies of two pancreatic cancer cell lines, which express the endogenous kisspeptin receptor, show that KP-54 induces the phosphorylation of p38 only in PANC-1 cells but not in AsPC-1 cells (Masui et al., 2004). Additionally, the kisspeptin receptor also signals to AKT in cell type-dependent manner. KP-10 stimulates the phosphorylation of AKT in NPA cells transfected with the human kisspeptin receptor (Stathatos et al., 2005), but not in ARO cells (Ringel et al., 2002). However, the mechanisms responsible for the cell type-dependent signalling of kisspeptins and the kisspeptin receptor and its biological functions remain to be demonstrated.

1.5 Ligand-induced receptor activation and signalling

1.5.1 The activation of GPCRs

Binding of a ligand to a GPCR induces a conformational change of the receptor. Determination of the structures of GPCRs in different states, such as the inactive (R) state and the active (R*) state, provides insights into some conformational rearrangements during the activation process of the receptor. For example, the comparison between the structure in R and R* state of β_2AR, shows an involvement of helical rearrangements (Bang and Choi, 2015). The helical rearrangements are primarily located in the cytoplasmic end of TM3, TM5, TM6 and TM7. A large outward movement of the cytoplasmic end of TM6, together with a moderate outward movement of the intracellular end of TM5 and a slightly inward movement of TM3 and TM7, result in an opening of the cytoplasmic ends of the TM bundle, which creates surface accessibility for the coupling of G proteins (Fig. 1.8A) (Bang and Choi, 2015). In addition to the conformational changes in the cytoplasmic side,

alternations in the extracellular end of the TM bundle are observed. For example, the extracellular tips of TM6 and TM7 in the agonist-bound P2Y purinergic receptor 12 structure move inward compared with that in antagonist-bound structure (Fig. 1.8B) (Zhang et al., 2015a).

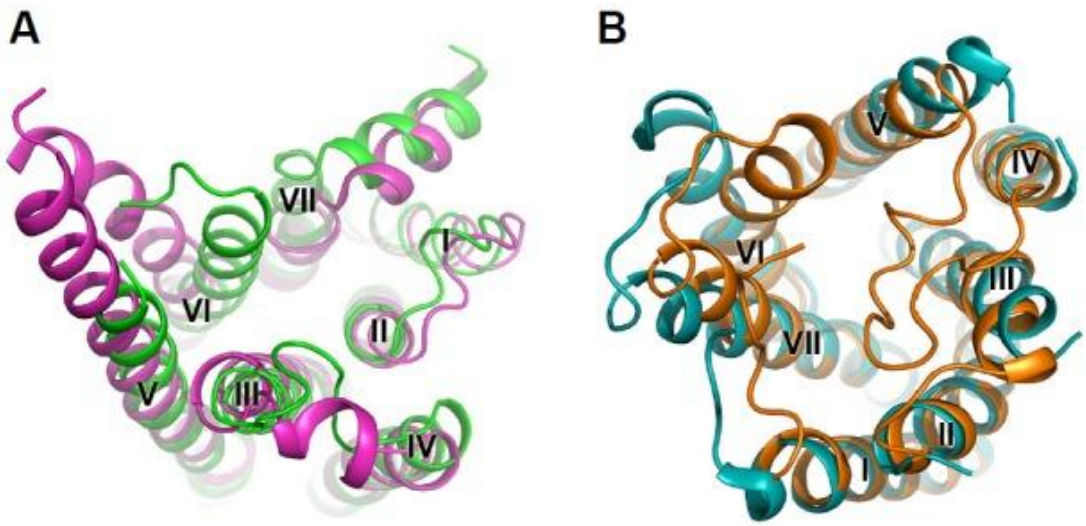


Figure 1.8 Comparison of the R and R* state structure of β_2AR and P2Y purinergic receptor 12. A) Intracellular view of the R* state structure (magenta) and R state structure (green) of β_2AR. B) Extracellular view of the R* state structure (orange) and R state structure (cyan) of P2Y purinergic receptor 12. The TM domains are labelled as I-VII. Figure is obtained from Zhang *et al*., 2015a.

However, the crystallographic studies are unable to provide full understanding of the activation process of GPCRs, since most crystalized structures may be trapped in a specific conformational state in a particular condition and in the relative stable conformations through protein engineering, such as fusion with T4 lysozyme (Rasmussen et al., 2011b, Shimamura et al., 2011, Chien et al., 2010) and removals of the flexible regions (part of N-, C-terminus and ECLs) (Park et al., 2008, Shimamura et al., 2011). Consistent with the note, several conformational intermediates have been

identified. One well characterized example is rhodopsin. Upon absorption of a photon, the chromophore of rhodopsin isomerizes from 11-*cis*-retinal to all-*trans*-retinal prior to any change in the protein portion of rhodopsin (opsin). Following that, a series of conformational changes occur in rhodopsin and several photointermediates are formed in tandem, containing photorhodopsin, bathorhodopsin, blue-shifted intermediate, lumirhodopsin, metarhodopsin I. Subsequently, a transition from metarhodopsin I to metarhodopsin II occurs and then the receptor is able to bind to the intracellular G proteins. In addition, a small portion of metarhodopsin II will decay to form metarhodopsin III (Palczewski, 2006). Another well-studied example is β_2AR. A large number of discrete conformational states are observed along with β_2AR activation pathway by using fluorescent-labelling on the cytoplasmic end of receptor TM6 (Bockenhauer et al., 2011). Even in the ligand-free state, β_2AR shifts between several inactive, partially active and fully active conformations that result in receptor basal activity (Bockenhauer et al., 2011). The discovery of intermediate states of GPCRs reveals the complexity of the conformational landscape during the activation process. These different conformational states of GPCRs are responsible for receptor interaction with distinct sets of downstream effectors and contribute to the biased signalling, including ligand-induced selective signalling (LiSS) as discussed below. Since the structures of the kisspeptin receptor and its downstream signalling pathways remain largely unknown, the understanding of the ligand-induced activation of other GPCRs and their biased signalling may provide some leads to elucidate the activation and signalling of the kisspeptin receptor.

1.5.2 Ligand-induced Selective Signalling (LiSS)

It is broadly accepted that some ligands of the same GPCR can preferentially activate one or some intracellular signalling pathways over the others. This concept is termed LiSS (Millar et al., 2008), biased agonism (Jarpe et al., 1998) or functional selectivity (Urban et al., 2007a). LiSS is used in this thesis. An increasing number of studies has demonstrated LiSS across a wide range of GPCRs such as μ-OR (Keith et al., 1996, Sternini et al., 1996, Thompson et al., 2015), 5-hydroxytryptamine receptor 2A and 5-hydroxytryptamine receptor 2C (Berg et al., 1998), D_2R (Mottola et al., 2002, Gay et al., 2004, Urban et al., 2007b), β_2AR (Azzi et al., 2003, Galandrin and Bouvier, 2006, Stallaert et al., 2012), CCR7 (Kohout et al., 2004), dopamine D_1 receptor (Ryman-Rasmussen et al., 2005), α_{1A}-adrenoceptor (Evans et al., 2011), CB_1 cannabinoid receptor (Khajehali et al., 2015) and adenosine A_1 receptor (Baltos et al., 2016). One early example of LiSS comes from the study on pituitary adenylyl cyclase-activating polypeptide (PACAP) type-I receptors that can activate adenylyl cyclases and PLC-β pathways (Spengler et al., 1993). Although PACAP-27 and PACAP-38, which are two agonists of PACAP type-I receptors, exhibit similar potency to activate adenylyl cyclases, PACAP-38 is more potent to stimulate the PLC-β-mediated signalling pathway than PACAP-27.

It has been proposed that some biased ligands can antagonize some signalling pathways and simultaneously stimulate the others (DeWire and Violin, 2011). It may contribute to some undesirable effects of clinical drugs (Pupo et al., 2016). One example is famotidine, which is used for the treatment of gastrointestinal ulcers by blocking the functions of histamine H_2 receptor (H_2R). Famotidine behaves as an

inverse agonist of H_2R that diminishes G_s-mediated accumulation of cAMP. However, it also acts as an agonist of H_2R to stimulate the dual phosphorylation of ERK1/2, and in turns increases the expression of histidine decarboxylase, which is responsible for the synthesis of histamine, an agonist of H_2R (Alonso et al., 2015). Therefore, the up-regulation of the expression of histidine decarboxylase may contribute to the rebound acid hypersecretion observed after withdrawal of famotidine. Hence, LiSS could be implicated in the development of drugs with improved therapeutics and fewer side effects. For example, TRV120027 [sarcosine (Sar-Arg-Val-Tyr-Ile-His-Pro-D-Ala-OH)] is a potential drug for the treatment of acute heart failure by targeting angiotensin II type 1 receptor (AT_1R). Similar as other classical angiotensin receptor blockers (ARBs), such as valsartan, losartan and telmisartan, TRV120027 behaves as an antagonist of AT_1R that antagonize $G_{q/11}$-mediated signalling pathways. In contrast to other ARBs, TRV120027 is also a β-arrestin-biased ligand of AT_1R, which can induce β-arrestin2-mediated activation of ERK1/2, c-SRC, endothelial nitric-oxide synthase, c-Jun and FAK (Violin et al., 2010). Consistent with the observations *in vitro*, TRV120027 displays a unique pharmacological profile *in vivo*. Like other ARBs such as telmisartan, TRV120027 reduces mean arterial pressure, which is regulated by $G_{q/11}$-stimulated calcium mobilization, in healthy male rats. By contrast, unlike telmisartan, which reduces cardiac performance and decreases stroke volume, TRV120027 also increases cardiac contractility and preserves stroke volume, which may be modulated by β-arrestin2-mediated signalling pathway (Violin et al., 2010).

The biased signalling of GPCRs can be mimicked by mutations in various regions of the receptor (Perez et al., 1996, Zuscik et al., 1998, Wei et al., 2003, Gaborik et al.,

2003, Koole et al., 2012, Sbai et al., 2014, Soto and Trejo, 2010). One of the early studies demonstrating mutation-induced signalling bias comes from the studies on α_{1B}-adrenergic receptor (α_{1B}AR) (Perez et al., 1996). Substitution of Cys^{128} in TM3 of α_{1B}AR with Phe (C128F) results in a conformational change that mimics an agonist-bound conformational of the receptor and, thus, creates a constitutively active α_{1B}AR mutant. However, the mutant receptor preferentially activates $G_{q/11}$/PLC-β signalling pathway in absence and presence of agonists, but not $G_{i/o}$/phospholipase A_2 pathway. In addition, Ala scanning mutagenesis of the ECL2 of glucagon-like peptide-1 receptor reveals that several mutants are able to induce receptor signalling toward ERK1/2 activation, but not cAMP accumulation nor Ca^{2+} mobilization (Koole et al., 2012). The discovery of mutation-induced signalling selectivity of GPCRs indicates the possibility of the presence of pathway-specific receptor conformations.

Indeed, several studies have demonstrated that binding of different ligands to the same receptor stabilizes distinct receptor conformations by using fluorescence resonance energy transfer (FRET)-based assays (Granier et al., 2007, Zurn et al., 2009, Reiner et al., 2010, Ziegler et al., 2011). For example, the binding of different ligands to fluorophore-tagged β_2AR results in distinct changes in the FRET signals (Granier et al., 2007). Briefly, the C-terminal tail of β_2AR is labelled with a donor fluorophore, fluorophore 4',5'-bis(1,2,3-dithioarsolan-2-yl)-fluorescein (FlAsH), by substituting the residues on either middle (residue 351-356; proximal site) or the end (last six residues; distal site) of the C-terminal tail with Cys-Cys-Pro-Gly-Cys-Cys to create the fluorophore binding sites, while the Cys^{265} at the cytoplasmic end of the TM6 is labelled with the acceptor fluorophore, Alex 568. At saturating concentrations,

although all tested ligands result in an increased in the FRET signal between Cys^{265} and the proximal site, they induce distinct changes in the FRET signal between Cys^{265} and the distal site. Ligands that induce the phosphorylation of ERK1/2, including the ICI-118551, isoproterenol, epinephrine and norepinephrine, cause a reduction in the FRET signal between Cys^{265} and the distal site. By contrast, dopamine leads to an increase in the FRET signal between Cys^{265} and the distal site, while the rest of the ligands, including salbutamol and alprenolol, have no effect on the FRET signal. The data indicate that the ligands inducing the activation of ERK1/2 stabilize a different conformation of β_2AR, which the end of C-terminus moves away from the central axis of the TM bundle, compared with other ligands. Further studies on the amplitudes and kinetics of the FRET changes in fluorophore-tagged β_2AR support the above suggestion of the formation of distinct receptor conformations in response to different ligands (Reiner et al., 2010). The fluorophore-tagged β_2AR is constructed by inserting the cyan fluorescent protein (CFP) into the ICL3 and fusing the yellow fluorescent protein (YFP) into the truncated end of C-terminus (at position 369). Either epinephrine or norepinephrine at saturating concentrations results in a decrease in the FRET signal, indicating an outward movement of the C-terminus from the ICL3 of β_2AR. However, the reduction in the FRET signal caused by norepinephrine stimulation is approximately half amount of that induced by epinephrine. Additionally, norepinephrine induces a significantly slower change in the FRET signal compared with that induced by epinephrine. Functionally, although norepinephrine is as potent as epinephrine to trigger G_s and adenylyl cyclase activation, it is less potent to recruit β-arrestin2 and to induce the internalization of β_2AR. Altogether, the data imply that norepinephrine stabilizes a distinct

conformation of β_2AR compared with that induced by epinephrine to preferentially activate different signalling pathways.

The comparison between the crystal structures stabilized by distinct ligands of the same GPCR provides some insights into the different conformational changes of the receptor. For example, four structures of thermostable turkey β_1AR bound to the full (carmoterol and isoprenaline) and partial (salbutamol and dobutamine) agonists are solved by crystallization (Warne et al., 2011). Comparisons among these structures reveal some subtle differences, although the overall structures of these crystal structures are similar. The differences could result from distinct ligand-receptor interactions. The full agonists form hydrogen bonds to the side chain of two conserved Ser residues within the TM5 of β_1AR (Ser^{212} and Ser^{215}) and, therefore, induces the rotamer conformational change of Ser^{212} and Ser^{215}. By contrast, the partial agonists make a hydrogen bond to the side chain of Ser^{212}, but not Ser^{215}, and, thus, only cause rotamer conformational change of Ser^{212}. The agonist-induced rotamer conformational change of Ser^{212} results in a strengthening of the interaction between the TM5 and TM6 by forming hydrogen bonds to Asn^{310}, whereas the rotamer conformation change of Ser^{215} causes the weakening of the interaction between the TM4 and TM5 by breaking the van der Waals interaction with Val^{172}. These differences may induce distinct receptor conformations that selectively activate different downstream signalling pathways.

Taken together, these studies indicate that different ligands may stabilize distinct conformations *via* common or differential ligand-receptor interaction, which

preferentially activate different downstream pathways (Lu et al., 2005, Lu et al., 2007). However, the molecular processes involved in the transition of ligand-induced conformational changes and the consequent receptor biased cellular signalling remain elusive. The understanding of LiSS may enhance the development of novel clinical drugs targeting GPCRs, such as the kisspeptin receptor, which exhibit increased potency and fewer side effects.

1.6 Aims

Aims of the thesis are to investigate the biological functions of kisspeptin analogues and the human kisspeptin receptor. Due to the important roles of kisspeptins and the kisspeptin receptor in the regulation of the development of puberty and cancer metastasis, the kisspeptin receptor may be a potential drug target. Kisspeptins, which are the endogenous ligands of the kisspeptin receptor, show low metabolic stability. The low stability of kisspeptins in serum may hinder the elucidation of the long-term physiological roles of kisspeptins and the kisspeptin receptor *in vitro* and *in vivo*. Therefore, in this thesis, three novel phosphinic peptides were designed and synthesised based on the amino acid sequence of KP-10, in order to improve its metabolic stability. Whether the synthetic peptides can bind to and activate the kisspeptin receptor and their ability to inhibit MMPs were tested. In addition, as described above, the precise signalling pathways activated by kisspeptins and the kisspeptin receptor remain to be demonstrated. The Pro-rich region within the C-terminal tail of the kisspeptin receptor might function as a SH3 binding motif to mediate interaction of the receptor with non-G protein effectors. In this thesis, the interaction of the kisspeptin receptor with two candidates, p85α or c-SRC, was

elucidated by using co-immunoprecipitation (co-IP) assays and their potential biological functions were examined. Moreover, two Cys residues, Cys^{338} and Cys^{340}, in the C-terminal tail of the kisspeptin receptor might be potential palmitoylation sites and be important for the signalling of the kisspeptin receptor. Therefore, the roles of Cys^{338} and Cys^{340} on the expression and function of the receptor were investigated by using mutagenesis studies.

Chapter 2

Materials and methods

2.1 Introduction

This chapter details the materials and the laboratory techniques used in the research presented within the thesis. All used materials that were received from external sources and any work that was not performed by me have been acknowledged.

2.2 Materials

The cDNA of flag-tagged kisspeptin receptor was kindly provided by Dr. Andy Babwah, The University of Western Ontario, Canada. The flag-tagged bovine p85α plasmid was kindly provided by Dr. Deborah Anderson, The University of Saskatchewan, Canada. The c-SRC reporter plasmids were kindly provided by Dr. Shu Chien, The University California, San Diego. All inhibitors used in the thesis were obtained from Selleck Chemicals (Houston, USA) with the exception of $G_{q/11}$ inhibitor YM-254890. YM-254890 was kindly provided by Dr. Masatoshi Taniguchi, Astella Pharma, Japan. The peptides were all synthesized from Chinese Peptides (Hangzhou, China) and the kisspeptin phosphinic analogues were designed and synthesized by Dr. Magdalini Matziari, Xi'an Jiaotong-Liverpool University, China. Anti-flag and anti-HA antibodies were purchased from Sigma-Aldrich (St. Louis, USA) and Roche (Basel, Switzerland) respectively. Other primary antibodies were obtained from Cell Signalling Technology (Boston, USA). Anti-HA and anti-flag agarose conjugated antibodies were purchased from Biotool (Houston, USA), and anti-c-SRC agarose conjugated antibody was purchased from Santa Cruz (Dallas, USA).

2.3 Transformation of competent cells

Competent cells (DH5α; Tiagen, Beijing, China) were thawed on ice and 100 µl of them were incubated with 10 µl of DNA ligation products on ice for 30 minutes. After incubation, the cells were heat shocked at 42^{o}C for 75 seconds and then were placed immediately on ice for 3 minutes. The cells were then incubated in 900 µL of Luria broth (LB) medium at 37^{o}C for 1 hour with constant agitation. After that, 200 µl of the culture medium was streaked out on a LB agar plate (containing 100 µg/mL of ampicillin or 50 µg/mL of kanamycin, as appropriate for the plasmid) and was incubated at 37^{o}C overnight. On the following day, single colonies were picked for further plasmid DNA amplification and DNA sequencing. The agar plates were sealed and stored at 4^{o}C.

2.4 Preparation of plasmid DNA

Single colonies were picked from streaked plates and then cultured in 5 mL of LB medium containing 100 µg/mL of ampicillin or 50 µg/mL of kanamycin. The culture was grown at 37^{o}C for 8 hours with constant shaking and 1 mL of the culture was added into 250 mL of fresh LB medium (containing the same concentration of antibiotics). The culture was further grown at 37^{o}C for 16 hours. Plasmid DNAs were extracted by using endofree plasmid maxiprep kit (Biotool, Houston, USA) according to the manufacturer's instructions and eluted in endofree elution buffer, which is supplied in the kit. The DNA concentration was determined using a Nanodrop spectrophotometer 2000 (Thermo Fisher Scientific, Waltham, USA).

2.5 Preparation of glycerol stocks

Glycerol stocks were prepared by adding 540 µl of sterile 100% (v/v) glycerol to 1.26 mL of transformed bacterial culture that had been grown overnight from a single colony. Following thorough mixing by inverting vials several times, they were then stored at -80^{o}C. To recover the bacteria, a sterile tip was used to scrape the surface of the frozen culture. Bacteria were grown, the culture expanded and DNA was purified (Section 2.4).

2.6 Agarose gel electrophoresis

Agarose gels with an appropriate concentration were prepared in TAE buffer (USB Corporation, Cleveland, USA) and stained with GelRed™ (Biosharp, Hefei, China). DNA samples were separated at 110 V for 90 minutes and visualised under ultraviolet (UV) light using Gel Doc^{TM} XR+ imager (Bio-rad, Hercules, USA). The DNA was recovered from the gel by using AxyPrep DNA gel extraction kit (Axygene, Tewksbury, USA) following the manufacturer's instructions.

2.7 Restriction enzyme digestion

Single restriction digestions of 1 µg of plasmid DNA were performed in a final volume of 50 µl using 1 unit of an appropriate enzyme (New England Biolabs, Ipswich, USA) in the buffer, which was chosen to give maximum enzymatic activity. Digestions were conducted at 37^{o}C for 1.5 hours. For low efficiency enzymes, the reaction time was extended to overnight. DNA was fractioned by agarose gel electrophoresis and the required products were recovered as described (Section 2.6). For the double

restriction digestions, the recovered products from the first digestion were used for another single restriction digestion.

2.8 Ligation of DNA

T4 DNA ligase (Promega, Madison, USA) was used to ligate inserts and linearized vectors with cohesive ends. A ligation mixture containing 1 unit of T4 ligase, 1 µl of 10 x T4 ligase buffer (300 mM Tris-HCl (pH 7.8), 100 mM $MgCl_2$, 100 mM dithiothreitol and 10 mM adenosine triphosphate) and 3:1 molar ratio of vector : insert was made. Ultrapure water was used to make a final concentration of 10 µl. The reaction was conducted at 4^{o}C overnight and the entire ligation mixture was used to transform DH5α competent cells (Section 2.3).

2.9 Site-directed mutagenesis

The flag-tagged kisspeptin receptor plasmids were constructed by a pEGFP-C3 backbone purchased from Invitrogen (Pampillo et al., 2009). Briefly, the open reading frame of the human kisspeptin receptor was amplified from a 1607-base pair cDNA of the receptor purchased from OriGene Technologies (Rockville, MD; NM_032551.3) and the flag-epitope was then introduced into the N-terminus of the receptor. Following that, the flag-tagged kisspeptin receptor was cloned into the *NheI* and *NotI* site of the pEGFP-C3 backbone.

All mutations were introduced into the flag-tagged kisspeptin receptor by polymerase chain reaction (PCR) site-directed mutagenesis. Since some mutation

sites are located in the Pro and Arg rich region of the kisspeptin receptor, the designed primers for these mutants possess very high CG content. In order to minimize non-specific binding, a pair of common primers was designed to generate a fragment of flag-kisspeptin receptor. The PCR product was purified and used as a template for the generation of all mutants.

A pair of overlapping primers for each mutation was designed and the mutation site was designed in the middle of each primer. Two pieces of PCR products containing mutated residues were created by using the combination of common primers and overlapping primers. These two PCR products were ligated by an overlap extension PCR using the common primer pair. The PCR products were then cloned into the *AflII* and *NotI* sites of flag-kisspeptin receptor plasmids. The plasmids containing the mutations were transformed into DH5α and purified using QIAprep® miniprep kit (Qiagen, Crawley, UK) according to the manufacturer's instructions. The purified plasmids were sent to Invitrogen (Shanghai, China) for sequencing.

2.10 Cell culture

The two cell lines used in this project are HEK293 and MCF-7 cells. Both were maintained in complete growth medium (Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% (v/v) fetal bovine serum (Bovogen, East Keilor, Australia), 2 nM L-glutamine, 0.2 unit/mL penicillin, 0.2 µg/mL streptomycin (Gibco, Waltham, USA) at 37^{o}C in a humidified 5% (v/v) CO_2 atmosphere. They were routinely passaged at 1:4 dilution twice weekly by enzyme dispersal with trypsin. Briefly, Ca^{2+}- and Mg^{2+}-free phosphate buffered saline (PBS; 0.0067 M PO_4, pH 7.2)

was used to wash the confluent cells prior to trypsinization. For cells cultured in 75 cm^3 cell culture flasks (T75s), 1 mL of trypsin (0.5 g/L trypsin, 0.2 g/L ethylene diamine tetraacetic acid (EDTA) and 0.85 g/L NaCl; Gibco, Waltham, USA) was applied and then the flask was returned back into the incubator for 1- to 5-minute incubation. After successful dissociation, 9 mL of complete growth medium was added to terminate further trypsin reaction. Cells were collected by centrifuging at 300 x g for 3 minutes and the cell pellet was then suspended in the complete growth medium. After 1:4 dilution, 10 mL of cell suspension was transferred into a T75 flask. A Nebauer haemocytometer was used to determine the cell number by taking the average of the four counting areas. The cell number was represented in 1 x 10^5/mL. The dilution factor was taken into the consideration.

2.11 Transient transfection by electroporation

The transient expression of required plasmid DNA in HEK293 and MCF-7 cells was achieved by electroporation. For both cell lines, cells with 10 µg (single plasmid transfection) or 15 µg (two plasmid transfection, 7.5 µg for each) DNA in Opti-MEM (Gibco, Waltham, USA) were cooled on ice for 5 minutes. After that cells were electroporated in a Gene Pulser Xcell™ instrument (Bio-rad, Hercules, USA). The HEK293 cells were electroporated using 300 volts and capacitance 950 µF. The MCF-7 cells were electroporated using 320 volts and capacitance 950 µF. Both cells were electroporated with 4 mm cuvettes (Bio-rad, Hercules, USA) with infinite resistance.

2.12 Preparation of pharmacological inhibitors and ligands

Stock solutions of inhibitors were prepared in anhydrous dimethyl sulfoxide (DMSO) as per the manufacturer's instructions. The stock solutions of KP-10 and Cy5-KP18 were prepared in 20% (v/v) propylene glycol. The stock solutions of phosphinic kisspeptin analogues were prepared in 20% (v/v) DMSO for compound A and B, and 50% (v/v) DMSO, 8% (v/v) propylene glycol for compound C. These stock solutions were stored in aliquots at -20^{o}C. On the day of experiments, peptide aliquots were thawed and diluted in culture mediums or buffers to a desired concentration by 1:10 serial dilution. All ligands and inhibitors were subjected to a maximum of two freeze/thaw cycles.

2.13 Fluorescence-based ligand binding assay

The fluorescence-based binding assay was established on intact cells transiently expressing the kisspeptin receptor (Section 2.11). After transfection, cells were seeded onto two 10-cm culture dishes. After 24-hour culture in the complete growth medium, cells were trypsinized and 8 x 10^4/well cells were seeded onto 96-well cell culture plates. On the following day, cells were washed once in PBS and then incubated with either vehicle (0.02% (v/v) propylene glycol) or various concentration of Cy5-KP18 in a serum-free medium (DMEM supplemented with 10 mM 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES), 0.1% (w/v) bovine serum albumin (BSA), 2 nM L-glutamine, 0.2 unit/mL penicillin and 0.2 µg/mL streptomycin) for 3 hours at 4^{o}C. The non-specific binding was determined in the presence of 10 µM unlabelled KP-10. After incubation, free ligands were removed by three rapid

washes with ice-cold PBS. The fluorescence intensity was measured by using a PHERAstar FS microplate reader (BMG LABTECH, Germany).

2.14 Agonist-induced internalization assay

The Cy5-KP18-induced internalization of the kisspeptin receptor was performed to determine the peptide binding properties. Briefly, cells were transiently transfected with the kisspeptin receptor by electroporation and seeded onto 96-well plates. After 48-hour culture in the complete growth medium, cells were incubated with a mixture of 50 nM Cy5-KP18 and various concentrations of tested peptides or corresponding vehicles in the complete growth medium for 30 minutes or a time range at 37^{o}C. After incubation, free ligands were removed by two rapid washes in ice-cold PBS. The non-internalized ligands were removed by one wash in ice-cold 0.1 M glycine-HCl buffer (pH 3.0) at 4^{o}C for 5 minutes. Further three rapid washes in ice-cold PBS were applied to wash off the free non-internalized ligands. The fluorescence intensity was measured by using PHERAstar FS microplate reader (BMG LABTECH, Germany).

2.15 Preparation of cellular extracts for western blotting

After appropriate treatments, the cell monolayers were placed on ice and rapidly washed twice with ice-cold PBS. Cells were then lysed in 2X sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) sample loading buffer (Beyotime, Shanghai, China) or Nonidet P-40 solubilisation buffer (250 nM NaCl, 50 mM HEPES, 0.5% (v/v) Nonidet P-40, 10% (v/v) glycerol, 2 mM EDTA, pH 8.0) supplemented with 2 mM sodium orthovanadate and complete^{TM} protease inhibitor cocktail (EDTA-free,

Roche, Basel, Switzerland). The nuclear contents were sheared by sonication. The samples lysed by Nonidet P-40 solubilisation buffer were clarified by centrifugation at 16,000 x g for 15 minutes and the protein concentrations were determined with the bicinchoninic acid (BCA) assay kit (Beyotime, Shanghai, China) following the manufacturer's instructions. The samples lysed by SDS-PAGE sample loading buffer were used directly for western blot (Section 2.17).

2.16 Co-IP assay

Transfected MCF-7 cells were seeded onto 6-well cell culture plates (at a density of 1.6 x 10^6 cells/well) and cultured for 24 hours in the complete growth medium. After that, the cells were washed twice in Ca^{2+}- and Mg^{2+}-free PBS prior to being incubated in the serum-free medium for 20 hours. The stimulations by agonists were performed in fresh serum-free medium at 37^{o}C in a humidified 5% (v/v) CO_2 atmosphere. Following appropriate agonist stimulations, cells were lysed in Nonidet P-40 solubilisation buffer (Section 2.15). The lysed cells were sonicated at 3 amplitude micron for 5 seconds and the supernatant was collected following centrifugation at 16,000 x g for 15 minutes. A volume of 20 µl of appropriate antibody conjugated beads was incubated with the supernatant on a rotating wheel overnight at 4^{o}C. After incubation, the beads were loaded into home-made microcolums (made from 200 µl pipette tips and the filter cartridges were obtained from 10 µl filtered pipette tips) and washed five times with 200 µl of PBS supplemented with 2 mM sodium orthovanadate and protease inhibitor cocktail at 4^{o}C. The proteins were eluted from beads by 2X SDS-PAGE sample loading buffer and ready for western blot (Section 2.17).

2.17 Western blotting

Solubilised protein samples were mixed with SDS-PAGE sample loading buffer and then boiled at 90^{o}C for 10 minutes and allowed to cool. Proteins were resolved by SDS-PAGE using 10% or 12% NuPAGE® Bis-Tris pre-cast gels (Invitrogen, Shanghai, China). Electrophoretic separation was performed at 170 volts for 70 minutes in NuPAGE® MOPS SDS Running buffer (Invitrogen, Shanghai, China).

Proteins were electroblotted onto polyvinylidene difluoride (PVDF) membranes (Millipore, Beijing, China) for protein immunoblotting. PVDF membranes were wetted in 100% methanol for few seconds and equilibrated in transfer buffer (25 mM Tris, 192 mM glycine, 20% (v/v) methanol and 0.03% (w/v) SDS) for at least 30 minutes. Meanwhile, two blotting papers (Bio-rad, Hercules, USA) and six sponge pads were soaked in the same transfer buffer. A sandwich was created following the instructions of XCell II^{TM} blot module (Novex, Shanghai, China). Proteins were transferred from the gel to the PVDF membrane at 25 voltages for 1.5 hours by using a XCell II^{TM} blot module.

Following electroblotting, the PVDF membranes were washed once in PBS-T (140 mM NaCl, 27 mM KCl, 10 mM phosphate (pH 7.4) and 0.05% (v/v) Tween-20) and blocked in blocking buffer (5% (w/v) BSA in PBS-T) for 1 hour. After blocking, the membranes were blotted by appropriate primary antibodies (1:1000 dilutions in PBS Odyssey® blocking buffer (LI-COR, Lincoln, USA) supplemented with 0.05% (v/v) Tween-20) at 4^{o}C overnight. On the following day, IRDye® goat anti-mouse or anti-

rabbit IgG (H + L) secondary antibody (LI-COR, Lincoln, USA) (1:10000 dilutions in PBS Odyssey® blocking buffer supplemented with 0.05% (v/v) Tween-20) was applied after three time washed in PBS-T for 10 minutes. After further three times washed in PBS-T for 10 minutes, the membranes were visualised in the infrared by using an Odyssey® infrared imaging system (LI-COR, Lincoln, USA). The band intensities were quantified using an Odyssey® application software (version 3.0).

2.18 Mobilization of intracellular calcium ion

Cells transiently expressing the kisspeptin receptor were seeded onto 96-well cell culture plates (at a density of 2 x 10^4 cells/well) and cultured for 40 hours in the complete growth medium. After that, the cells were washed twice in Ca^{2+}- and Mg^{2+}-free PBS and the intracellular calcium flux was measured by using Fluo-4 Direct™ calcium assay kits (Invitrogen, Shanghai, China) according to the manufacturer's instructions. Briefly, an equal volume of 2 X Fluo-4 Direct™ calcium reagents was loaded into each well and then the plate was incubated at 37°C, 5% (v/v) CO_2 for 50 minutes. Then, the intracellular calcium flux was detected by using a PHERAstar FS microplate reader (BMG LabTech, Ortenberg, Germany) under the fast kinetic mode at 37°C. Readings were taken every 0.5 second over total 90 seconds and a final concentration of 100 nM KP-10 was injected into each well by onboard fluidics after the first-20-second reading. The calcium response is calculated using following formula. The baseline is calculated by taking the average over first 20 seconds. The maximal response was the maximum signal between 20 and 30 seconds.

$$\left[\frac{\text{Max response} - \text{Baseline}}{\text{Baseline}}\right] * 100 = \text{percentage response}$$

2.19 Measurement of matrix metalloproteinase (MMP) activities

The activities of MMPs were measured by using MMP colorimetric drug discovery kits (Enzo Life Sciences, New York, USA) in a 96-well microtiter plate according to the manufacturer's instructions. Different concentrations of the phosphinic analogues or 1.3 µM N-Isobutyl-N-(4-methoxyphenylsulfonyl)glycyl hydroxamic acid (NNGH, a commercial MMP-2 and -9 inhibitor) were added to an assay mixture containing MMP enzyme (MMP-2 or MMP-9; 9 mU/µL). The plates were incubated at 37°C for 1 hour to allow enzyme/inhibitor interaction. After that, a final concentration of 100 µM colorimetric substrate (Ac-PLG-[2-mercapto-4-methyl-pentanoyl]-LGOC2H5) was added and plates were then read at 412 nm every minute for at least 20 minutes by using a PHERAstar FS microplate reader (BMG LabTech, Ortenberg, Germany). A full enzyme activity (control) was measured without addition of any inhibitor or tested sample. The reaction velocity (V) was determined by calculating the slop of the fitted line to the linear portion of the data collected. The slop of blank (without the addition of MMP enzymes) was subtracted from all samples and remaining activity of the enzyme is calculated using following formula.

$$\text{Inhibitor \% activity remaining} = \frac{\text{V(inhibitor)}}{\text{V(control)}} * 100$$

2.20 Measurement of protein-protein interactions by FRET assay

Cells transiently co-expressing enhanced YFP (eYFP)-tagged kisspeptin receptor and enhanced CFP (eCFP)-tagged calmodulin were seeded onto 96-well cell culture plates (glass-bottom; at a density of 2 x 10^4 cells/well) and cultured for 20 hours in the complete growth medium. For FRET controls, cells were co-transfected with flag-kisspeptin receptor and pcDNA3.1+. In addition, eCFP only cells (co-transfected with flag-kisspeptin receptor and eCFP-calmodulin and eYFP only cells (co-transfected with eYFP-kisspeptin receptor and pcDNA3.1+) were used. The cells were washed twice in Ca^{2+}- and Mg^{2+}-free PBS and then cultured in serum-free medium for 20 hours. The FRET signal was detected by using a PHERAstar FS microplate reader (BMG LabTech, Ortenberg, Germany) with simultaneously monitoring 480 nm and 530 nm dual emissions at 37^{o}C. Readings were first taken every 6 seconds over 90 seconds and then every 20 seconds over further 240 seconds by using the fast kinetic mode. A final concentration of 100 nM KP-10 was injected into each well by onboard fluidics after the first-30-second reading. The eCFP/eYFP emission ratio is calculated.

2.21 Statistical analysis

All experiments were repeated independently at least three times and all assays were at least duplicated. Data are presented as mean value ± the standard deviation (SD). GraphPad Prism 6.0 (GraphPad Software, San Diego, USA) was used for the data analysis. Statistical significance is calculated using one-way or two-way ANOVA according to the experimental designs and indicated by asterisks in figures.

Chapter 3

Functional examination of kisspeptin analogues

3.1 Outline

All native functional human kisspeptins contain the conserved last 10 amino acids (45–54) at their C-termini, which are important for receptor binding and functional activation, and can be inactivated by the cleavage of the peptide bond between Gly^{51} and Leu^{52}. The cleavage is mediated by matrix metalloproteinases (MMPs) including membrane-type 1-MMP, membrane-type 3-MMP, membrane-type 5-MMP, MMP-2 and MMP-9. Due to the important roles of kisspeptins and the kisspeptin receptor in the regulation of cancer and the reproduction system development, MMP-resistant kisspeptin analogues may provide a better therapeutic efficacy. However, studies in this area are lacking. Therefore, three phosphinic kisspeptin analogues were designed and synthesized. In this chapter, the kisspeptin receptor-agonistic activities of the peptides were evaluated by measuring the stimulation of phosphorylation of ERK1/2 using western blot (Section 2.17). Additionally, the kisspeptin receptor-antagonist activities of the peptides were evaluated by measuring their inhibition on the KP-10-induced phosphorylation of ERK1/2 (Section 2.17). Moreover, the binding of the peptides to the kisspeptin receptor was measured by using a Cy5-KP18-induced internalization assay (Section 2.14). Furthermore, ability of the peptides to inhibit MMP-2 and MMP-9 was tested by using MMP colorimetric drug discovery kits (Section 2.19).

3.2 Introduction

Kisspeptins and their cognate receptor play important roles in the development of puberty (Section 1.3.1) and the regulation of cancer metastasis (Section 1.3.2) (Roseweir et al., 2009, Cho et al., 2012). Therefore, the kisspeptin receptor may be a potential drug target. Kisspeptins share a common 10-amino-acid sequence of a shorter decapeptide (KP-10) at the C-terminus (Fig. 1.4) (Section 1.2.3.1) (Ohtaki et al., 2001, Kotani et al., 2001). However, they are metabolically unstable in serum and can be metabolized by various serum-containing proteases, such as trypsin-like proteases (Asami et al., 2012a). In addition, kisspeptins are also sensitive to MMPs, including membrane-type 1-MMP, membrane-type 3-MMP, membrane-type 5-MMP, MMP-2 and MMP-9 (Takino et al., 2003). They can be inactivated by the MMP-mediated cleavage of the peptide bond between Gly^{51}-Leu^{52}. MMPs are a family of zinc- and calcium-dependent proteolytic enzymes. They play critical roles in the degradation of extracellular matrix (ECM) by digesting various components of ECM, such as collagen, entacin, fibronectin and proteoglycans (Vargova et al., 2012). The degradation of ECM results in alternations of cell-matrix and cell-cell interaction, and the release of ECM-coupled growth factors (Visse and Nagase, 2003, Sternlicht and Werb, 2001, Page-McCaw et al., 2007, Nagase et al., 2006). Dysregulated activity of MMPs is implicated in the development of tumorigenesis and cancer metastasis (Stetler-Stevenson, 2001). Among various MMPs, MMP-2 and MMP-9 play key roles in the progression of invasion and metastasis of tumours by depredating type IV collagen and gelatin, which are two main components of ECM (Curran and Murray, 1999, Stamenkovic, 2003). The secretion of MMP-2 and MMP-9 has been reported in

many human tumours, including ovarian, breast and prostate cancers (Roomi et al., 2009). Since kisspeptins and the kisspeptin receptor play important role in the suppression of cancer metastasis, the development of MMP-resistant analogues of kisspeptins may provide better therapeutic efficacy.

Two main approaches are used to improve the metabolic stability of kisspeptins, including reductions of the peptide length (Niida et al., 2006) and/or substitutions of the cleavage sites with nonhydrolyzable isosteres (Asami et al., 2012a, Tomita et al., 2008). Few MMP-resistant kisspeptin analogues have been designed and synthesized, so far, based on the amino acid sequence of a pentapeptide (4-fluorobenzoyl-Phe-Gly-Leu-Arg-Trp-NH_2), which exhibits potent kisspeptin receptor-agonistic activity (Tomita et al., 2008). The Gly-Leu dipeptide of the analogues is substituted by *(E)*-Alkene- and hydroxyethylene-type isosteres. They retain the kisspeptin receptor-agonistic activity and are resistant to MMP-2- and MMP-9-mediated digestions with improved stability in murine serum. Therefore, the introduction of nonhydrolyzable isosteres and downsizing the length of peptides might be a possible approach to develop MMP-resistant and metabolically stable kisspeptin analogues. Accordingly, Dr. Magdalini Matziari designed and synthesized two quadrapeptides and an octapeptide based on the primary structure of KP-10 (Fig. 3.1). The peptide bond between Gly-Leu of the analogues is replaced by a phosphinic acid moiety, -PO_2-CH_2-. In this chapter, the kisspeptin receptor-agonistic and -antagonistic activities of the analogues were examined. The peptides contain a substitution of one peptide bond with a phosphinic acid moiety are termed phosphinic peptides. Phosphinic peptides have been proposed as potent and

selective inhibitors of various proteases, such as the human immunodeficiency virus protease (Dreyer et al., 1989, Grobelny et al., 1990) and MMPs (Dive et al., 2004, Georgiadis and Dive, 2015), as their chemical structure could mimic that of intermediates formed during the hydrolysis of peptides by proteases. Therefore, the inhibition of the kisspeptin analogues on MMP-2 and MMP-9 were also tested.

3.3 Results

3.3.1 Design and synthesis of kisspeptin analogues

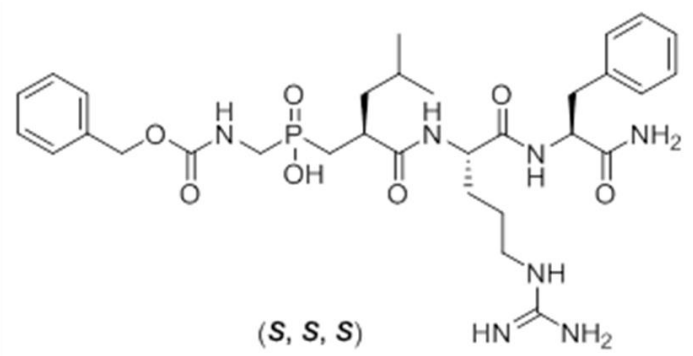
Three kisspeptin analogues are named as compound A, B and C (Fig. 3.1). For these analogues, the peptide bond between Gly^{51} and Leu^{52} of kisspeptin is replaced by a phosphinic acid moiety ($-PO_2-CH_2-$). It was found that the last five residues on the C-terminal of KP-10 are essential for its binding and activation of the kisspeptin receptor (Niida et al., 2006). Therefore, two shorter phosphinic pseudopeptides, compound A and B, were initially synthesized as pilot compounds. Later, a 10-amino-acid analogue, which is named as compound C, was also synthesized.

Compound A and B are stereoisomers and were separated by reversed phase high performance liquid chromatography (RP-HPLC). The first eluent was named as compound A, which is predicted as an *S*-isomer at the pseudo-Leu residue (Fig. 3.1b), and compound B was given to the following eluent (Fig. 3.1c). However, the structures of compound A and B had not been determined and further NMR experiments are required to confirm of their structures. Compound A and B contain the last four amino acids of KP-10 and the Leu^{52} is substituted by a pseudo-Leu residue. A benzyl carbamate group was added to the N-terminus of these two phosphinic peptides to increase their stability for storage. The benzyl carbamate group mimics the structure of Phe^{50} of KP-10 and, therefore, compound A and B may retain the kisspeptin receptor-agonistic activity. Compound C contains all 10 residues of KP-10 and is a mixture of two diastereoisomers, which were not subjected to RP-HPLC separation.

a) H-Tyr^{45}-Asn^{46}-Trp^{47}-Asn^{48}-Ser^{49}-Phe^{50}-Gly^{51}-Leu^{52}-Arg^{53}-Phe^{54}-NH_2

KP-10

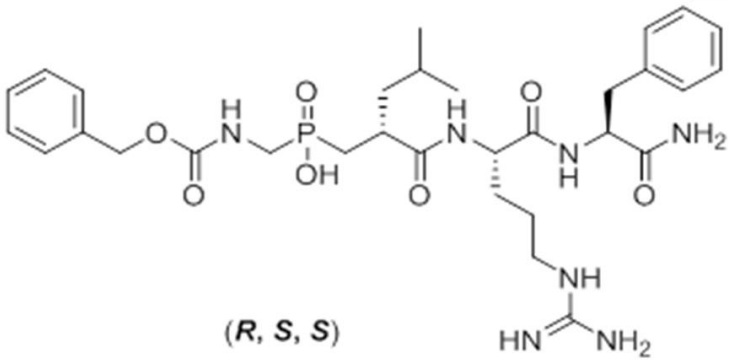
b)



(***S, S, S***)

Compound A

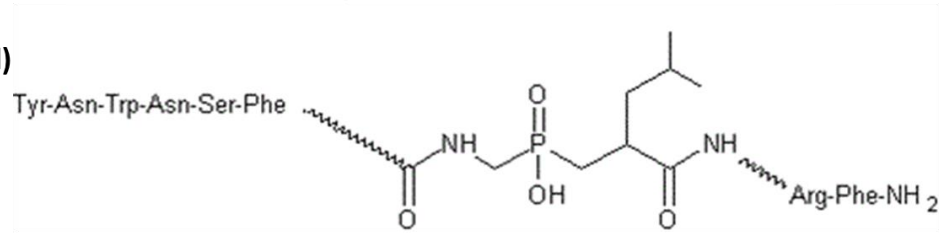
c)



(***R, S, S***)

Compound B

d)



(***R/S, S, S***)

Compound C

Figure 3.1 Predicted structures of KP-10 phosphinic analogues. The amino-acid sequence of KP-10 is shown in panel a). The predicted structure of compound A, B and C are shown in b), c) and d) respectively. Compound A and B are diastereoisomers and their structures needed further confirmation. Compound C is a mixture of two 10-amino-aicd long diastereoisomers.

3.3.2 Determination of the kisspeptin receptor-agonistic activities of kisspeptin analogues

In order to determine whether these peptides retain the kisspeptin receptor-agonistic activity, a series of functional assays were conducted. For these assays, the dual phosphorylation of ERK1/2 was measured by western blot and the band intensity was quantified by Odyssey® application software (version 3.0). β-actin is used as a loading control and the mean fold over control (as indicated in each figure legend) for each sample is calculated and plotted. HEK293 cells transiently expressing the kisspeptin receptor were used as a model for these assays.

At the beginning, compound A or B at a final concentration of 100 nM was used to stimulate cells for 3 minutes, when the maximum response was detected (as shown in Fig. 3.5). The same concentration of KP-10 was used as a positive control. Stimulation of 100 nM KP-10 brought about a 1.75 ± 0.267-fold increase in the level of phosphorylation of ERK1/2. However, no increase in the level of phosphorylation of ERK1/2 was identified in response to either 100 nM compound A or 100 nM compound B (Fig. 3.2; A, white bar; B, grey bar). Then, a dose-dependence dual phosphorylation of ERK1/2 was measured. Three different concentrations of compound A (Fig. 3.3) or B (Fig. 3.4) were used to stimulate the cells for 3 minutes. Cells treated with 100 nM KP-10, which could lead to a saturable phosphorylation of ERK1/2 (Szereszewski et al., 2010), were used as a positive control. Neither 1 μM nor 10 μM of compound A was able to induce the phosphorylation of ERK1/2. By contrast, stimulation with 10 μM compound B brought about a 1.53 ± 0.175-fold

increase in the level of phosphorylation of ERK1/2. Lower concentration of compound B (1 μM) did not lead to the significant increase in the level of phosphorylation of ERK1/2.

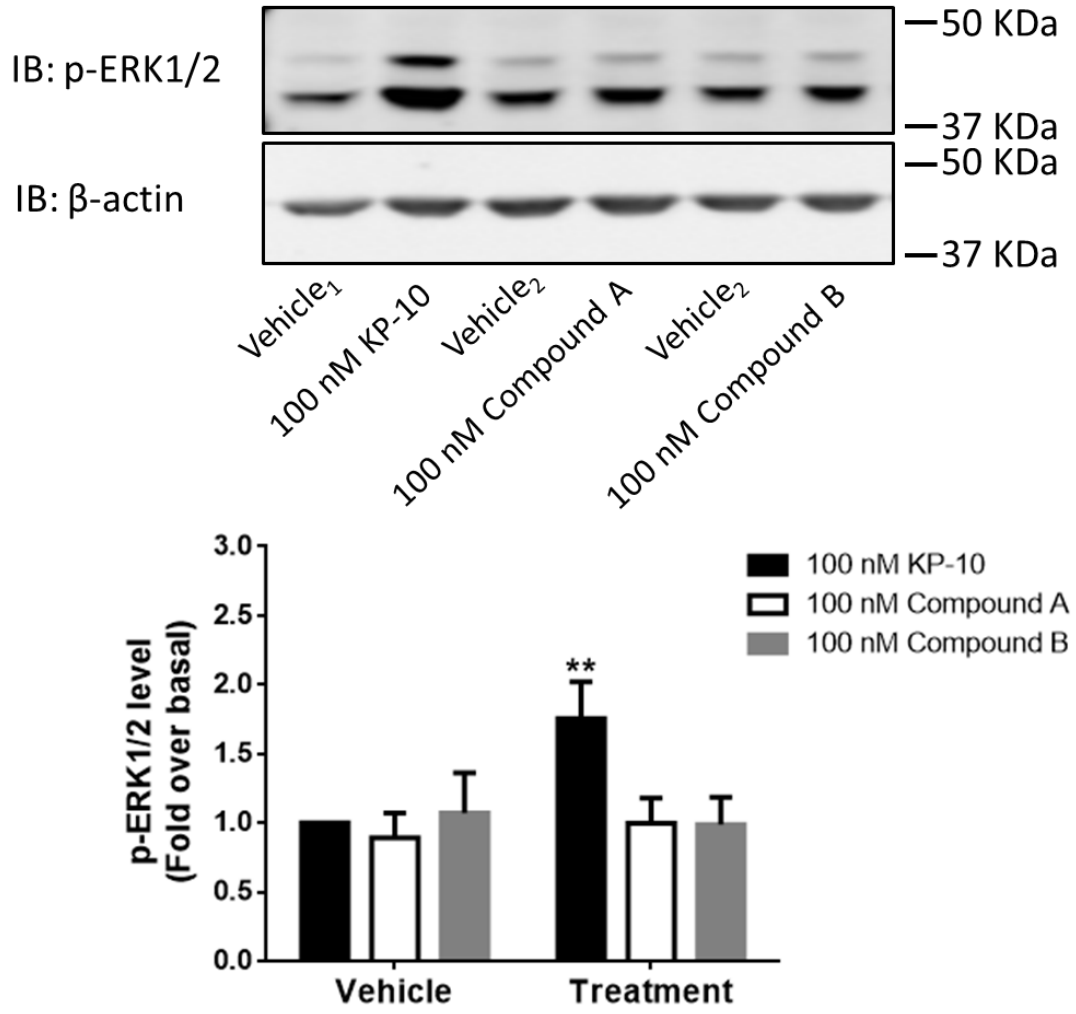


Figure 3.2 Immunoblots depicting the KP-10, compound A or B induced phosphorylation of ERK1/2 using HEK293 cells. HEK293 cells transiently transfected with flag-kisspeptin receptor cDNA were serum-starved for 20 hours prior to being treated with vehicle (vehicle$_1$, 0.02% (v/v) propylene glycol; vehicle$_2$, 0.0002% (v/v) DMSO), 100 nM KP-10 (black bar), compound A (white bar) or compound B (grey bar) for 3 minutes. Representative blots are shown. Data from three independent experiments were quantified (using β-actin as a loading control) and the mean fold over vehicle$_1$ treated control ± SD for the phosphorylation of ERK1/2 is presented. *p<0.01* (**) represents statistical significance from vehicle$_1$ treated control.

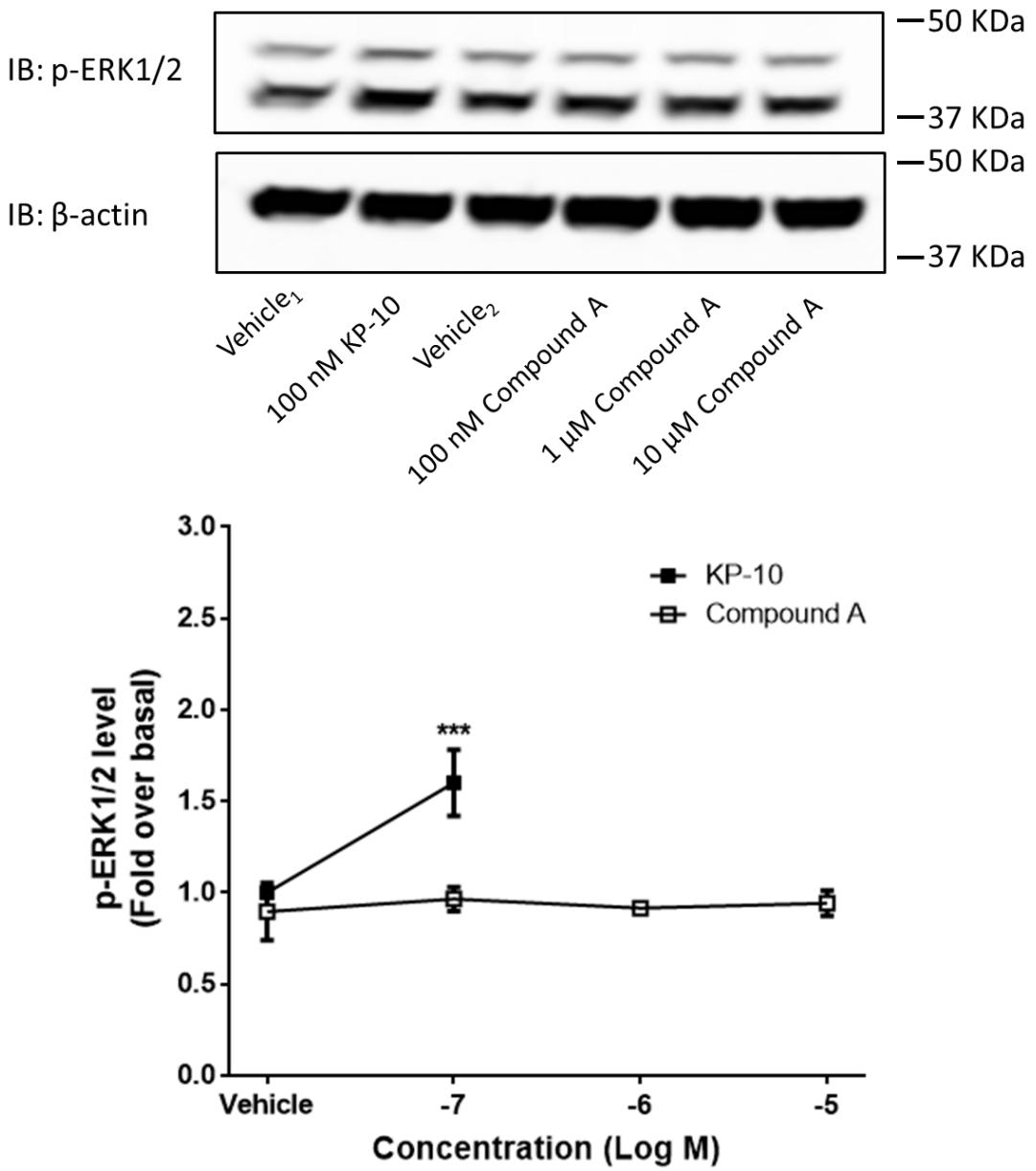


Figure 3.3 Immunoblots depicting a dose-dependent response of compound A-induced phosphorylation of ERK1/2 using HEK293 cells. HEK293 cells transiently transfected with HA-kisspeptin receptor cDNA were serum-starved for 20 hours prior to being treated with vehicle (vehicle$_1$, 0.02% (v/v) propylene glycol; vehicle$_2$, 0.02% (v/v) DMSO), 100 nM KP-10 (■), increasing does of compound A (0.1 μM, 1 μM and 10 μM; □) for 3 minutes. Representative blots are shown. Data from three independent experiments were quantified (using β-actin as a loading control) and the mean fold over vehicle$_1$ treated control ± SD for the phosphorylation of ERK1/2 is presented. *p<0.005* (***) represents statistical significance from vehicle$_1$ treated control.

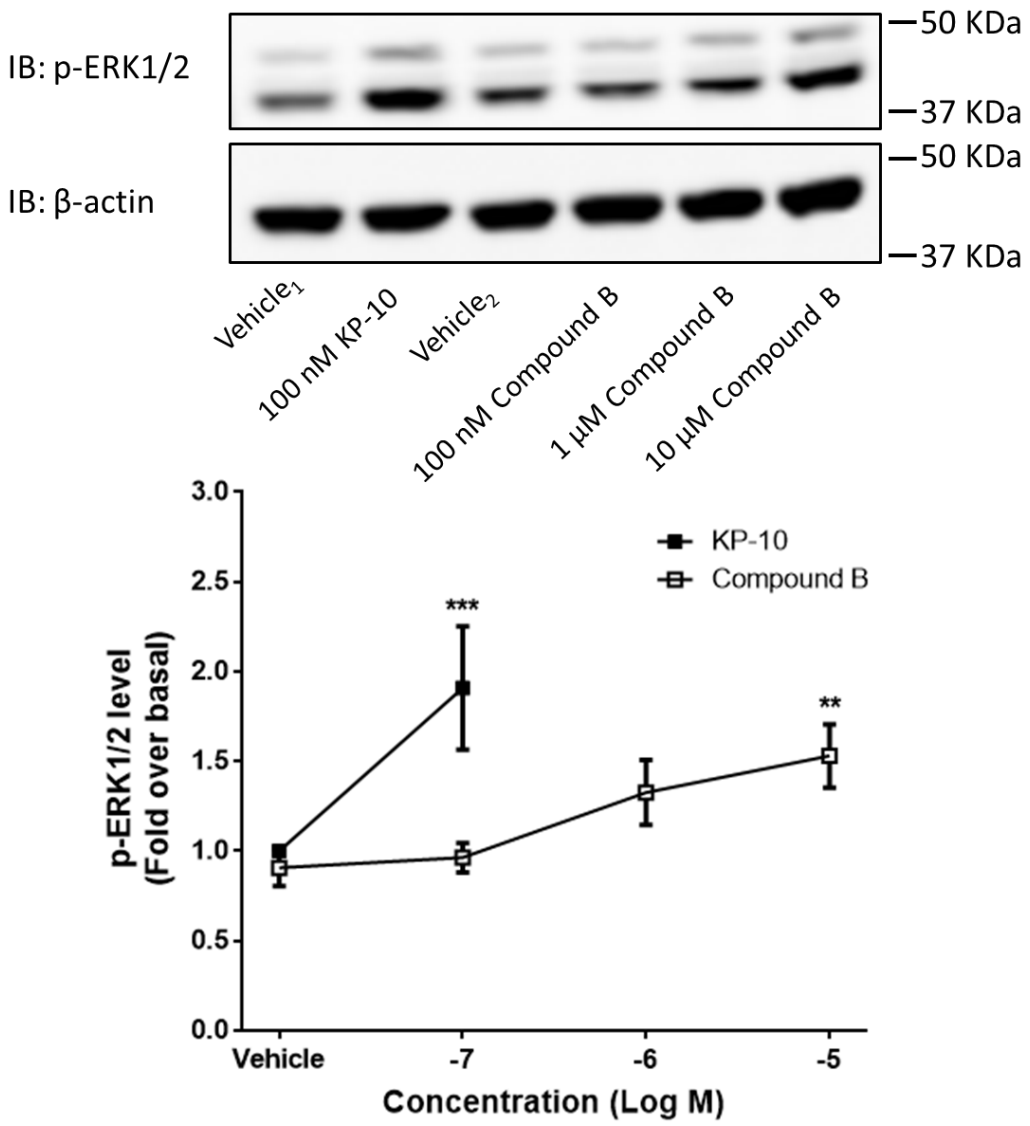


Figure 3.4 Immunoblots depicting a dose-dependent response of compound B-induced phosphorylation of ERK1/2 using HEK293 cells. HEK293 cells transiently transfected with HA-kisspeptin receptor cDNA were serum-starved for 20 hours prior to being treated with vehicle (vehicle$_1$, 0.02% (v/v) propylene glycol; vehicle$_2$, 0.02% (v/v) DMSO), 100 nM KP-10 (■), increasing does of compound B (0.1 μM, 1 μM and 10 μM; □) for 3 minutes. Representative blots are shown. Data from three independent experiments were quantified (using β-actin as a loading control) and the mean fold over vehicle$_1$ treated control ± SD for the phosphorylation of ERK1/2 is presented. *p<0.01* (**) and *p<0.005* (***) represent statistical significance from vehicle$_1$ treated control.

Since compound B can induce the phosphorylation of ERK1/2 only at the highest concentration tested, it decided to determine if the 10-amino-acid phosphinic peptide, compound C, could also activate the kisspeptin receptor. Therefore, the kisspeptin receptor-agonistic activity of compound C was tested in both time- and dose-dependent manners. For the positive control, 100 nM KP-10 was used for the stimulation. Compound C could not elicit an increase in the levels of the phosphorylation of ERK1/2 at any time (Fig. 3.5) or dose tested (Fig. 3.6). Compared with KP-10 vehicle (0.02% (v/v) propylene glycol) treated group, a decrease in the level of phosphorylation of ERK1/2 was detected after the treatment of compound C vehicle (0.625% (v/v) DMSO and 0.1% (v/v) propylene glycol) or 10 μM compound C (Fig. 3.6). By contrast, the level of phosphorylation of ERK1/2 remained the same as KP-10 vehicle treated group upon stimulation with 100 nM or 1 μM compound C. In order to rule out an effect of high concentration of solvent on the phosphorylation of ERK1/2, a further set of individual vehicle treated controls was done for the 100 nM and 1 μM compound C treated groups (Fig. 3.7). None of them affected the level of phosphorylation of ERK1/2. The results confirmed that compound C did not stimulate the phosphorylation of ERK1/2 at any of the doses tested.

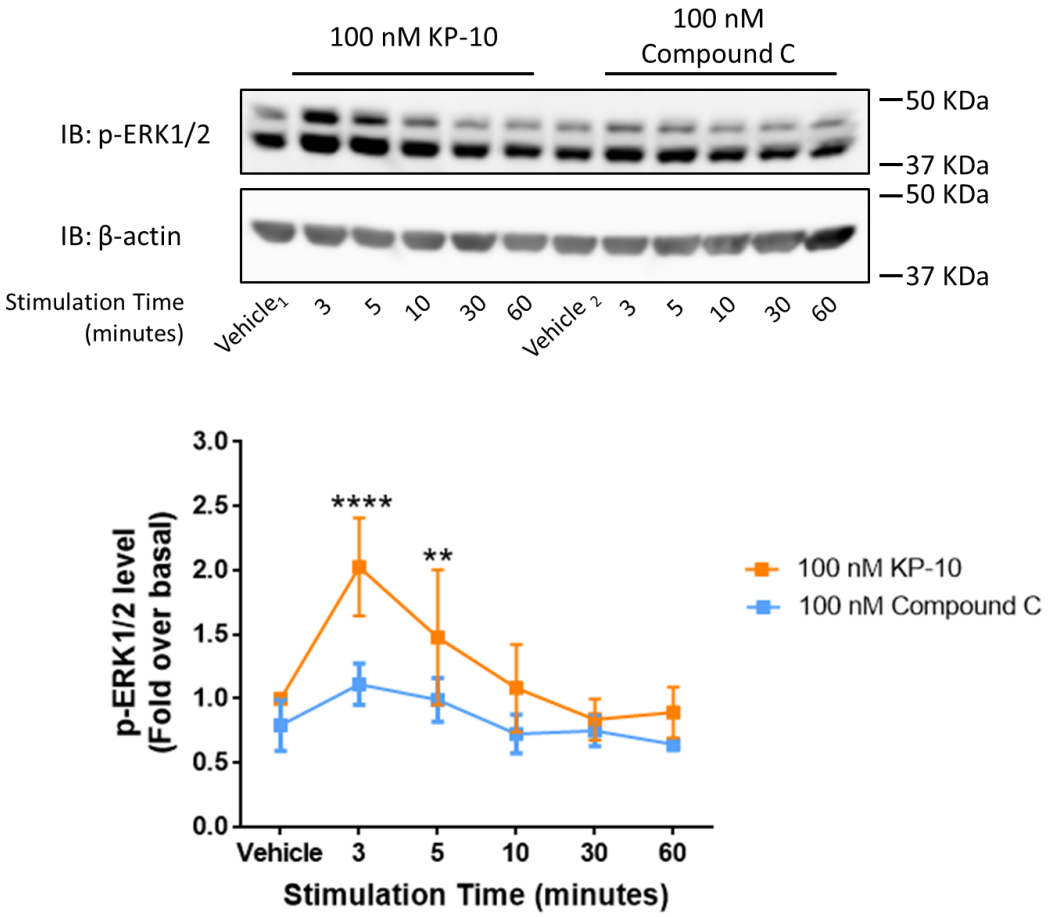


Figure 3.5 Immunoblots depicting time-dependent responses of the KP-10- or compound C-induced phosphorylation of ERK1/2 using HEK293 cells. HEK293 cells transiently transfected with flag-kisspeptin receptor cDNA were serum-starved for 20 hours prior to being treated with vehicle (vehicle$_1$, 0.02% (v/v) propylene glycol; vehicle$_2$, 0.00625% (v/v) DMSO and 0.001% (v/v) propylene glycol), 100 nM KP-10 (orange square) or 100 nM compound C (blue square) for the indicated times. Representative blots are shown. Data from three independent experiments were quantified (using β-actin as a loading control) and the mean fold over vehicle$_1$ treated control ± SD for the phosphorylation of ERK1/2 is presented. *p<0.01* (**) and *p<0.001* (****) represent statistical significance from vehicle$_1$ treated control.

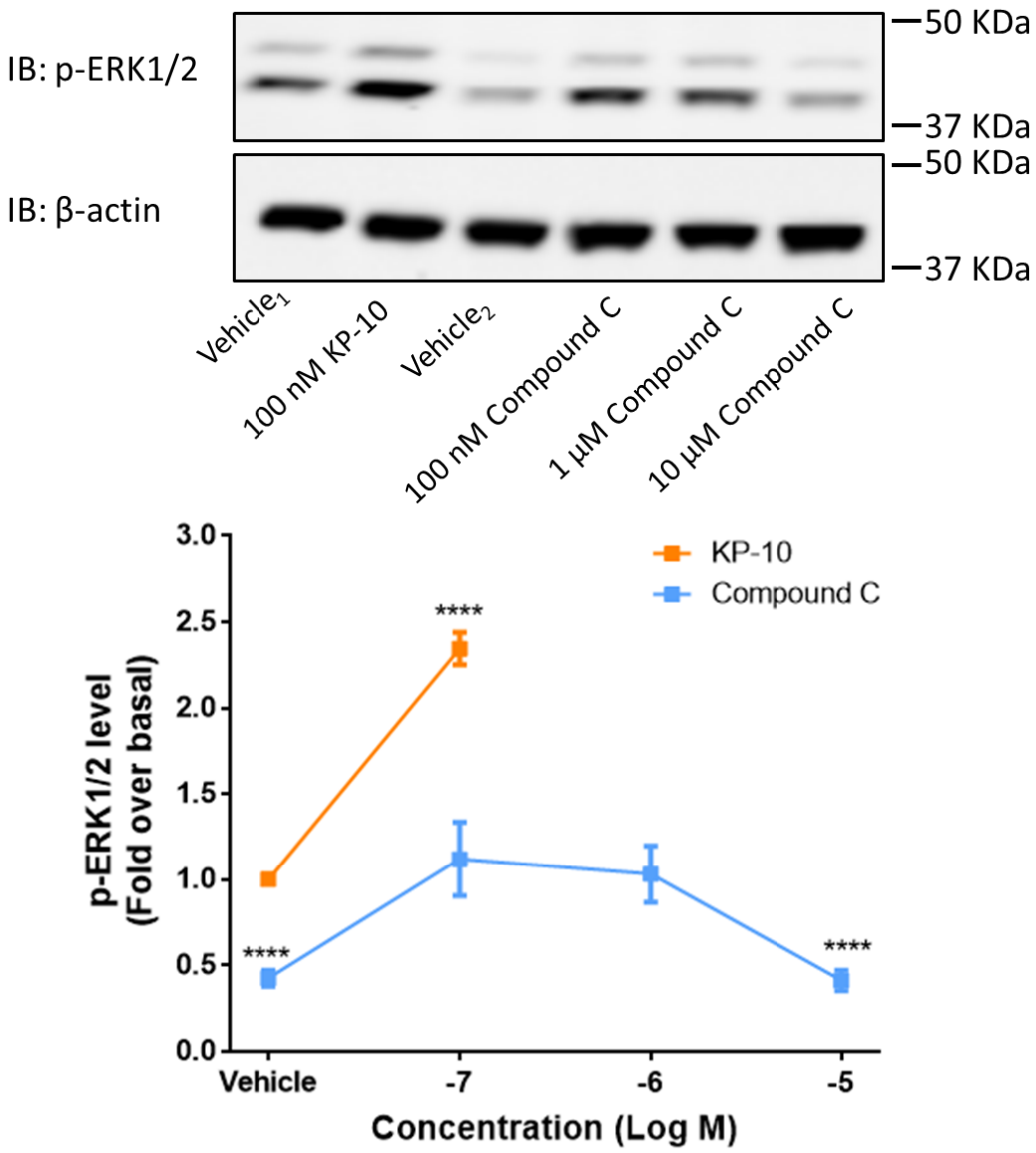


Figure 3.6 Immunoblots depicting a dose-dependent response of compound C-induced phosphorylation of ERK1/2 using HEK293 cells. HEK293 cells transiently transfected with HA-kisspeptin receptor cDNA were serum-starved for 20 hours prior to being treated with vehicle (vehicle$_1$, 0.02% (v/v) propylene glycol; vehicle$_2$, 0.625% (v/v) DMSO and 0.1% (v/v) propylene glycol), 100 nM KP-10 (orange square), or increasing does of compound C (0.1 µM, 1 µM and 10 µM; blue square) for 3 minutes. Representative blots are shown. Data from three independent experiments were quantified (using β-actin as a loading control) and the mean fold over vehicle$_1$ treated control ± SD for the phosphorylation of ERK1/2 is presented. *p<0.001* (****) represents statistical significance from vehicle$_1$ treated control.

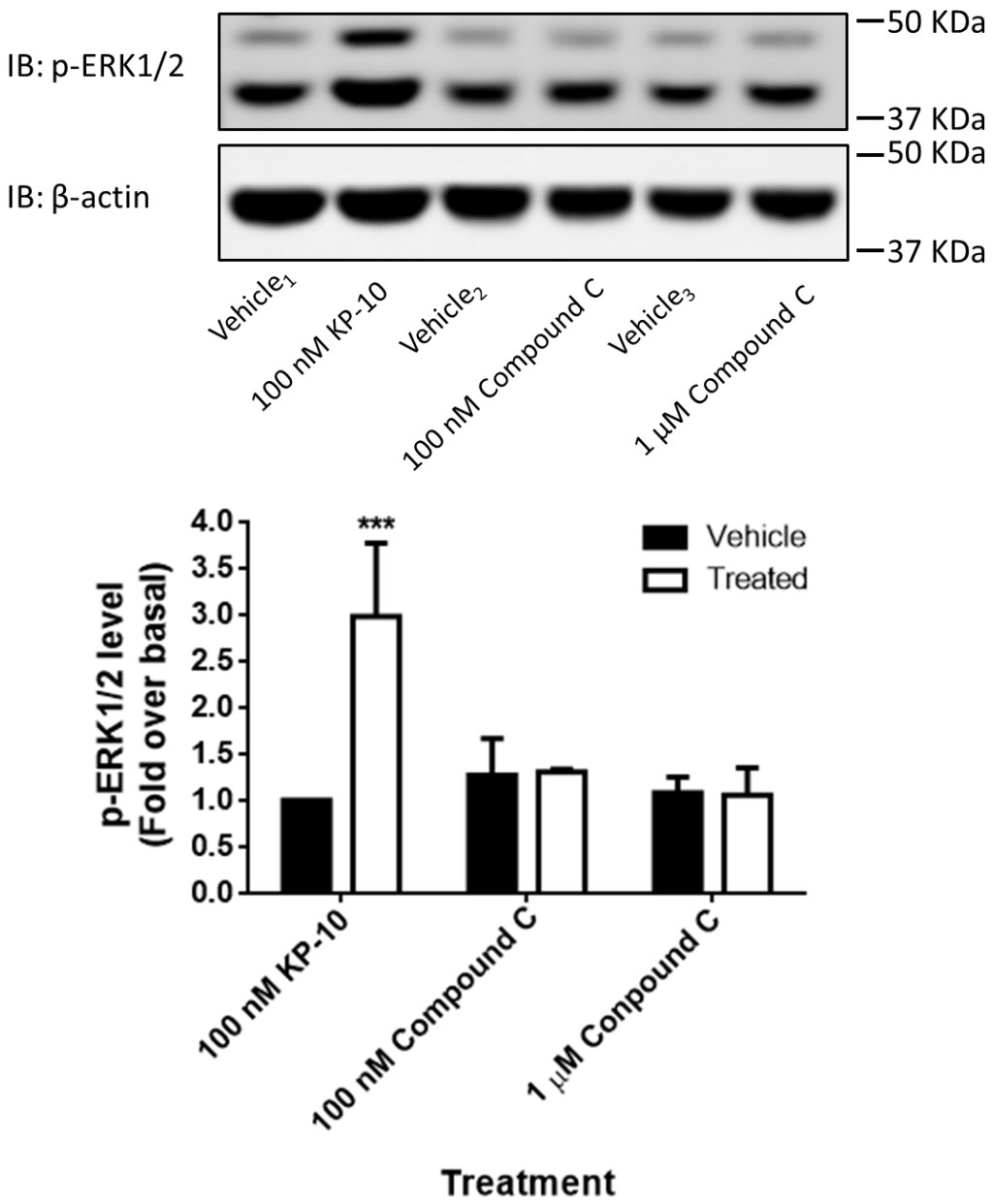


Figure 3.7 Immunoblots depicting a dose-dependent response of compound C-induced phosphorylation of ERK1/2 using HEK293 cells. HEK293 cells transiently transfected with HA-kisspeptin receptor cDNA were serum-starved for 20 hours prior to being treated with vehicle (vehicle$_1$, 0.02% (v/v) propylene glycol; vehicle$_2$, 0.00625% (v/v) DMSO and 0.001% (v/v) propylene glycol; vehicle$_3$, 0.0625% (v/v) DMSO and 0.01% (v/v) propylene glycol; black bar), 100 nM KP-10, or increasing does of compound C (0.1 μM and 1 μM) for 3 minutes. Representative blots are shown. Data from three independent experiments were quantified (using β-actin as a loading control) and the mean fold over vehicle$_1$ treated control ± SD for the phosphorylation of ERK1/2 is presented. *p<0.005* (***) represents statistical significance from vehicle$_1$ treated control.

The kisspeptin receptor-agonistic activities of these phosphinic peptides were then tested in MCF-7 cells transiently expressing the receptor. In the assays, KP-10 at a final concentration of 100 nM was used as a positive control. As seen in the previous results, neither 100 nM compound A nor compound B can stimulate the phosphorylation of ERK1/2 after 10-minute stimulation, when the maximum response was measured (Fig. 3.8). In addition, stimulation of 100 nM compound C did not induce increase in the level of phosphorylation of ERK1/2 at any tested time point (Fig. 3.9).

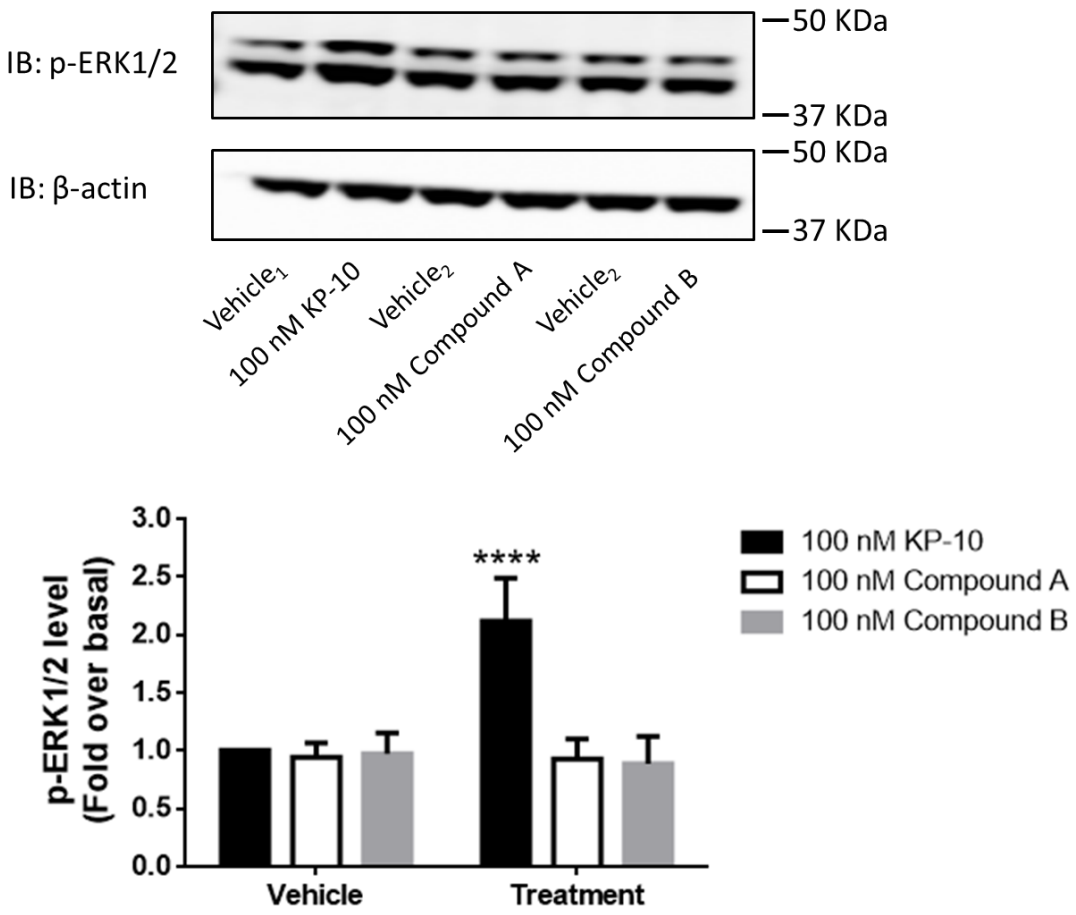


Figure 3.8 Immunoblots depicting the compound A- or B-induced phosphorylation of ERK1/2 using MCF-7 cells. MCF-7 cells transiently transfected with flag-kisspeptin receptor cDNA were serum-starved for 20 hours prior to being treated with vehicle (vehicle$_1$, 0.02% (v/v) propylene glycol; vehicle$_2$, 0.0002% (v/v) DMSO), 100 nM KP-10 (black bar), compound A (white bar) or compound B (grey bar) for 10 minutes. Representative blots are shown. Data from three independent experiments were quantified (using β-actin as a loading control) and the mean fold over vehicle$_1$ treated control ± SD for the phosphorylation of ERK1/2 is presented. *p<0.001* (****) represent statistical significance from vehicle$_1$ treated control.

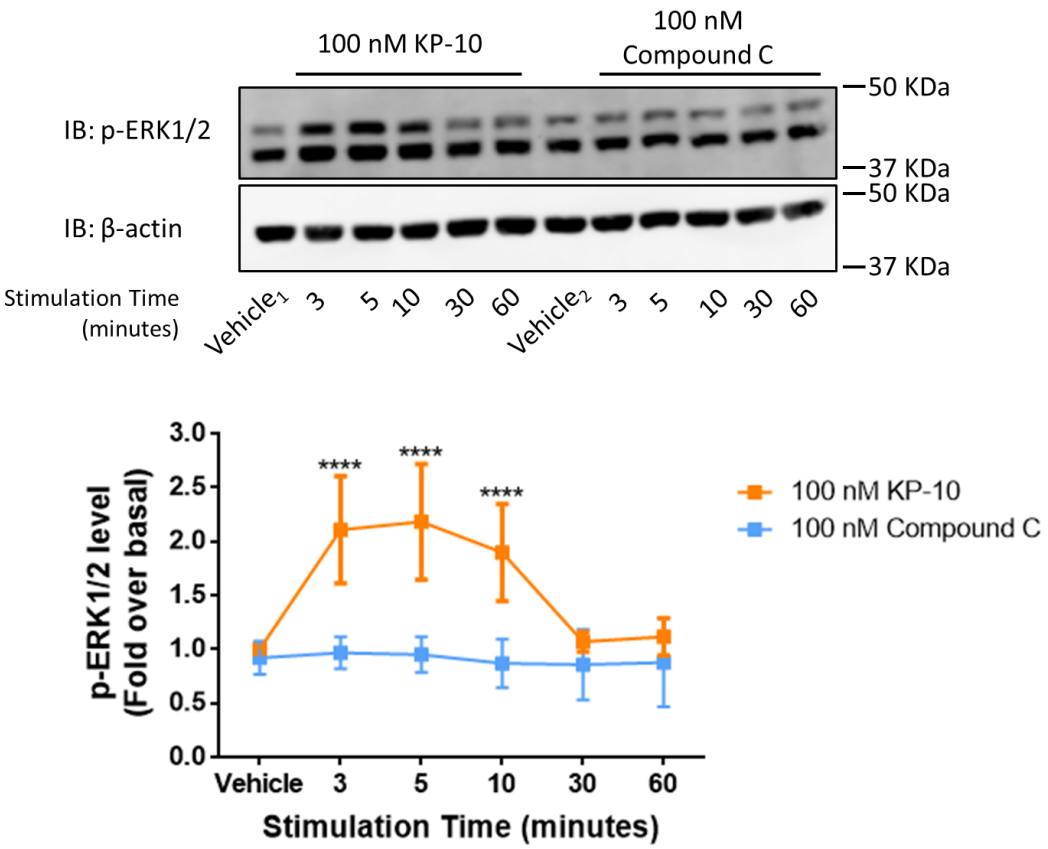


Figure 3.9 Immunoblots depicting time-dependent responses of the KP-10- and compound C-induced phosphorylation of ERK1/2 using MCF-7 cells. MCF-7 cells transiently transfected with flag-kisspeptin receptor cDNA were serum-starved for 20 hours prior to being treated with vehicle (vehicle$_1$, 0.02% (v/v) propylene glycol; vehicle$_2$, 0.00625% (v/v) DMSO and 0.001% (v/v) propylene glycol), 100 nM KP-10 (orange square) or 100 nM compound C (blue square) for the indicated times. Representative blots are shown. Data from four independent experiments were quantified (using β-actin as a loading control) and the mean fold over vehicle$_1$ treated control ± SD for the phosphorylation of ERK1/2 is presented. *p<0.001* (****) represents statistical significance from vehicle$_1$ treated control.

3.3.3 Determination of the kisspeptin receptor-antagonistic activities of kisspeptin analogues

Since compound A and C did not possess detectable kisspeptin receptor-agonistic activities, and compound B can induce the phosphorylation of ERK1/2 only at a very high concentration, their antagonistic activities were then examined. In order to do this, the ability of these phosphinic peptides to inhibit the KP-10-induced phosphorylation of ERK1/2 was tested. MCF-7 cells transiently expressing the kisspeptin receptor was used as a model. For these assays, the cells were first treated with 10 μM of the peptides for 30 minutes. After that, 100 nM KP-10 was added for further 10 minutes to stimulate the phosphorylation of ERK1/2, which was measured as before (Section 3.3.3). β-actin is used as a loading control and the mean fold over control (as indicated in each figure legend) for each sample is calculated and plotted. The treatment of 10 μM compound A or B did not affect KP-10-induced phosphorylation of ERK1/2 (Fig. 3.10). By contrast, the level of KP-10-induced phosphorylation of ERK1/2 was slightly increased in the 10 μM compound C treated group compared with the vehicle treated control (Fig. 3.11).

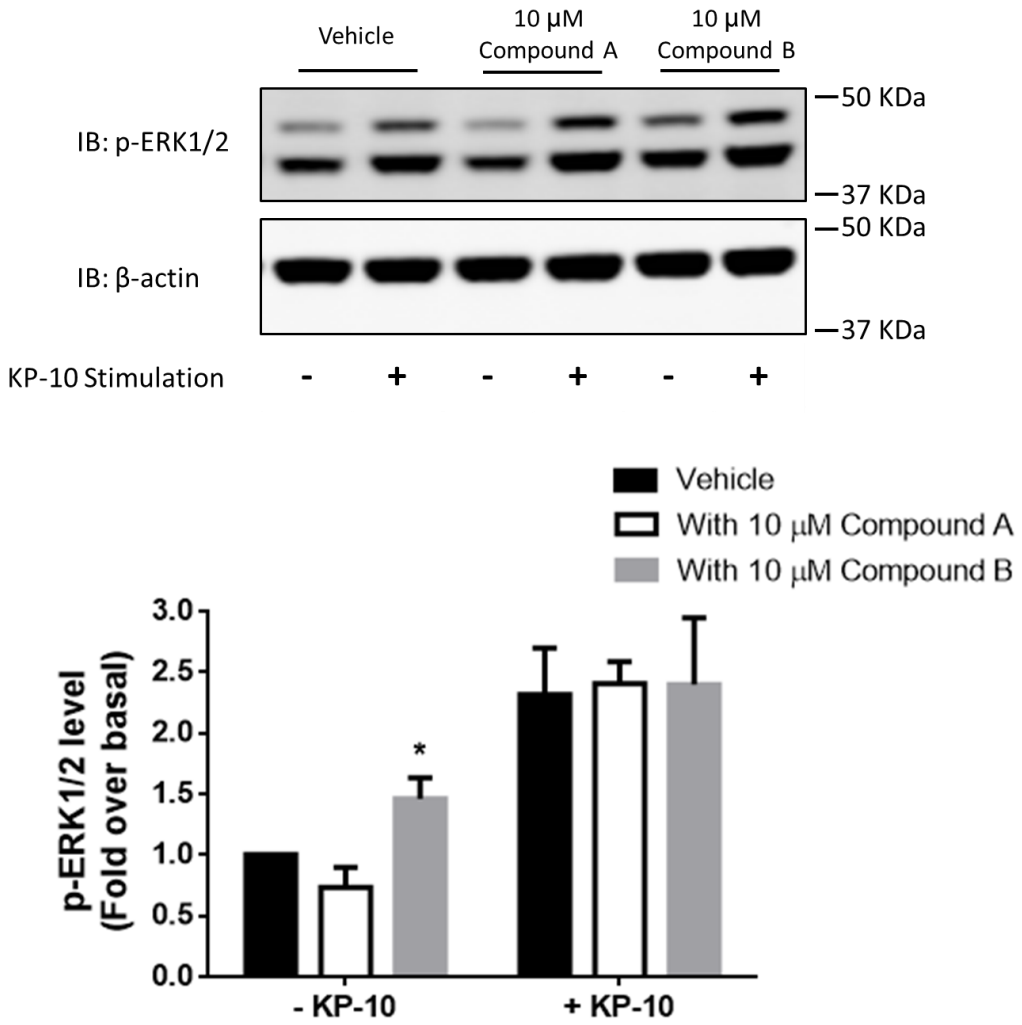


Figure 3.10 Immunoblots depicting the effect of compound A and B on KP-10-induced phosphorylation of ERK1/2 using MCF-7 cells. MCF-7 cells transiently transfected with flag-kisspeptin receptor were serum-starved for 20 hours. After that cells were treated with vehicle (0.02% (v/v) DMSO; black bar), 10 µM compound A (white bar) or 10 µM compound B (grey bar) as indicated for 30 minutes. After that, 100 nM KP-10 was added (+) for further 10-minute stimulation. For the group without KP-10 stimulation (-), 0.02% (v/v) propylene glycol (KP-10 vehicle) was added. Representative blots are shown. Data from three independent experiments were quantified (using β-actin as a loading control) and the mean fold over control (KP-10 vehicle treated) ± SD for the phosphorylation of ERK1/2 is presented. *p<0.05* (*) represents statistical significance from KP-10 vehicle treated control.

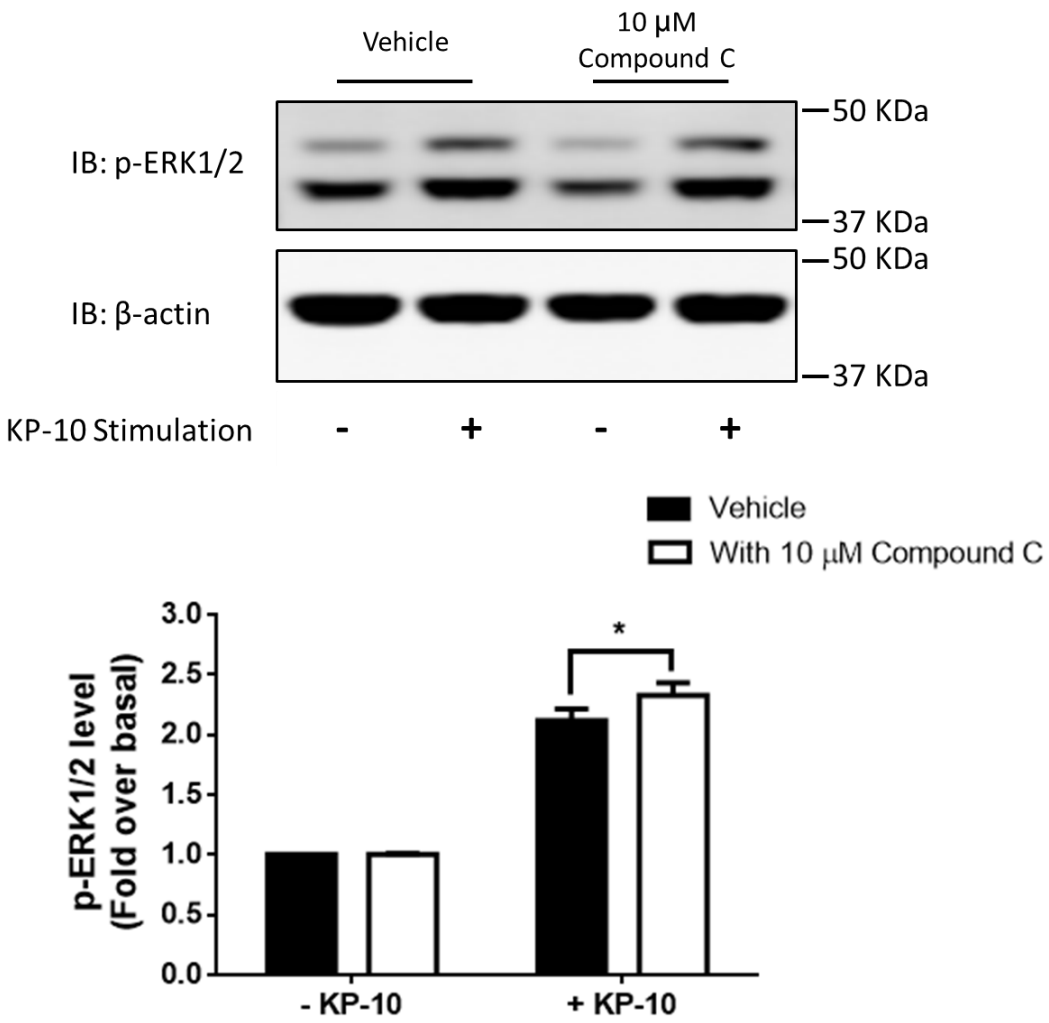


Figure 3.11 Immunoblots depicting the effect of compound C on KP-10-induced phosphorylation of ERK1/2 using MCF-7 cells. MCF-7 cells transiently transfected with flag-kisspeptin receptor were serum-starved for 20 hours. After that cells were treated with vehicle (0.625% (v/v) DMSO and 0.1% (v/v) propylene glycol; black bar) or 10 μM compound C (white bar) as indicated for 30 minutes. After that, 100 nM KP-10 was added (+) for further 10-minute stimulation. For the group without KP-10 stimulation (-), 0.02% (v/v) propylene glycol (KP-10 vehicle) was added. Representative blots are shown. Data from three independent experiments were quantified (using β-actin as a loading control) and the mean fold over control (KP-10 vehicle treated) ± SD for the phosphorylation of ERK1/2 is presented. *$p<0.05$* (*) represents statistical significance from vehicle treated control.

3.3.4 Determination of the binding of Cy5-KP18 and kisspeptin analogues to the kisspeptin receptor

Due to the limitation in our laboratory with respect to using radioactivity, an attempt was made to establish a fluorescence-based ligand-binding assay using PHERAstar FS microplate reader (BMG LABTECH, Germany). The assay was conducted using sulfo-cyanine5 (Cy5) labelled KP-18 peptide (Cy5-KP18). The peptide was synthesized by Chinese Peptides (Hangzhou, China). A saturation binding assay was tried to detect the binding of Cy5-KP18 to the kisspeptin receptor (Section 2.13). Briefly, the total binding was measured by incubating a fixed number of HEK293 cells expressing the kisspeptin receptor with increasing concentrations of Cy5-KP18 from 1 nM to 1 µM and the non-specific binding was determined at each concentration of Cy5-KP18 by co-incubating with 100 µM unlabeled KP-10. Fig. 3.12 represents the binding curve of Cy5-KP18. The specific binding (green curve) of Cy5-KP18 is calculated by subtracting the non-specific binding (purple curve) from the total binding (orange curve). A significant specific binding was detected at 50 nM or higher concentrations of Cy5-KP18. The saturation binding of Cy5-KP18 was not observed even using very high concentration (up to 3 µM; data were not shown). Therefore, the Kd value of Cy5-KP18 cannot be calculated using a Langmuir isotherm model (Klotz, 1982).

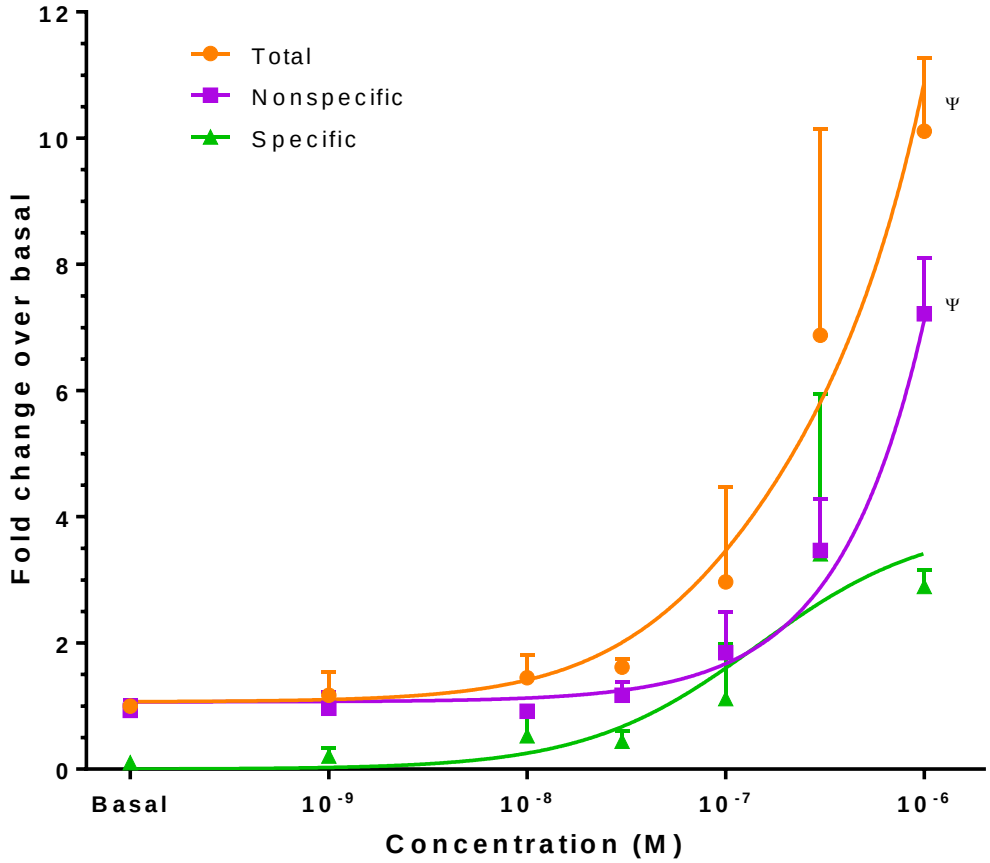


Figure 3.12 Binding of Cy5-KP18 to the kisspeptin receptor. HEK293 cells were transiently transfected with flag-kisspeptin receptor and cultured for 48 hours. Cells were incubated with increasing concentrations of Cy5-KP18 as indicated. The amount of ligands bound to the kisspeptin receptor expressed in living cells was measured by fluorescence intensity. The fold change over basal is calculated and plotted. Total binding (orange circle and line) and non-specific binding (purple square and line) is calculated from three independent experiments. The amount of specific binding (green triangle and line) is calculated by subtracting non-specific binding from the total. The mean fold over individual baseline ± SD for the level of bound Cy5-KP18 is presented. Ψ, data are calculated from two independent experiments.

Since the significant binding of Cy5-KP18 could only be observed at 50 nM or higher concentrations, it is impossible to use competition binding assay to determine the binding affinity of the phosphinic peptides. Hence an agonist-induced receptor internalization assay was used to detect if the phosphinic peptides could bind to the kisspeptin receptor. Considering the potentially low binding affinity of these peptides, 50 nM Cy5-KP18 was initially used. Their binding affinity was determined by measuring the competition abilities of the unlabelled peptides on the Cy5-KP18-induced internalization of the kisspeptin receptor in MCF-7 cells. Unlabeled KP-10 was used as a positive control, which led to a maximum 22.28% ± 7.960 inhibition (at concentration of 5 μM) on Cy5-KP18-induced internalization of the kisspeptin receptor. By contrast, compound A, B or C cannot inhibit the internalization of Cy5-KP18 at any concentration tested (Fig. 3.13). The failure of the inhibition of the peptides on the internalization of Cy5-KP18 may be due to the high concentration of Cy5-KP18 used and/or low binding affinity of the phosphinic peptides to the kisspeptin receptor.

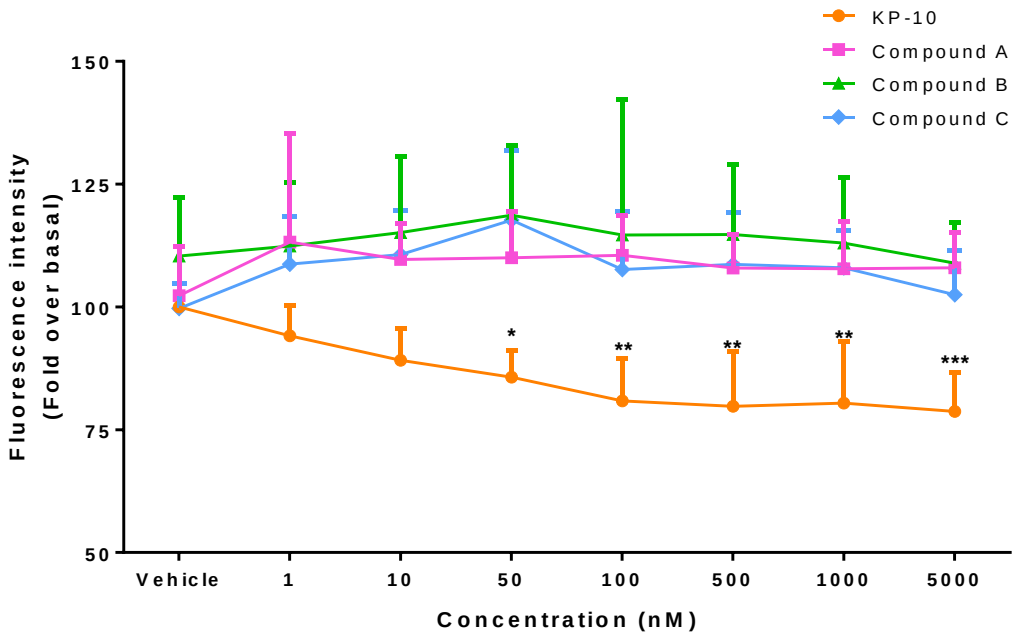


Figure 3.13 Effect of the kisspeptin analogues on the Cy5-KP18-induced internalization of the kisspeptin receptor using MCF-7 cells. MCF-7 cells were transiently transfected with flag-kisspeptin receptor and cultured for 48 hours. Cells were co-incubated with 50 nM Cy5-KP18 and different concentrations of unlabeled KP-10 (orange circle) or the kisspeptin analogues (0-5 µM), including compound A (pink square), B (green triangle) and C (blue diamond) for 30 minutes. The baseline activity was measured in the absence of KP-10 or the kisspeptin analogues. Data from four independent experiments were quantified (compared with the baseline) and the mean fold over the baseline ± SD for the level of internalized Cy5-KP18 is presented. *p<0.05* (*), *p<0.01* (**) and *p<0.005* (***) represent statistical significance from vehicle treated control.

3.3.5 Inhibition of kisspeptin analogues on MMPs

In addition to the above functional and binding assays, the inhibition of these peptides on the enzyme activity of MMP-2 and MMP-9 was tested by using MMP-2 and MMP-9 colorimetric drug discovery kits (Section 2.19) (Enzo Life Sciences, USA). For the control group, the enzyme activity of MMP-2 (Fig. 3.14) and MMP-9 (Fig. 3.15) was measured without addition of any inhibitor. NNGH is a potent inhibitor of MMPs and used as positive control. The MMP activities were tested by measuring the hydrolysis activities of a colorimetric substrate of MMPs via continuously reading the absorbance at 412 nm. The slope is calculated and the mean fold over control is plotted in bar chart. Only compound B can inhibit 35.98% ± 10.22 of MMP-2 activity at 10 μM. However, lower concentration of compound B (1 μM) was unable to inhibit MMP-2 activity (Fig. 3.14). None of these peptides showed inhibition activity on MMP-9 (Fig. 3.15).

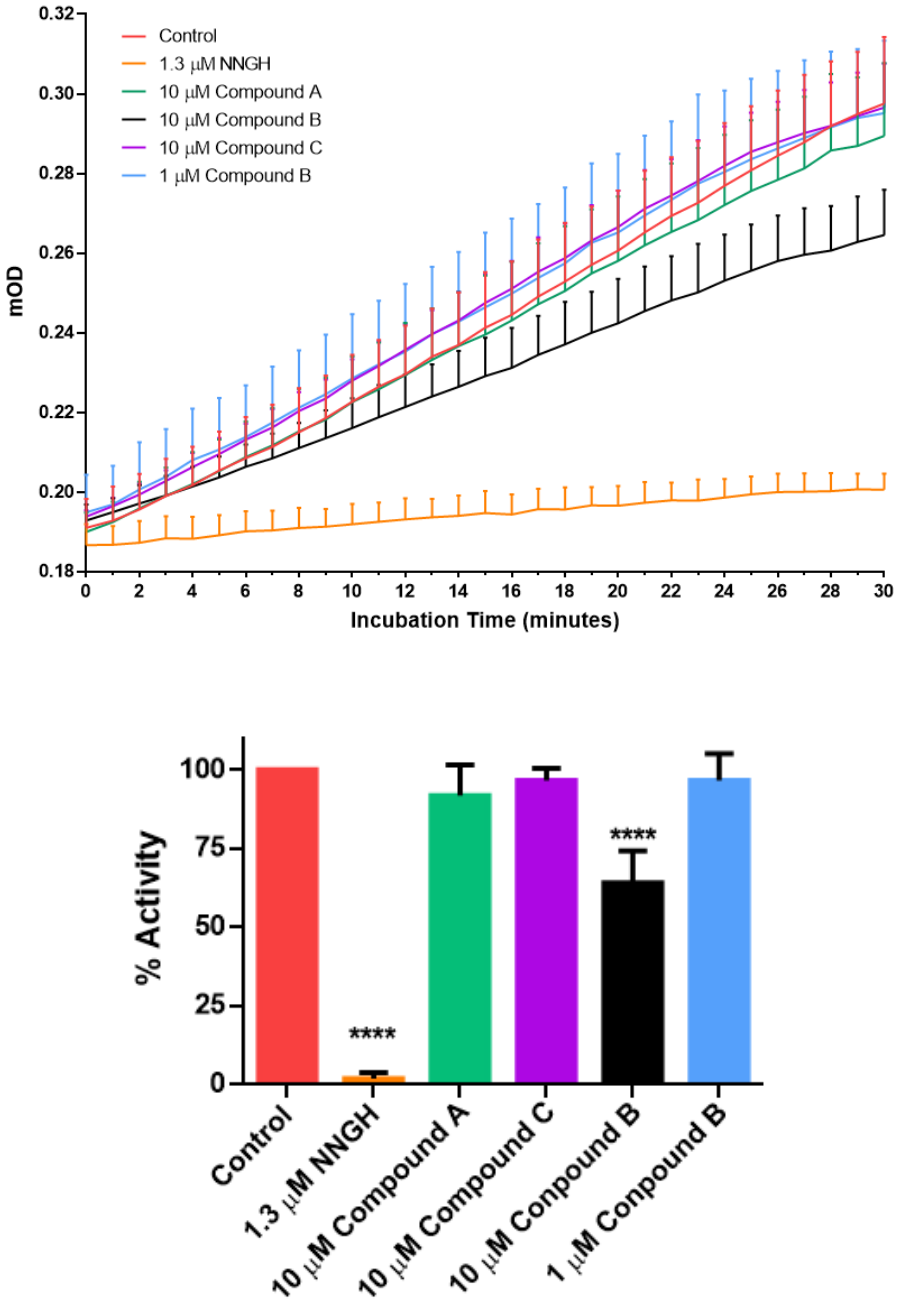


Figure 3.14 Effect of the kisspeptin analogues on MMP-2 enzyme activities. The MMP-2 inhibition assay was conducted using a kit based assay in a 96-well microtiter plate as per the manufacturer's instructions. The kisspeptin analogues, including 10 µM compound A (green), B (black), C (purple) and 1 µM compound B (blue), or a positive control (NNGH, orange) as indicated were added to an assay mixture containing MMP-2 enzyme. The baseline activity (red) was measured in the absence of inhibitors. The plates were read at 412 nm in a microplate reader for 30 minutes. Data from at least three independent experiments were quantified (compared with the baseline activity) and the mean fold over control ± SD for the inhibition of MMP-2 activity is presented. *p<0.001* (****) represents statistical significance from control.

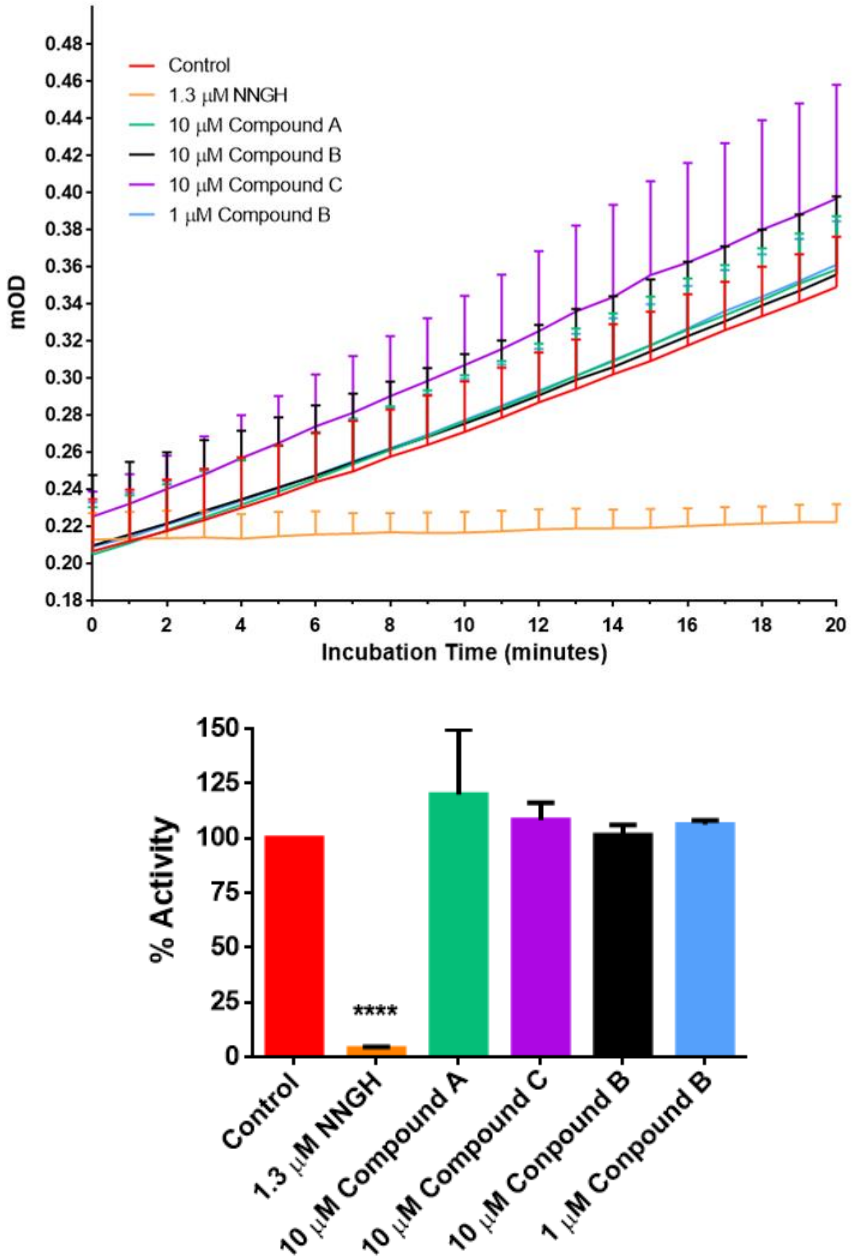


Figure 3.15 Effect of the kisspeptin analogues on MMP-9 enzyme activities. The MMP-9 inhibition assay was conducted using a kit based assay in a 96-well microtiter plate as per the manufacturer's instructions. The kisspeptin analogues, including 10 µM compound A (green), B (black), C (purple) and 1 µM compound B (blue), or a positive control (NNGH, orange) as indicated were added to an assay mixture containing MMP-9 enzyme. The baseline activity (red) was measured in the absence of inhibitors. The plates were read at 412 nm in a microplate reader for 20 minutes. Data from at least three independent experiments were quantified (compared with the baseline activity) and the mean fold over control ± SD for the inhibition of MMP-9 activity is presented. *p<0.001* (****) represents statistical significance from control.

3.4 Discussion

In this chapter, the biological functions of three novel kisspeptin analogues were assessed by the stimulation of phosphorylation of ERK1/2. The results showed that compound B possesses the kisspeptin receptor-agonistic activity at a high concentration (10 μM). The agonistic activity cannot be detected in lower concentrations of compound B or any tested concentration of its diastereoisomer, compound A. It has been shown that the last five amino acids at C-terminus of KP-10 are stereochemically essential for its binding and activation of the kisspeptin receptor (Niida et al., 2006). Substitution of each of the residues with their D-enantiomers leads to complete loss of the kisspeptin receptor-agonistic activity. Therefore, it is not surprising that only one of the synthesized enantiomers possesses the kisspeptin receptor-agonistic activity. However, among them, compound B, which is predicated as an *R*-isomer at the pseudo-Leu residue, exhibited the kisspeptin receptor-agonistic activity. The mechanisms underlying it remain unclear and the structures of the enantiomers need to be determined. Since compound B exhibited the kisspeptin receptor-agonistic activity at a high concentration, a 10-amino-acid phosphinic peptide, which is termed compound C, was synthesized to improve its agonistic activity. However, compound C cannot stimulate the phosphorylation of ERK1/2 at any concentration tested. Since compound C is a mixture of the stereoisomer pair, it is possible that one of two enantiomers may possess the kisspeptin receptor-agonistic activity. The kisspeptin receptor-agonistic activity is masked by the properties of the other isomer, which may possess the kisspeptin receptor-antagonistic activities and/or higher binding affinity to the kisspeptin receptor. As a consequence, the kisspeptin receptor-agonistic activity cannot be

detected by the method. In this regard, the kisspeptin receptor-antagonistic activities of the phosphinic peptides were then examined by detecting their inhibition on KP-10-induced phosphorylation of ERK1/2. The results showed that none of them can inhibit KP-10-induced phosphorylation of ERK1/2 and, therefore, these peptides might not be antagonists of the kisspeptin receptor. Additionally, it is also possible that the kisspeptin receptor-antagonistic activity of these peptides cannot be detected by current method. The binding affinity of the peptides to the kisspeptin receptor may not be high enough to allow them competitively bind to the receptor with 100 nM KP-10.

In order to detect the kisspeptin receptor binding properties of kisspeptin analogues, a fluorescence-based ligand-binding assay was developed. In order to minimize the effects of a conjugation of fluorophore on the properties of kisspeptin, Cy5 was introduced to the N-terminus of KP-18. To assess whether the fluorescent-labelled KP-18 retains its ability to bind to the kisspeptin receptor, a saturation binding assay was carried out in living cells. The specific binding of Cy5-KP18 can only be detected at 50 nM or higher concentrations. Such a high concentration of ligands precludes the use of a standard competition binding assay to determine of the binding properties of unknown and unlabeled peptides. Alternatively, it was found that the fluorescent agonist can be co-internalised with the receptor upon its stimulation (Arttamangkul et al., 2000). Based on this, competition by these peptides for Cy5-KP18-induced internalization of the kisspeptin receptor in living cells was used to indirectly measure their binding properties. Cy5-KP18-induced internalization of the kisspeptin receptor was significantly reduced after co-incubation with the same

concentration of KP-10. However, none of the kisspeptin analogues can affect Cy5-KP18-induced internalization of the receptor at any concentration tested. Therefore, whether these kisspeptin analogues can bind to the kisspeptin receptor cannot be determined by this method. It is possible that these peptides possess binding affinity to the kisspeptin receptor, but affinity is lower than KP-10 and Cy5-KP18 and so beyond the limit of detection of the assays used here.

Since phosphinic peptides have been found to be potent inhibitors of MMPs (Dive et al., 2004, Georgiadis and Dive, 2015), the inhibition of the kisspeptin analogues on MMP-2 and MMP-9 was detected. Among these peptides, compound B inhibited MMP-2 activity at a high concentration, but not MMP-9 activity, indicating that compound B could be a weak and selective inhibitor toward MMP-2. In addition, the results also suggest that compound B may form a stable complex with MMP-2 and, thus, may be resistant to the MMP-2-mediated hydrolysis. For the rest of the peptides, none were able to inhibit MMP-2 or MMP-9. Whether the phosphinic peptides are resistant to MMP-mediated digestion and have inhibitory activity towards other MMPs remains to be elucidated.

In conclusion, among synthesized peptides, only compound B possesses the kisspeptin receptor-agonistic activities and could function as a selective inhibitor of MMP-2. Due to the limitation of current methods, the kisspeptin receptor-antagonistic activities and binding properties of the synthesized peptides cannot be detected. To date, there have been very few studies focused on the development of kisspeptin analogues with increased potency and metabolic stability. One main

approach to develop more stable kisspeptin analogues is based on the substitution of key residues of kisspeptin, which are susceptible to enzyme digestion. These residues include Trp^{47}, Gly^{51} (Asami et al., 2012b) and Arg^{53} (Asami et al., 2012a). In addition, only one publication was found that has been successfully developed MMP-2 and MMP-9-resistant kisspeptin analogues by a replacement of Gly^{51}-Leu^{52} with non-hydrolysable dipeptide isostere (Tomita et al., 2008). Here, we proposed a new option to develop novel kisspeptin analogues that may target both MMP-2 and the kisspeptin receptor by replacing the peptide bound between Gly^{51} and Leu^{52} with a phosphinic acid moiety. The results provide an insight for the further development of this kind of kisspeptin analogues.

Chapter 4

Identification of novel kisspeptin receptor-interacting proteins and their biological functions

4.1 Outline

In addition to G proteins, a wide range of GIPs have been reported. Direct associations of GPCRs through their Pro-rich motif with SH3 domain-containing proteins are observed in some GPCRs. The kisspeptin receptor contains a Pro-rich motif within its C-terminal tail, which may be able to mediate the interaction of the receptor with PP2A. In this chapter, the interaction of the human kisspeptin receptor with two potential interacting proteins, the p85α subunit of PI3K and c-SRC were investigated *in vitro* by using co-IP assays (Section 2.16). In addition, the potential biological functions of these interactions were examined by western blot (Section 2.17). Furthermore, mutant receptors with single Ala substitution on the predicted sites (Pro^{342}, Pro^{345}, Pro^{348} and Pro^{353}), which may mediate the kisspeptin receptor/p85α and the kisspeptin receptor/c-SRC interactions, was conducted by site-directed mutagenesis (Section 2.9) for further examinations.

4.2 Introduction

Conventionally, signalling by GPCRs has been considered to be mediated solely by their coupled G proteins. Upon ligand stimulation, the activated GPCRs act as GEFs for heterotrimeric G protein and promote the dissociation of G_{α} subunits from $G_{\beta\gamma}$ subunits (Neer, 1995). Subsequently, the free G_{α} subunit and the Gβγ complex are able to transmit intracellular signals *via* the activation of various target effectors (Neer, 1995). However, recent work has discovered that GPCRs can interact with numerous non-G proteins, such as spinophilin (Wang et al., 2004), calmodulin (Labasque et al., 2008) and protein phosphatases (Evans et al., 2008), to mediate G protein-independent signalling pathways (Luttrell, 2008). Like other GPCRs, the kisspeptin receptor has been shown to interact with both G and non-G proteins (Fig. 1.6). In general, the kisspeptin receptor interacts with $G_{q/11}$ subfamilies to activate PLC-βs and, consequently, the mobilization of intracellular calcium and the activation of PKC (Section 1.4.1) (Muir et al., 2001, Kotani et al., 2001). In addition, the kisspeptin receptor can also interact with non-G proteins (Section 1.4.2). The kisspeptin receptor has been shown to interact with β-arrestins to activate signalling pathways, such as MAPKs cascades (Luttrell et al., 1999). In addition to β-arrestins, the kisspeptin receptor has three Pro-Arg-Arg repeats within the C-terminal tail, which have been shown to mediate the interaction of the receptor with PP2A-B and PP2A-C subunits (Evans et al., 2008). Moreover, the Pro-Arg-Arg repeats are also predicted by motif scanning to mediate the interactions of the kisspeptin receptor with other SH3-domain containing proteins, such as amphiphysin, p85 and SRC. However, whether the kisspeptin receptor in fact interacts with the SH3-domain

containing proteins and their biological functions remain to be elucidated in cellular studies.

P85α is one of the regulatory subunits (p85) of class IA PI3K and has a p110 binding motif flanking by two SH2 domains and a N-terminal SH3 domain (Hawkins et al., 2006). P85 interacts with the catalytic subunit of PI3K, p110, to inhibit the activity of p110. After extracellular signal stimulation, the SH2 domains of p85 mediates the interaction with phosphorylated Tyr residues in activated receptors or adaptors to release p85α from p110 and, consequently, relieves the inhibition on p110 (Geering et al., 2007). In addition, the SH3 domain of p85 also plays an important role of the regulation of PI3K signalling (Pleiman et al., 1994). For example, p85 is shown to be co-immunoprecipitated with obscurins in MCF-10A cells (Shriver et al., 2014). The GST-pull down assay using GST fusion protein containing obscurin-PH domain and His-tagged p85-SH3 domain reveals that the association between obscurins and p85 is mediated by PH-SH3 domain interaction. Knockdown of obscurin by shRNA in MCF-10A cells results in increased level of phosphorylation of all tested downstream effectors of PI3K including AKT, PDK1 and glycogen synthase kinase 3β (GSK3β), in the absence of any stimulation. These data suggest that obscurins regulate the PI3K/AKT pathway through the direct interaction with SH3 domain of p85 and the loss of obscurins in breast epithelial cells may impair the regulatory activity of p85 on p110. By contrast, influenza A virus infection triggers PI3K activation *via* the interaction between encoding protein NS1 and p85 (Shin et al., 2007). Co-IP and GST-pull down assays reveal that NS1 formed a complex with p85 subunit mediated by

both SH3 and the C-terminal SH2 domain of p85. NS1 strongly interacts with the SH3 domain of p85, while NS1 couples to the C-terminal SH2 domain of p85 with a lesser degree. Mutations on both SH2- and SH3-binding sites within NS1 abolish the interaction with p85 and impair the phosphorylation of AKT in response to the infection, suggesting that NS1 associates with p85 through the SH3- and SH2-binding to activate PI3K/AKT pathway. The kisspeptin receptor is able to activate PI3K/AKT signalling pathway in a cell-dependent manner (Section 1.4.4) (Stathatos et al., 2005, Luque et al., 2011). An inhibitor of PI3K abolishes kisspeptin-induced LH stimulation in baboon pituitary cells, indicating an important role of PI3K/AKT pathway in the signalling of kisspeptins and the kisspeptin receptor (Luque et al., 2011). However, detailed the kisspeptin receptor/PI3K/AKT signalling pathway has not been fully elucidated.

C-SRC is a non-receptor tyrosine kinase and encoded by *src*, which is one of the most investigated proto-oncogenes, in humans (Martin, 2001). It has been shown that c-SRC is involved in the regulating the internalization of some GPCRs. For example, the level of dynamin-mediated internalization of M_1 muscarinic acetylcholine receptor is reduced by either impairing c-SRC kinase activity through the K298M mutation or substituting Tyr residues on dynamin, which are the target sites of c-SRC, upon agonist stimulation (Werbonat et al., 2000). Consistently, the agonist-induced internalization of β_2AR is reduced either by impairing c-SRC kinase activity (K298M) or in the presence of the c-SRC kinase inhibitor (Ahn et al., 1999). Besides, c-SRC has also been demonstrated to mediate the signalling of GPCRs. Some GPCRs, such as

β_2AR (Luttrell et al., 1999), can recruit c-SRC *via* β-arrestins to phosphorylate ERK1/2, while some GPCRs, including β_3AR (Cao et al., 2000), can directly interact with c-SRC to trigger the phosphorylation of ERK1/2, or to induce the phosphorylation of c-SRC and other receptors such as platelet-derived growth factor receptor and EGFR (Liu et al., 2004). The kisspeptin receptor has been shown to activate c-SRC-dependent signalling pathways. In the presence of c-SRC inhibitor, reductions in KP-10-induced phosphorylation of ERK1/2 and glycogen synthase kinase 3β were observed in HTR8SVneo immortalized extravillious trophoblast-derived cells (Roseweir et al., 2012). The c-SRC-dependent signalling of the kisspeptin receptor may be mediated by β-arrestins, since β-arrestins are shown to assist the recruitment of c-SRC to the receptor (Section 1.4.2) (Luttrell et al., 1999). However, whether the kisspeptin receptor can directly interact with c-SRC is unclear.

Therefore, the studies of the interaction of the kisspeptin receptor with p85α and c-SRC predicted by motif scanning might provide more details of kisspeptin/the kisspeptin receptor-regulated cellular responses. In this chapter, the kisspeptin receptor/p85α and the kisspeptin receptor/c-SRC interactions were investigated by co-IP assays in MCF-7 cells, a breast cancer cell line, and their potential functions were explored.

4.3 Results

4.3.1 Prediction on GIPs for the human kisspeptin receptor

The potential GIPs for the human kisspeptin receptor were initially predicted by using the NEW Scansite 3 (Obenauer, 2003). Binding motifs for 70 mammalian kinases/domains provided by the software were scanned on the amino acid sequence of the human kisspeptin receptor (GenBank, AAK83235.1) with high stringency level. The New Scansite 3 assigns scores to indicate how the primary sequences of an enquired protein match the binding motifs of potential interacting proteins. A score-value of 0.000 is assigned to the optimal match. A higher score-value indicates a poorer match. High stringency only reports the matching sequences of an enquired protein, when their scores falls within the top 0.2% of all matching sequences contained in vertebrate proteins in the SwissProt database. As summarised in table 4.1, six SH3 binding motifs, which are specific to amphiphysin and p85, and a kinase binding site group for ERK D-domain were identified. In order to find a wider range of potential binding sites, a medium stringency level, which reduces the threshold to 1%, was applied. Additional potential interactions were PLK1 kinase, protein kinase A, 14-3-3 model 1 and SRC SH3 binding motifs (Table 4.2). When the surface accessibility is considered, highly possible interacting proteins were amphiphysin, p85 and SRC *via* the SH3 binding motif on the C-terminal tail of the human kisspeptin receptor. Another interacting site with high surface accessibility is basophilic Ser/Thr kinase for protein kinase A. However, the phosphorylation site is located on the ECL1 of the human kisspeptin receptor and so is most unlikely to be phosphorylated by PKA. Since p85α and c-SRC are pivotal in cellular pathophysiology, they are selected as candidate proteins.

Table 4.1 Prediction of motif sites on the human kisspeptin receptor by using the New Scansite 3 with high stringency. The table presents details of the predicted interacting proteins and the potential binding sites within the human kisspeptin receptor. The GenBank accession number of the human kisspeptin receptor used for prediction is AAK83235.1. The score indicates how the amino acid sequence of the receptor matches the binding motif. The optimal match is assigned a score-value of 0.000 and higher scores indicate poorer matches. The percentile shows the percentile that the score of matching sequences of the kisspeptin receptor falls into compared with all matching sequences contained in vertebrate proteins in the SwissProt database. The surface accessibility indicates the accessibility of matching sequences of the kisspeptin receptor. A higher value of surface accessibility indicates a higher accessibility of the matching sequence.

Predicted Motif Sites (Table)

Please allow popups in your browser settings to make links in the table work properly!

Score	Percentile	Motif	▲ Motifgroup	Site	Sequence	Surface Accessibility
0.309	0.008%	Erk D-domain (ErkDD)	Kinase binding site group (Kin_bind)	L160	RRTPRLAlAVSLSIW	0.1582
0.465	0.122%	Amphiphysin SH3 (Amphi_SH3)	Src homology 3 group (SH3)	P342	RRVCPCApRRPRRPR	1.1383
0.407	0.034%	Amphiphysin SH3 (Amphi_SH3)	Src homology 3 group (SH3)	P345	CPCAPRRpRRPRRPG	8.0636
0.482	0.169%	Amphiphysin SH3 (Amphi_SH3)	Src homology 3 group (SH3)	P348	APRRPRRpRRPGPSD	8.0636
0.459	0.092%	p85 SH3 mode1 (p85_SH3_m1)	Src homology 3 group (SH3)	P353	RRPRRPGpSDPAAPH	1.8764
0.466	0.089%	p85 SH3 mode2 (p85_SH3_m2)	Src homology 3 group (SH3)	P342	RRVCPCApRRPRRPR	1.1383
0.453	0.066%	p85 SH3 mode2 (p85_SH3_m2)	Src homology 3 group (SH3)	P348	APRRPRRpRRPGPSD	8.0636

Table 4.2 Prediction of motif sites on the human kisspeptin receptor by using the New Scansite 3 with medium stringency. The table presents details of the predicted interacting proteins and the potential binding sites within the human kisspeptin receptor. The GenBank accession number of the human kisspeptin receptor used for prediction is AAK83235.1. The score indicates how the amino acid sequence of the receptor matches the binding motif. The optimal match is assigned a score-value of 0.000 and higher scores indicate poorer matches. The percentile shows the percentile that the score of matching sequences of the kisspeptin receptor falls into compared with all matching sequences contained in vertebrate proteins in the SwissProt database. The surface accessibility indicates the accessibility of matching sequences of the kisspeptin receptor. A higher value of surface accessibility indicates a higher accessibility of the matching sequence.

Predicted Motif Sites (Table)

Please allow popups in your browser settings to make links in the table work properly!

Score	Percentile	Motif	▲ Motifgroup	Site	Sequence	Surface Accessibility
0.307	0.511%	PLK1 Kinase (PLK1)	Acidophilic serine/threonine kinase group (Acid_ST_kin)	T90	NLAATDVtFLLCCVP	0.2414
0.422	0.626%	Protein Kinase A (PKA_Kin)	Basophilic serine/threonine kinase (Baso_ST_kin)	T155	LRALHRRtPRLALAV	3.1689
0.309	0.008%	Erk D-domain (ErkDD)	Kinase binding site group (Kin_bind)	L160	RRTPRLAlAVSLSIW	0.1582
0.567	0.549%	Erk D-domain (ErkDD)	Kinase binding site group (Kin_bind)	V236	RHLGRVAvRPAPADS	0.3902
0.404	0.540%	14-3-3 Mode 1 (1433_m1)	Phosphoserine/threonine binding group (pST_bind)	S368	AELHRLGsHPAPARA	0.5328
0.547	0.648%	Amphiphysin SH3 (Amphi_SH3)	Src homology 3 group (SH3)	P147	RWYVTVFpLRALHRR	0.3716
0.465	0.122%	Amphiphysin SH3 (Amphi_SH3)	Src homology 3 group (SH3)	P342	RRVCPCApRRPRRPR	1.1383
0.407	0.034%	Amphiphysin SH3 (Amphi_SH3)	Src homology 3 group (SH3)	P345	CPCAPRRpRRPRRPG	8.0636
0.482	0.169%	Amphiphysin SH3 (Amphi_SH3)	Src homology 3 group (SH3)	P348	APRRPRRpRRPGPSD	8.0636
0.449	0.478%	Src SH3 (Src_SH3)	Src homology 3 group (SH3)	P353	RRPRRPGpSDPAAPH	1.8764
0.498	0.205%	p85 SH3 mode1 (p85_SH3_m1)	Src homology 3 group (SH3)	P339	QAFRRVCpCAPRRPR	0.1181
0.591	0.916%	p85 SH3 mode1 (p85_SH3_m1)	Src homology 3 group (SH3)	P342	RRVCPCApRRPRRPR	1.1383
0.459	0.092%	p85 SH3 mode1 (p85_SH3_m1)	Src homology 3 group (SH3)	P353	RRPRRPGpSDPAAPH	1.8764
0.466	0.089%	p85 SH3 mode2 (p85_SH3_m2)	Src homology 3 group (SH3)	P342	RRVCPCApRRPRRPR	1.1383
0.557	0.456%	p85 SH3 mode2 (p85_SH3_m2)	Src homology 3 group (SH3)	P345	CPCAPRRpRRPRRPG	8.0636
0.453	0.066%	p85 SH3 mode2 (p85_SH3_m2)	Src homology 3 group (SH3)	P348	APRRPRRpRRPGPSD	8.0636

4.3.2 Validation of the kisspeptin receptor/p85α and the kisspeptin receptor/c-SRC interactions

To test the above bioinformatics predictions, co-IP assays were conducted in MCF-7 cell line, which was used as a model, to detect the interactions of the human kisspeptin receptor with p85α and c-SRC. The interaction between the human kisspeptin receptor and p85α was initially measured. Cells were transiently co-transfected with flag-tagged bovine p85α and HA-tagged human kisspeptin receptor. For a co-IP negative control, cells were co-transfected with flag-p85α and flag-tagged human kisspeptin receptor. After immunoprecipitation by bead-conjugated HA antibody, a very weak non-specific binding was detected in the negative controls (Fig. 4.1). A much stronger band for co-immunoprecipitated p85α was detected in samples treated either with or without KP-10 stimulation (Fig. 4.1). However, no significant difference in the level of immunoprecipitated p85α was observed in the presence and absence of KP-10 stimulation. The same approach was used to detect potential interactions between the human kisspeptin receptor and c-SRC. Since endogenous expression of c-SRC was observed in MCF-7 cells, cells were only transiently transfected with flag-tagged human kisspeptin receptor. For a co-IP negative control, cells were transfected with pcDNA3.1+. After immunoprecipitation with c-SRC antibody-conjugated beads, the flag-kisspeptin receptor was detected in samples treated either with or without KP-10 stimulation, rather than in a negative control (Fig. 4.2). The level of precipitated flag-kisspeptin receptor remained a similar level in cells with and without KP-10 treatment. These results suggest that the predicted interactions of the kisspeptin receptor with p85α and c-SRC may occur in the cells. Although the level/concentration of the complex was not influenced by KP-

10 stimulation, KP-10 induced rearrangement of the quaternary structure, which mediates activation of p85a and c-SRC, could not be excluded.

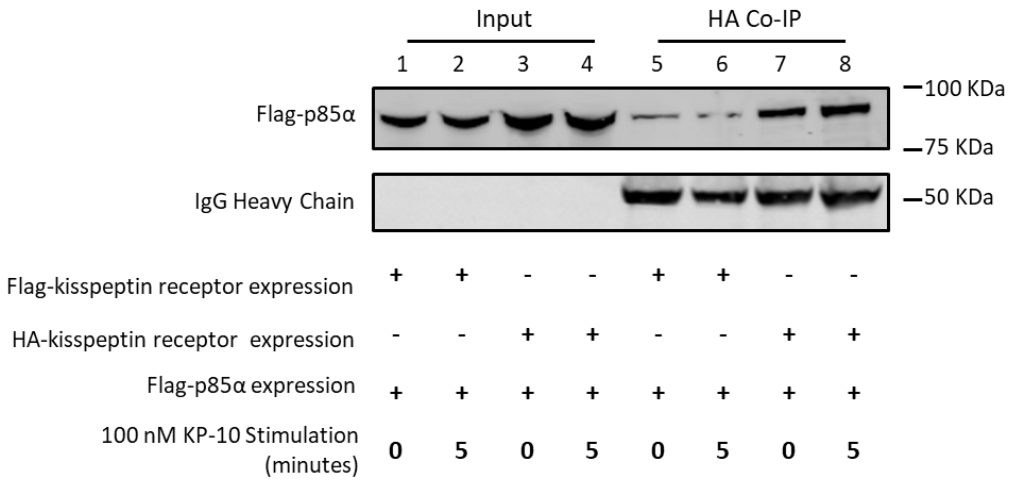


Figure 4.1 Co-IP assays depicting a direct interaction between the human kisspeptin receptor and p85α using MCF-7 cells. MCF-7 cells were transiently co-transfected with the combination between flag-p85α, HA-kisspeptin receptor or flag-kisspeptin receptor construct as indicated with "+" for with and "-" for without. The cells were then cultured for 24 hours followed by a 20 hours serum starvation. After that, 100 nM KP-10 was used for the stimulation for 5 minutes. The cell lysate were then incubated with HA antibody conjugated Sepharose bead slurry. Cells co-transfected with flag-p85α and flag-kisspeptin receptor was served as a negative Co-IP control. The heavy chain of IgG served as a loading control and the amount of cDNA transfected remained constant. Three independent expriments were conducted, and one representative immunoblot was shown here.

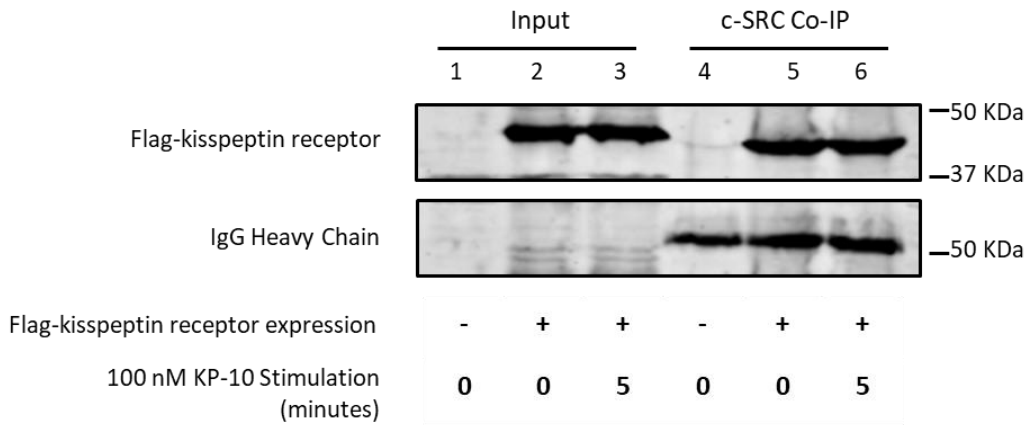


Figure 4.2 Co-IP assays depicting a direct interaction between the human kisspeptin receptor and c-SRC using MCF-7 cells. MCF-7 cells were transiently transfected with or without flag-kisspeptin receptor construct as indicated with "+" for with and "-" for without. The cells were then cultured for 24 hours followed by a 20 hour serum starvation. After that, 100 nM KP-10 was used to stimulate cells for 5 minutes. The cell lysate were then immunoprecipitated with c-SRC antibody conjugated agarose bead slurry. Cells transfected with pcDNA3.1+ was served as a negative Co-IP control. The heavy chain of IgG served as a loading control and the amount of cDNA transfected remained constant. Three independent experiments were conducted, and one representative immunoblot is shown here.

As an alternative method to measure the interaction between the kisspeptin receptor and non-G protein efforts, attempts were made to establish a FRET-based method in living cells. The method also provides an opportunity to monitor real-time protein interactions upon kisspeptin stimulation. The assay was established in HEK293 cells, because of their ease of transfection. Since a direct interaction between the human kisspeptin receptor and calmodulin was identified in our lab (data not shown), cells co-expressing the eYFP-tagged human kisspeptin receptor and eCFP-calmodulin served as a positive control for FRET (termed FRET). For negative controls, cells were co-transfected with flag-kisspeptin receptor and eCFP-calmodulin (termed eCFP only), eYFP-kisspeptin receptor and pcDNA3.1+ (termed eYFP only) or flag-kisspeptin receptor and pcDNA3.1+ (termed control). The fluorescent intensity was measured in a PHERAstar FS microplate reader and the FRET signals are calculated as the CFP/YFP ratio. No differences were found among negative controls and FRET samples before or after addition of 100 nM KP-10 at any tested time (Fig.4.3). Several optimization steps were conducted in order to improve the method. Unfortunately, a FRET signal was unable to be detected in any of them.

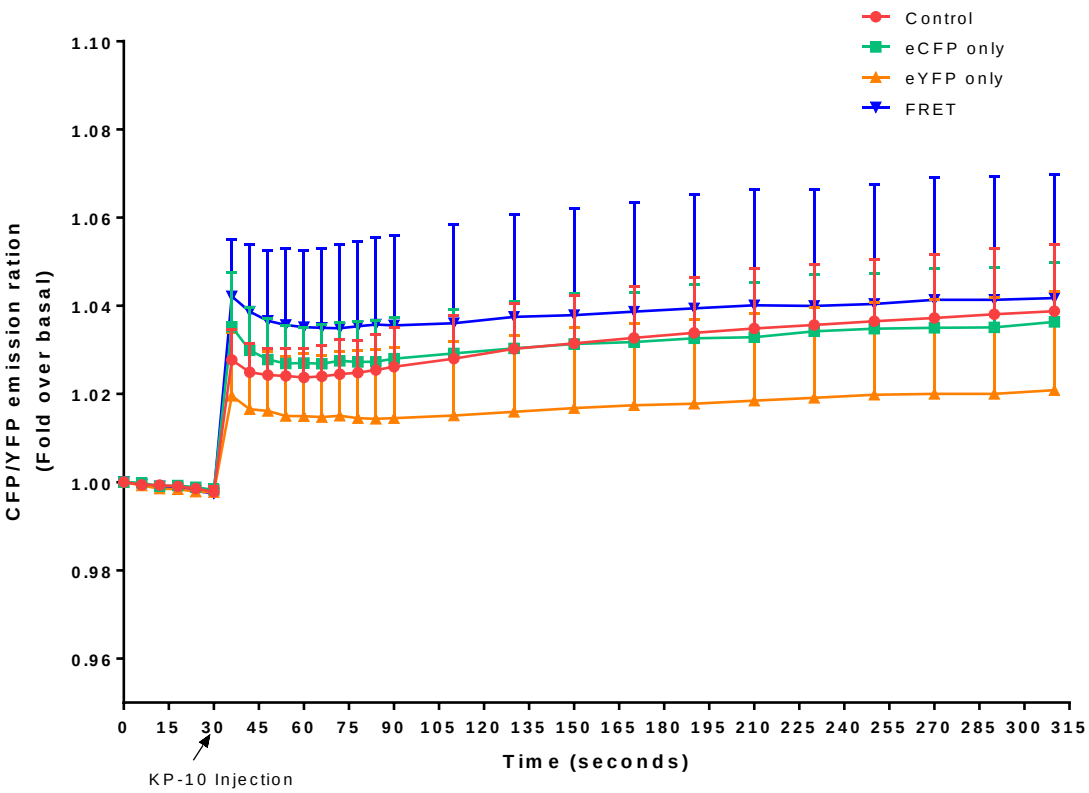


Figure 4.3 Test of an interaction between the kisspeptin receptor and calmodulin by FRET assay in HEK293 cells. The FRET assays were established using HEK293 cell line as a model. Cells co-transfected with flag-kisspeptin receptor and pcDNA3.1+ were severed as a negative control. In addition, eCFP only (co-transfected with flag-kisspeptin receptor and eCFP-calmodulin) and eYFP only (co-transfected with eYFP-kisspeptin receptor and pcDNA3.1+) were used in the control group. A FRET group, which the cells were co-transfected with eYFP-kisspeptin receptor and eCFP-calmodulin, was used to measure the kisspeptin receptor/calmodulin interaction. The cells were cultured for 24 hours post-transfection followed by a 20-hour serum starvation. After that, cells were stimulated with 100 nM KP-10 after 30-second reading of the baseline. Measurements are given in relative fluorescent units (RFU). The ratio of CFP/YFP is calculated and the mean over the baseline ± SD is plotted. Three independent expriments were conducted, and only one of them is plotted here.

4.3.3 Functional investigation of the human kisspeptin receptor/p85α interaction

In order to investigate the biological functions of the interaction between the human kisspeptin receptor and p85α, a series of functional assays were conducted. The activation of the PI3K signalling pathway was measured by measuring the phosphorylation of AKT, which is a key downstream effector of PI3K and regulates cell proliferation and survival. The phosphorylation of AKT in MCF-7 cells transiently expressing the kisspeptin receptor was examined by western blot. A final concentration of 100 nM KP-10 was used to stimulate cells for different times. Stimulation by KP-10 elicited an increase in the phosphorylation of AKT with a maximal response of 1.99 ± 0.0644-fold that of vehicle treated control after 10-minute incubation. The level of phosphorylation of AKT slightly dropped to 1.64 ± 0.175-fold after 20-minute stimulation (Fig. 4.4).

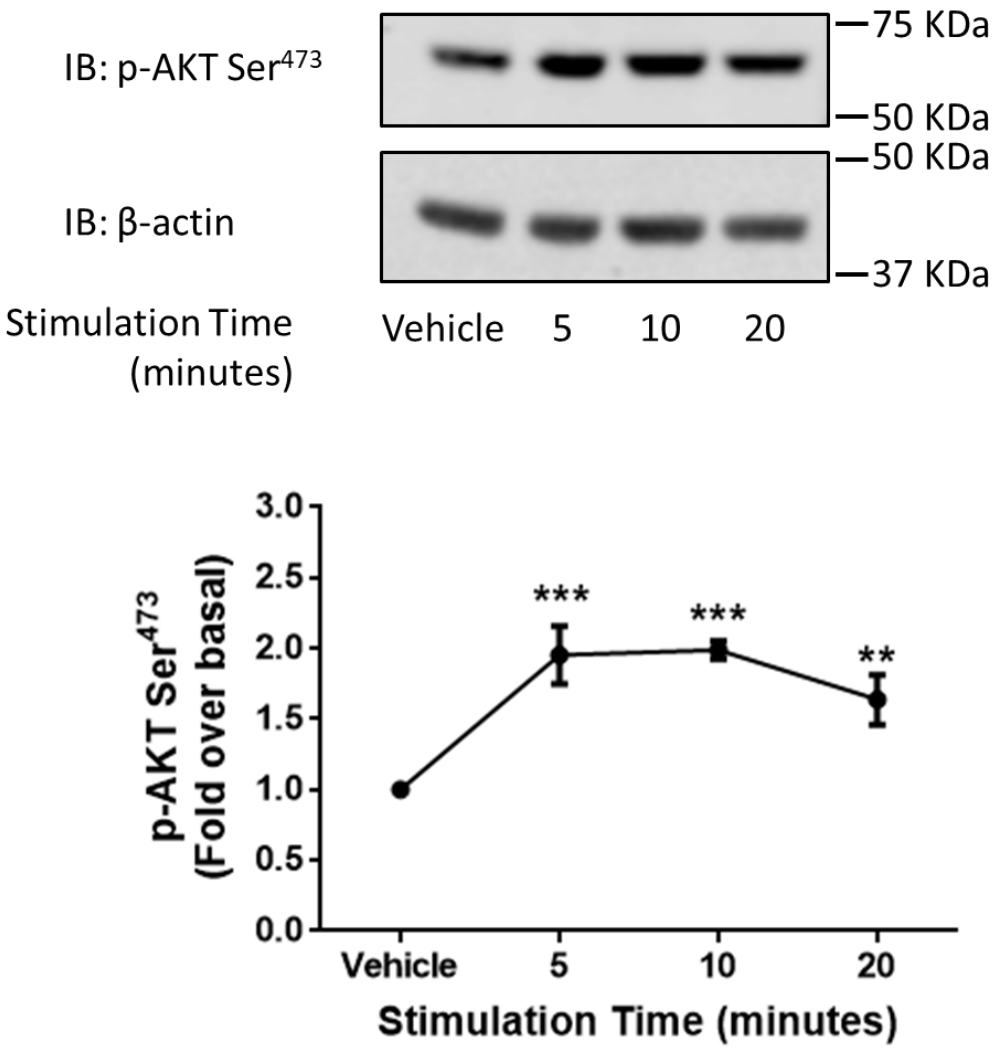


Figure 4.4 Immunoblots depicting the KP-10-induced phosphorylation of AKT in MCF-7 cells. MCF-7 cells transiently transfected with flag-kisspeptin receptor cDNA were serum-starved for 20 hours prior to being treated with vehicle (0.02% (v/v) propylene glycol) or 100 nM KP-10 for indicated time. Representative blots are shown. Data from three independent experiments were quantified (using β-actin as a loading control) and the mean fold over vehicle treated control ± SD for the phosphorylation of AKT is presented. *p<0.01 (**)* and *p<0.005* (***) represent statistical significance from vehicle treated control.

In order to elucidate whether the PI3K/AKT signalling could be mediated in a $G_{q/11}$-independent manner, the phosphorylation of AKT was then detected in the presence and absence of YM-254890. YM-254890 is a $G_{q/11}$-selective inhibitor and was able to inhibit P2Y purinergic receptor 1/$G_{q/11}$-mediated intracellular Ca^{2+} mobilization with IC_{50} values of 0.18 μM in C6-15 cells, which is a rat glioma cell line (Takasaki et al., 2004). The Ca^{2+} mobilization was completely inhibited by pre-treatment of cells with 100 nM YM-254890 for 5 minutes. Therefore, in this experiment, MCF-7 cells were treated with 100 nM YM-254890 for 1 hour and then the phosphorylation of AKT was measured after stimulation with 100 nM KP-10 by western blot. Significant increases in the phosphorylation of AKT were observed upon the stimulation of KP-10 in the absence of YM-254890 (Fig. 4.5; black bars). A maximal response of 2.95 ± 0.774-fold was obtained after 3-minute stimulation and the phosphorylation of AKT was detectable up to 60-minute stimulation (Fig. 4.5; black bars). In the presence of YM-254890, KP-10 induced a maximal response of 1.74 ± 0.181-fold increase in the phosphorylation of AKT after 10-minute stimulation. After that, KP-10 was unable to stimulate the phosphorylation of AKT (Fig. 4.5; grey bars). Compared with vehicle treated samples, the level of phosphorylation of AKT was markedly reduced in the presence of YM-254890 within 10-minute stimulation and completely abolished after 30-minute or longer time stimulation

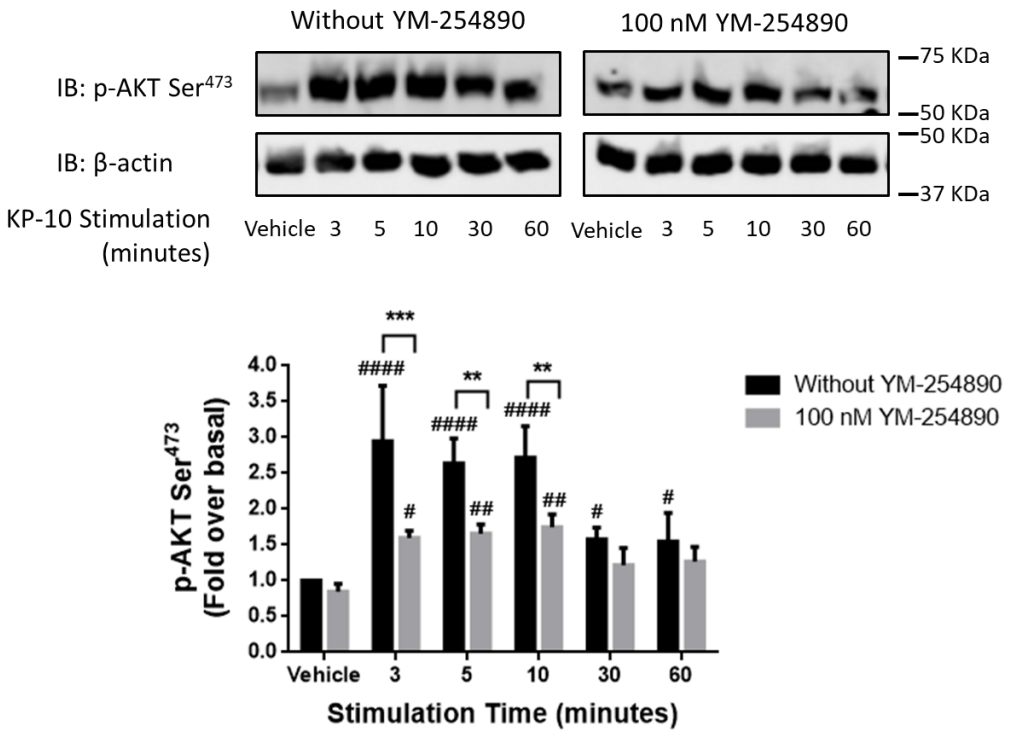


Figure 4.5 Immunoblots depicting the effects of a $G_{q/11}$ inhibitor on KP-10-induced phosphorylation of AKT in MCF-7 cells. MCF-7 cells transiently transfected with flag-kisspeptin receptor cDNA were serum-starved for 20 hours prior to being treated without (black bars) or with 100 nM YM-254890 (grey bars) for 1 hour. After that, cells were stimulated by vehicle (0.02% (v/v) propylene glycol) or 100 nM KP-10 for indicated time. Representative blots are shown. Data from three independent experiments were quantified (using β-actin as a loading control) and the mean fold over vehicle treated control (in the absence of YM-254890) ± SD for the phosphorylation of AKT is presented. *p<0.05 (#)*, *p<0.01* (##) and *p<0.001* (####) represent statistical significance from vehicle treated control (in the absence of YM-254890). *p<0.01* (**) and *p<0.005* (***) represent statistical significance from the sample as indicated.

In contrast to above results, it has been observed that KP-10 can inhibit the EGF- and insulin-induced phosphorylation of AKT *via* EGFR and the insulin receptor in HEK293 cells expressing the kisspeptin receptor (Navenot et al., 2009). To investigate whether KP-10 is also able to abolish the insulin-induced phosphorylation of AKT in cancer cells, the experiments were conducted in MCF-7 cells. Cells transfected with the human kisspeptin receptor were incubated with 10 μg/mL of insulin in the presence and absence of 100 nM KP-10. The basal level of phosphorylation of AKT in cells was measured in the absence of both insulin and KP-10. Briefly, the insulin was added to the cells and incubated for 30 minutes. Simultaneously, 100 nM KP-10 was added at indicated time points (Fig. 4.6). For the vehicle group, 0.02% (v/v) propylene glycol was added. Insulin caused a significant increase in the phosphorylation of AKT. However, no significant differences were observed when KP-was added at any of the tested times (Fig. 4.6). As an alternative method, cells were firstly exposed to 100 nM KP-10 for the indicated time (Fig. 4.7). After that, cells were co-treated with KP-10 and 10 μg/mL of insulin for another 30 minutes. Pre-treatment with KP-10 caused a slightly decrease in the level of phosphorylation of AKT after 5-minute pre-treatment with KP-10 (0.75 ± 0.0391-fold; Fig. 4.7).

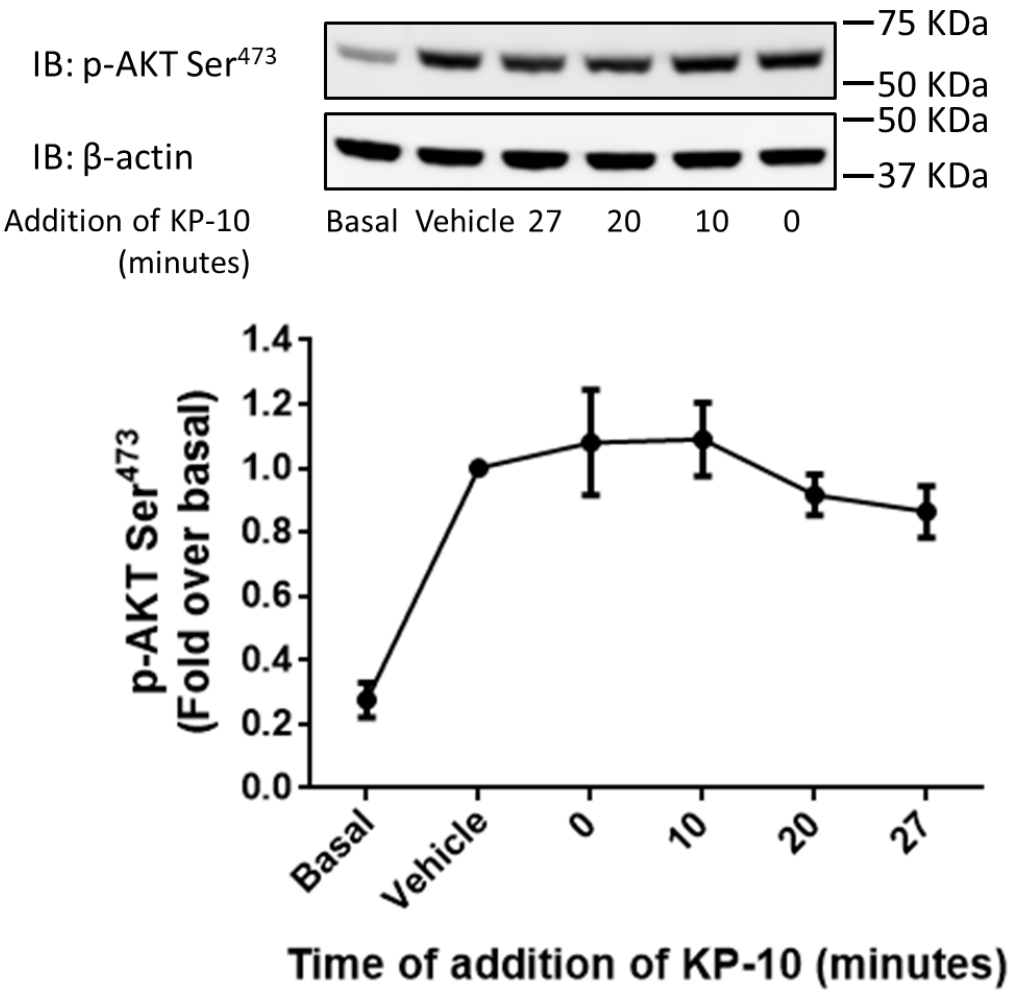


Figure 4.6 Immunoblots depicting the effects of KP-10 on insulin-induced phosphorylation of AKT in MCF-7 cells. MCF-7 cells transiently transfected with flag-kisspeptin receptor cDNA were serum-starved for 20 hours prior to being treated with 10 µg/mL insulin for 30 minutes. Simultaneously, 100 nM KP-10 were added at indicated time. Vehicle (0.02% (v/v) propylene glycol) was added for the vehicle group. Basal level of phosphorylation of AKT was measured in the absence of KP-10 and insulin stimulation. Representative blots are shown. Data from three independent experiments were quantified (using β-actin as a loading control) and the mean fold over control (treated with insulin only) ± SD for the phosphorylation of AKT is presented.

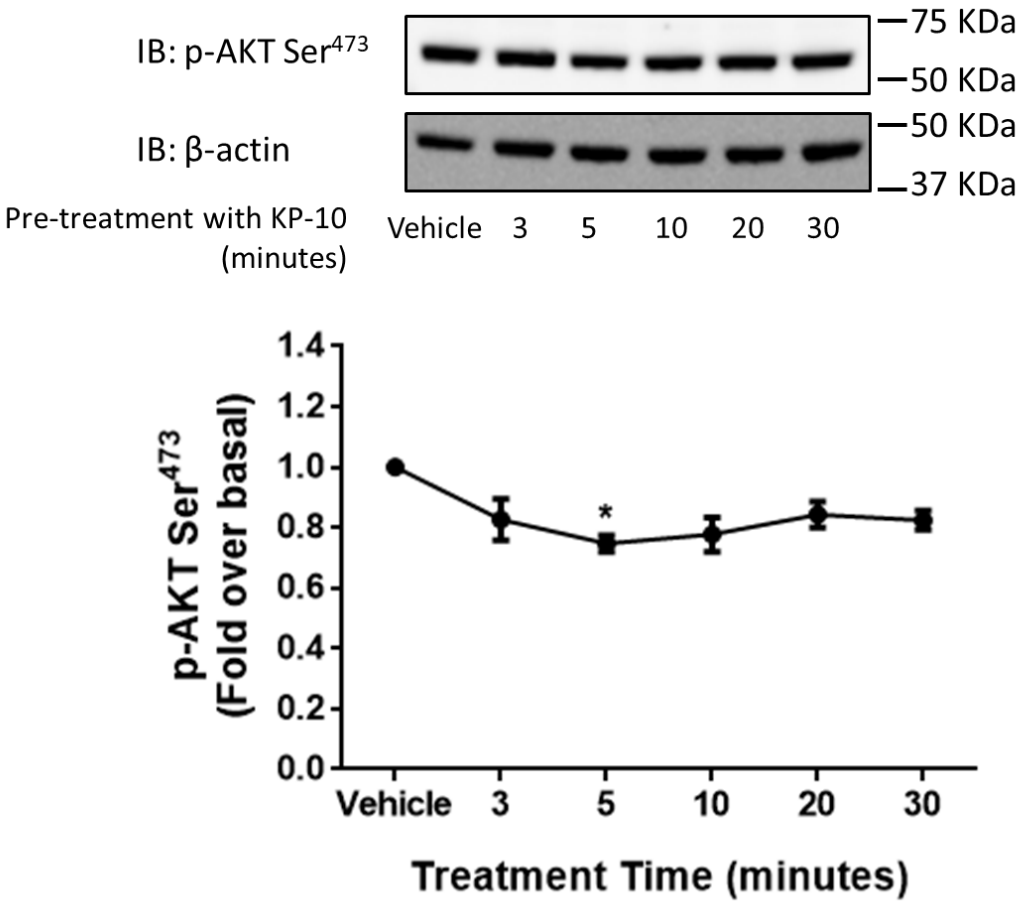


Figure 4.7 Effects of KP-10 pretreatment on insulin-induced phosphorylation of AKT in MCF-7 cells. MCF-7 cells transiently transfected with flag-kisspeptin receptor cDNA were serum-starved for 20 hours prior to being treated with vehicle (0.02% (v/v) propylene glycol) or 100 nM KP-10 for indicated time. After that, insulin at a final concentration of 10 µg/mL was added to stimulate cells for a further 30 minutes. Representative blots are shown. Data from two independent experiments were quantified (using β-actin as a loading control) and the mean fold over vehicle treated control ± range for the phosphorylation of AKT is presented. *p<0.05 (*)* represents statistical significance from vehicle treated control.

4.3.4 Functional investigation of the human kisspeptin receptor/c-SRC interaction

In order to investigate the biological function of the interaction between the human kisspeptin receptor and c-SRC, a series of assays were conducted. The phosphorylation of c-SRC was measured by detecting the autophosphoylation of the Tyr^{416} residue within the kinase domain of the protein (Martin, 2001). A final concentration of 100 nM KP-10 was used for the stimulation over different times in MCF-7 cells transiently expressing the kisspeptin receptor. No significant increase in the phosphorylation of Tyr^{416} was identified in response to KP-10 (Fig. 4.8). Alternatively, a FRET-based SRC reporter was used to monitor c-SRC kinase activity in HEK293 cells. The c-SRC reporter is composed of an eCFP, a SH2 domain, a linker, a specific substrate peptide of c-SRC and an eYFP from N-terminus to C-terminus (Fig. 4.9). In the ground status, the juxtaposition of eCFP and eYFP yield a FRET signal. Activated c-SRC phosphorylates the Tyr^{662} and Tyr^{664} residues located in the substrate region of the c-SRC reporter. The phosphorylated Tyr residues lead to the interaction of substrate peptide with the SH2 domain of the c-SRC reporter. The interaction then induces a conformational change, which causes a separation of eCFP from eYFP and eliminates the FRET signal. For a negative control, the Arg^{175} residue in the SH2 domain of c-SRC reporter is substituted by Val (R175V). The substitution abolishes the ability of SH2 domain binding to the phosphorylated substrate and, thus, retains the conformation in the ground status even in the presence of c-SRC kinase activity (Wang et al., 2005). However, a decrease in the FRET signal was observed in HEK293 cells transfected with the c-SRC reporter or R175V mutant, in response to a final concentration of 50 ng/µl of EGF (data not shown). After sequencing the original

stock of plasmids, unknown contaminations of DNA were identified, which may contribute to the unexplainable results.

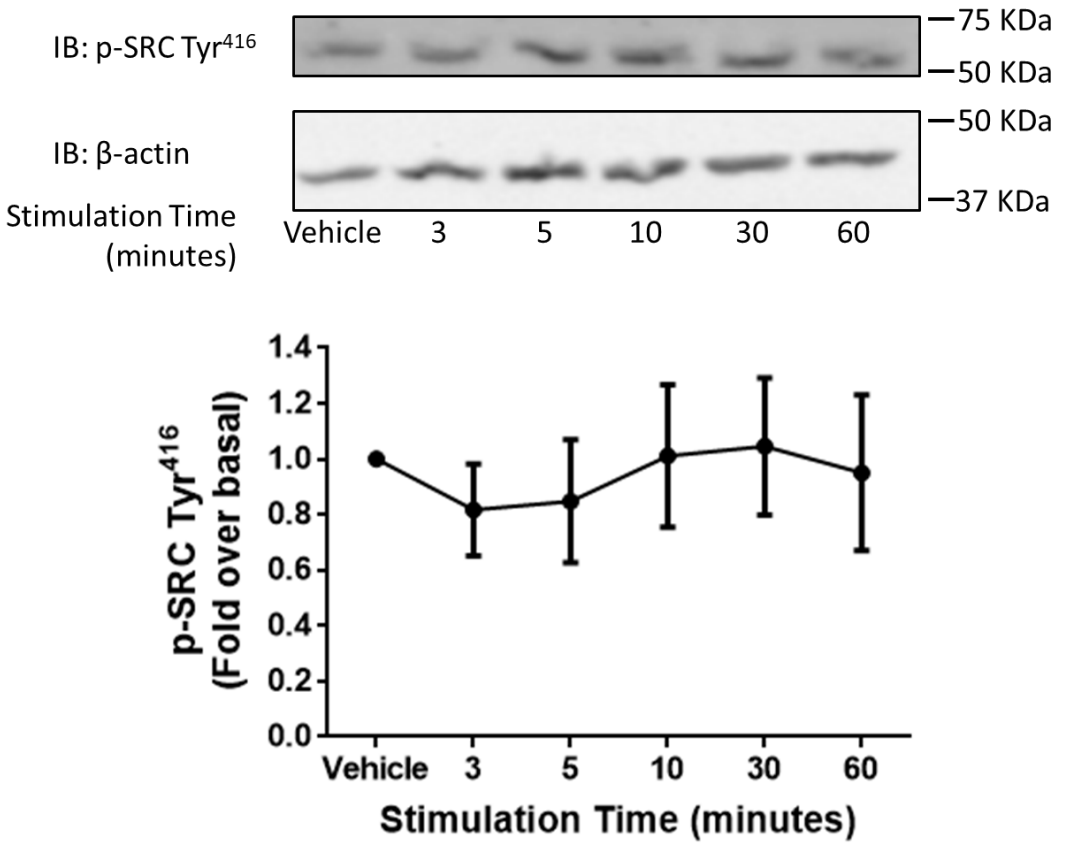


Figure 4.8 Effect of KP-10 on the phosphorylation of SRC Tyr^{416} in MCF-7 cells. MCF-7 cells transiently transfected with flag-kisspeptin receptor cDNA were serum-starved for 20 hours prior to being treated with vehicle (0.02% (v/v) propylene glycol) or 100 nM KP-10 for indicated time. Representative blots are shown. Data from three independent experiments were quantified (using β-actin as a loading control) and the mean fold over vehicle treated control ± SD for the phosphorylation of c-SRC is presented.

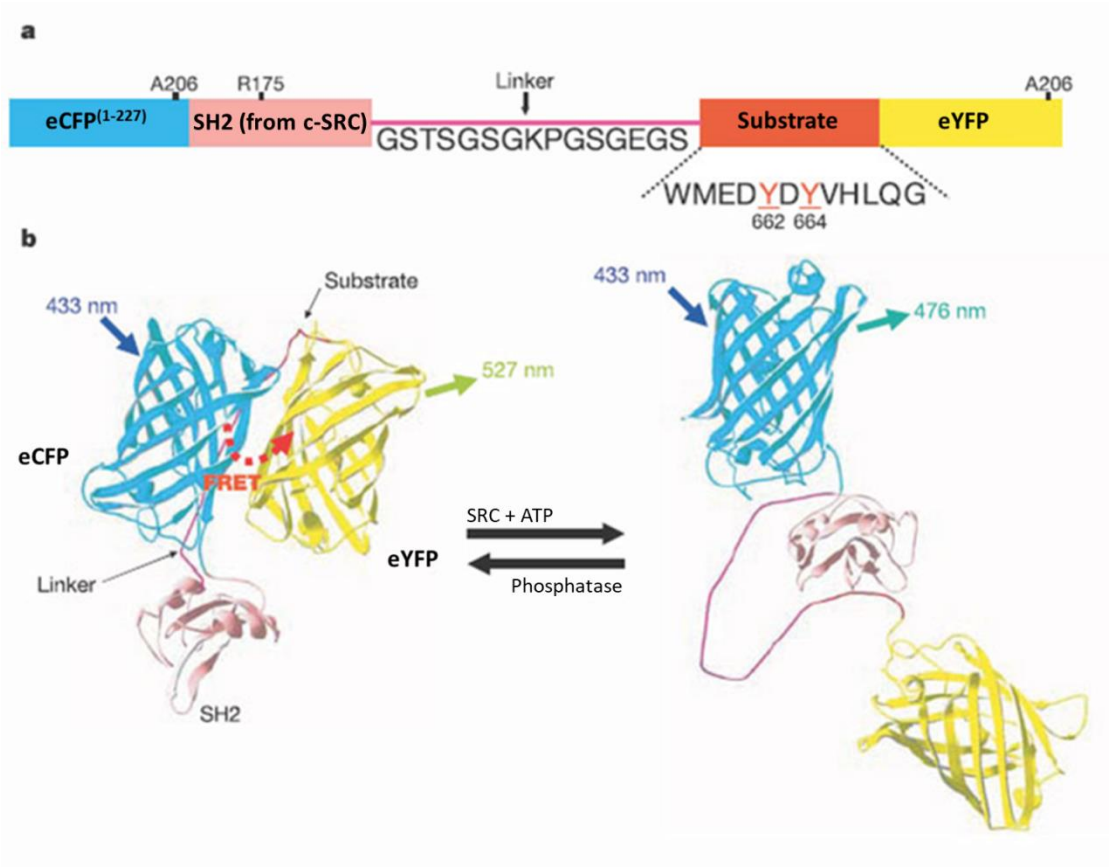


Figure 4.9 The structure of c-SRC reporter. a, The c-SRC reporter is composed of an eCFP, a SH2 domain, a flexible linker, a specific substrate of c-SRC and an eYFP from N-terminal to C-terminal. b, Cartoon to illustrate the FRET response of c-SRC reporter upon SRC kinase and phosphatase activity. Figure is adapted from Wang *et al*., 2005.

The KP-10-induced phosphorylation of ERK1/2 was examined further in the presence and absence of bosutinib (also termed SKI-606), in order to elucidate whether c-SRC is involved in the kisspeptin receptor signalling. Bosutinib is a potent dual inhibitor for SRC and ABL1 with an IC_{50} value of 1.2 nM (Boschelli et al., 2001) and 1 nM (Golas et al., 2003) in enzyme assays respectively. A rapid inhibition of autophosphoylation of c-SRC and phosphorylation of its downstream effectors was identified in the presence of bosutinib for 10 minutes in breast cancer cell lines including MCF-7 (Vultur et al., 2008). Therefore, cells were treated with 120 nM bosutinib for 1 hour and then the level of phosphorylation of ERK1/2 was measured after stimulation with 100 nM KP-10 for indicated time (Fig. 4.10). In the absence of bosutinib, a maximal response of 2.78 ± 0.732-fold was observed after 10-minute incubation and remained high after 30-minute stimulation (1.93 ± 0.166-fold; Fig. 4.10; black bars). In the presence of bosutinib, KP-10 stimulation brought a maximal response of 2.41 ± 0.797-fold increase in the phosphorylation of ERK1/2 after 5-minute incubation and the level of phosphorylation of ERK1/2 was decreased thereafter (Fig. 4.10; grey bars). No significant increase in the phosphorylation of ERK1/2 was observed after 30-minute stimulation in the presence of bosutinib. Compared with that in the absence of bosutinib, the level of phosphorylation of ERK1/2 was significantly reduced after 10-and 30-minute stimulation in the presence of bosutinib.

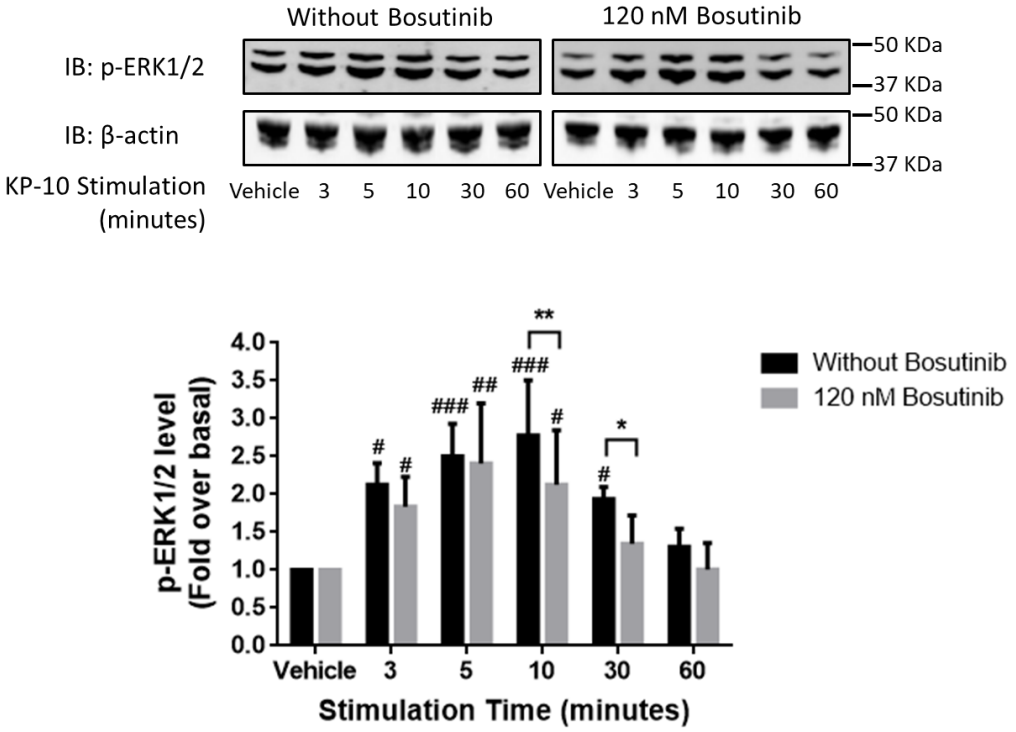


Figure 4.10 Immunoblots depicting the effects of a SRC inhibitor on KP-10-induced phosphorylation of ERK1/2 in MCF-7 cells. MCF-7 cells transiently transfected with flag-kisspeptin receptor cDNA were serum-starved for 20 hours prior to being treated without (0.0006% (v/v) DMSO; black bars) or with 120 nM bosutinib (grey bars) for 1 hour. After that, cells were stimulated by vehicle (0.02% (v/v) propylene glycol) or 100 nM KP-10 for indicated time. Representative blots are shown. Data from three independent experiments were quantified (using β-actin as a loading control) and the mean fold over vehicle treated control (in the absence of bosutinib) ± SD for the phosphorylation of ERK1/2 is presented. *p<0.05 (#), p<0.01* (##) and *p<0.005* (###) represents statistical significance from vehicle treated control (in the absence of bosutinib). *p<0.05* (*) and *p<0.01* (**) represents statistical significance from the sample as indicated.

4.3.5 Generation of the flag-tagged human kisspeptin receptor mutant constructs

The NEW Scansite 3 results showed that the predicted sites mediating the interaction of the human kisspeptin receptor with p85α and c-SRC are Pro^{342}, Pro^{345}, Pro^{348} and Pro^{353} (Table 4.1 and 4.2). To determine whether these residues are involved in the protein-protein interactions, their Ala mutants were constructed (P342A, P345A, P348A and P353A) (Fig. 4.11). The mutants were generated by using PCR site-directed mutagenesis approach. Briefly, a fragment PCR product of the flag-kisspeptin receptor was generated by using common primers and used as a template for the further mutagenesis (Table 4.3). The primers used for each mutant are listed in Table 4.3 and the PCR products containing mutated residues were subcloned into the flag-kisspeptin receptor between the *AflII* and *NotI* sites. The mutated kisspeptin receptor plasmids were sent to Invitrogen (Thermo Fisher Scientific, USA) for sequencing. Functional examinations of these mutant receptors will be carried out in the future.

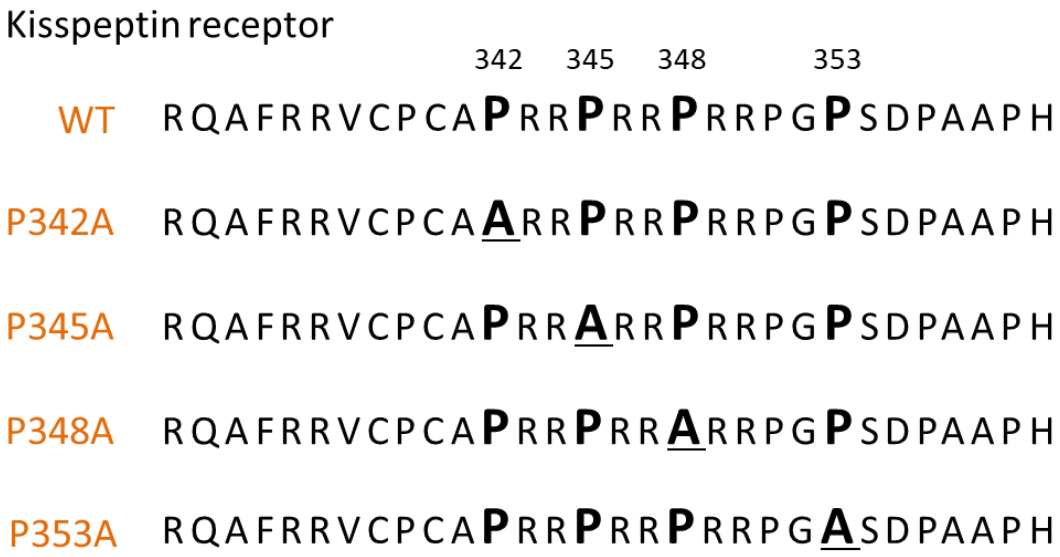


Figure 4.11 Partial protein sequences for wild type and mutated kisspeptin receptor. The amino acid sequences of mutated sites of four mutants were indicated. The mutated residues are enlarged and underlined.

Table 4.3 Primers for PCR site-directed mutagenesis. This table shows the sequences of primers used for the generation of four mutant receptors. The nucleotides encoding mutated residues are underlied and the mutated sites are labeled in bold.

Primer	Sequence (5' to 3')
Common Forward	TTCGCACTGTACAACCTGCT
Common Reverse	CCTCTACAAATGTGGTATGGCTG
P342A Forward	CTGCGCG**GCA**CGCCGCCCC
P342A Reverse	GGGGCGGCG**TGC**CGCGCAG
P345A Forward	CGCGCCGA**GCA**CGCCGCCC
P345A Reverse	GGGCGGCG**TGC**TCGGCGCG
P348A Forward	CCCGCCGA**GCA**CGCCGGCC
P348A Reverse	GGCCGGCG**TGC**TCGGCGGG
P353A Forward	GGCCCGGA**GCA**TCGGACCC
P353A Reverse	GGGTCCGA**TGC**TCCGGGCC

4.4 Discussion

Bioinformatics analysis of 75 human GPCRs reveals 424 putative protein binding motifs on the intracellular portions of receptors (Tovo-Rodrigues et al., 2014). Among them, one of the most observed motifs is the SH3 domain-binding site, which is found in more than 50% of the receptors. SH3 domain recognises and binds to short amino acid sequences, which are rich in Pro residues (Ren et al., 1993, Yu et al., 1994). Indeed, interactions between GPCRs and SH3 domain-containing proteins have been reported be mediated by the Pro-rich regions located in ICL3 (Cao et al., 2000, Oldenhof et al., 1998) and the C-terminal tail of GPCRs (Cao et al., 2000, Liu et al., 2004) (Section 1.4.2). Three Pro-Arg-Arg repeats are identified within the C-terminal tail of the kisspeptin receptor and are able to mediate the interaction with PP2A, which is a SH3 domain-containing protein (Evans et al., 2008). However, studies on other interacting proteins thought the Pro-Arg-Arg repeats are lacking. Bioinformatics analysis by the NEW Scansite 3 program shows that this region is a putative SH3 domain-binding motif for p85 and c-SRC. In this chapter, the direct interaction of the kisspeptin receptor with p85α or c-SRC was shown in the first time by co-IP in a breast cancer cell line.

In addition, attempts to establish CFP/YFP-based FRET assay in living cells were made, which would allow the measurement in real-time the protein-protein interactions. Unfortunately, no difference in FRET signals was observed in positive control compared with that in negative controls. CFP/YFP is a widely used fluorescent protein pair in FRET assay, and has been applied in the detection of the protein-protein interactions (Ma et al., 2014). However, certain properties of eCFP and eYFP

can cause difficulties in the development of FRET assays in living cells, such as reversible photobleaching of eCFP and eYFP at physiological pH (Sinnecker et al., 2005), photoconversion of eYFP to eCFP-like species (Raarup et al., 2009) and phototoxic of extended eCFP excitation (Dixit and Cyr, 2003). Besides, other factors including the orientation of eCFP and eYFP, pH, temperature and electrolyte concentration are able to affect the eCFP/eYFP FRET (Felber et al., 2004). In addition to these factors, the microplate reader monitors changes in cell cluster rather than an individual cell, which may result in a low signal-to-noise ratio compared with confocal microscopy- or flow cytometry-based assays and hence exhibits low sensitivity. Further optimizations are necessary for the establishment of our FRET assay using the microplate reader.

Then, the biological functions of the kisspeptin receptor/p85α interaction were examined. Some GPCRs, such as somatostatin receptor type 2, have been shown to inhibit the PI3K signalling pathway by disrupting the interaction of the receptor and p85α upon agonist stimulation (Bousquet et al., 2006). A similar mechanism may be applied by the kisspeptin receptor to suppress the PI3K/AKT signalling. To test so, the influence of KP-10 on the kisspeptin receptor/p85α interaction and the stimulation of phosphorylation of AKT were examined. However, the interaction between the kisspeptin receptor and p85α retained at a similar level as that in the basal conditions following 5-minute KP-10 stimulation. In addition, the level of phosphorylation of AKT was increased in response to KP-10 stimulation and remained high up to 60-minute stimulation. It has been shown that some GPCRs can activate the PI3K/AKT pathway by $G_{q/11}$, especially Ca^{2+} and PKC (Seatter et al., 2004,

Wang and DeFea, 2006). Therefore, to elucidate whether the KP-10-induced phosphorylation of AKT could be mediated by a $G_{q/11}$-independent manner, a specific inhibitor of $G_{q/11}$, YM-254890, was used. In the presence of YM-254890, KP-10-induced phosphorylation of AKT was rapidly reduced within 10 minutes and was completely abolished after 30 and 60 minutes. However, a weak $G_{q/11}$-independent phosphorylation of AKT could still be observed, suggesting that the kisspeptin receptor mediates PI3K/AKT pathway in both $G_{q/11}$-dependent and -independent manners. Indeed, other signal effectors, such as β-arrestins, are able to mediate the activation of PI3K/AKT pathway (Goel and Baldassare, 2002). Whether the kisspeptin receptor/p85α interaction could be involved in the activation of PI3K/AKT remains to be elucidated.

A negative crosstalk between the kisspeptin receptor with CXCR4 (Navenot et al., 2005), EGFR and the insulin receptor (Navenot et al., 2009) was identified (Section 1.4.3). The KP-10-induced activation of the kisspeptin receptor can completely impair SDF-1/CXCL12-induced phosphorylation of AKT in CHO cells (Navenot et al., 2005), and EGF- and insulin-triggered phosphorylation of AKT in HEK293 cells (Navenot et al., 2009). To test whether the same occurred in the MCF-7 cells, the effect of KP-10 on insulin-induced AKT activation was measured. However, KP-10 treatment only led to a slight decrease in insulin-induced phosphorylation of AKT activation in MCF-7 cells. The differences may result from the fact that different cell types were used, suggesting that KP-10 inhibition of the stimulation of phosphorylation by other effectors is not a general phenomenon.

The KP-10-induced PI3K/AKT signalling presented here was measured in the early stage. Since metastasis is a long-term progress, to fully understand the kisspeptin/the kisspeptin receptor-mediated PI3K/AKT pathway in the suppression of metastasis, a longer stimulation time of kisspeptin is necessary. It has been shown that in some cancer cells, such as NPA cells, kisspeptin can trigger the phosphorylation of AKT within 30 minutes, but suppress the activity of AKT by, for example, upregulating the expression of *tribble 3* after longer time stimulation (up to 24 hours) (Stathatos et al., 2005). Instead, the activation of PI3K/AKT signalling pathway by kisspeptins and the kisspeptin receptor in the early stage might be involved in the regulation of the reproductive axis. Kisspeptins can activate PI3K/AKT pathway in GnRH neuronal cell lines, including GT1-7 (Terasaka et al., 2013, Novaira et al., 2009) and GN11 (Novaira et al., 2009). Inhibition of PI3K activity by a PI3K selective inhibitor attenuated kisspeptin-induced GnRH secretion (Novaira et al., 2009). In addition, inhibition of either PI3K or mTOR activity completely impaired KP-10-induced LH secretion in baboon pituitary cells (Luque et al., 2011).

The biological functions of the interaction between the kisspeptin receptor and c-SRC were further examined by measuring the kisspeptin-stimulated phosphorylation of the Tyr^{416} residue of c-SRC, which reflects the kinase activity of c-SRC (Martin, 2001). However, no change in the level of phosphorylation of Tyr^{416} was detected following KP-10 stimulation, indicating kisspeptin may be unable to activate c-SRC. Since a basal association between the kisspeptin receptor and c-SRC was observed, another explanation is that c-SRC may be activated in the absence of kisspeptin and the addition of kisspeptin may not further activate c-SRC. Moreover, it has been shown

that the interaction of some GPCRs, such as progesterone receptor, with the SH3 domain of c-SRC is required for the activation of c-SRC (Boonyaratanakornkit et al., 2001). Therefore, the kisspeptin receptor may be able to activate c-SRC through its interaction with the SH3 domain of c-SRC without affecting the phosphorylation of Tyr^{416} of c-SRC. As an alternative approach to measure the kisspeptin-induced activation of c-SRC in living cells, a FRET-based c-SRC reporter was used. The assay is currently under construction. Functionally, the effect of c-SRC upon kisspeptin-induced phosphorylation of ERK1/2 was measured, since some GPCRs, including β_3AR (Cao et al., 2000), has been identified to activate ERK1/2 signalling pathway through the interaction with c-SRC. The inhibition of c-SRC activity markedly decreased the KP-10-induced phosphorylation of ERK1/2 in a later stage (after 10-minute stimulation of KP-10), suggesting that the kisspeptin receptor may bind to c-SRC to transduce c-SRC-dependent cell signalling.

Kisspeptin was found to inhibit vascular endothelial growth factor (VEGF)-induced human umbilical vein endothelial cell migration by impairing VEGF-induced c-SRC activation and, consequently, c-SRC-mediated FAK activation. However, KP-10 showed little effect on VEGF-mediated FAK autophosphorylation, indicating that KP-10 may inhibit c-SRC activity to suppress cell migration and invasion (Cho et al., 2009b). However, the kisspeptin-induced c-SRC activation presented here was only measured at the early stage and may not be involved in the suppression of cancer metastasis, as metastasis is a progress that occurs over long time. Since the ERK1/2 activation is important for KP-10-induced LH (Luque et al., 2011) and GnRH (Castellano et al., 2006) release, the interaction of the kisspeptin receptor with c-

SRC, which is involved in KP-10-induced phosphorylation of ERK1/2 as demonstrated here and in other studies (Roseweir et al., 2012), may be important in transducing downstream signalling in the reproductive axis. Consistently, inhibition of c-SRC activity by using PP2, which is a selective inhibitor of SRC-family kinase, completely impaired kisspeptin-induced inward TRPC4 current in GnRH slices prepared from female mice. However, blockade of the downstream effector of c-SRC, such as MEK1/2, by an inhibitor (U-0126) did not influence kisspeptin-mediated TRPC4 response, implying that kisspeptin activates the TRPC4 channels to excite GnRH neuron through c-SRC-dependent signalling pathways (Zhang et al., 2013). Thus, the kisspeptin receptor may directly couple to c-SRC to excite GnRH neurons.

In the further studies, to identify which Pro residues within the Pro-Arg-Arg repeats of the kisspeptin receptor may mediate the interaction of the kisspeptin receptor with p85α and c-SRC, site-directed mutagenesis was used to generate mutant receptors. Ala substitution of individual Pro residue, Pro^{342}, Pro^{345}, Pro^{348} and Pro^{353}, has been successfully introduced, but the co-IP and functional assays have yet to be conducted.

In summary, the results within the chapter constitute the first observation of direct interaction between the kisspeptin receptor/p85α and the kisspeptin receptor/c-SRC. Additionally, they indicate that the coupling of receptor to these proteins regulates a variety of cellular responses including the phosphorylation of AKT and ERK1/2. Besides, the data also suggest that some cross-regulation of the kisspeptin receptor signalling with other receptor tyrosine kinases may occur. The integration of these

signalling events will then determine the signalling output and the cell fate such as cell survival, migration and apoptosis. The studies presented here provide a basis for the further investigation into the kisspeptin receptor signalling.

Chapter 5

Investigation of the roles of Cys^{338} and Cys^{340} in the C-terminal tail of the kisspeptin receptor on receptor expression and function

5.1 Outline

One lipid modification, known as *S*-palmitoylation, has been reported in some GPCRs. The post-translational modification occurs on the Cys residues of the C-terminal tail of GPCRs, about 10-amino-acid away from the intracellular end of TM7. The modification introduces a saturated 16-carbon palmitoyl group on the Cys residues, which may be involved in the anchoring of H8 in the inner surface of the cell membrane. *S*-palmitoylation is a reversible post-translation modification and the cycle of palmitoylation and depalmitoylation has been proposed to play an important role in GPCRs signalling, trafficking and stability. However, the palmitoylation status of the kisspeptin receptor has not been determined yet. There are two Cys residues (Cys^{338} and Cys^{340}) on the C-terminal tail of the kisspeptin receptor predicted as palmitoylation sites by bioinformatics analysis. In this chapter, Ser substitutions on the Cys residues were conducted by site-direct mutagenesis (Section 2.9) to generate two single (C338S and C340S) and one double (C338S-C340S) mutant receptors. Additionally, a truncated mutant to the C-terminal tail of the human kisspeptin receptor at the residue Arg^{331} (R331X), which is a natural mutation identified in the patients with IHH, was also created. The levels of the expression of the mutant receptors were examined by western blot (Section 2.17). In addition, the effects of the mutations on ligand binding (Section 2.13) and the internalization of the receptor (Section 2.14) were measured. Moreover, the effects of the mutations on receptor signalling were examined by measuring agonist-induced dual phosphorylation of ERK1/2 (Section 2.17) and intracellular calcium flux (Section 2.18),

5.2 Introduction

Palmitoylation is a post-translational modification on the Cys residues of a protein by the addition of a palmitoyl group derived from palmitic acid. The consequence of the presence of the long-hydrophobic chain of the fatty acid is that it can favour the attachment of targeted proteins to the cell membrane and, therefore, promotes their membrane localization. Various proteins have been observed to undergo this lipid modification such as monomeric G proteins, H-Ras and N-Ras (Rocks et al., 2005, Escriba et al., 2007), heterotrimeric G proteins (Chisari et al., 2007), and GPCRs (Percherancier et al., 2001, Adams et al., 2011). Two types of palmitoylation have been identified based on the locations of Cys residues. In most cases, palmitate can be attached to Cys residues *via* a reversible thioester linkage (*S*-palmitoylation). However, if the Cys residues are located at the N-terminus of a protein, the thioester linkage rearranges due to the proximity of the primary amine, leading to a stable amide linkage (*N*-palmitoylation) (Linder and Deschenes, 2007).

Some GPCRs have been reported to be *S*-palmitoylated. The *S*-palmitoylation sites in GPCRs, such as β_1AR (Zuckerman et al., 2011), endothelin receptors A and B (Stannard et al., 2003), and D_2R (Ebersole et al., 2015), are mainly found on their cytoplasmic tail. Alternative palmitoylation sites are found on the ICLs of some GPCRs such as μ-OR (Ebersole et al., 2014, Zheng et al., 2012), β_1AR (Zuckerman et al., 2011) and β_2AR (Adachi et al., 2016). Since the *S*-palmitoylation is a reversible lipid modification, the turnover of this type of palmitoylation is widely hypothesized to be involved in regulating GPCR signalling by modulating structure, trafficking and stability of the receptor (Goddard and Watts, 2012, Norskov-Lauritsen and Brauner-

Osborne, 2015, Zhang and Kim, 2017). As discussed in Section 1.2.2, the human kisspeptin receptor contains two Cys residues that are highly possible to be palmitoylated, but it is not known if the Cys residues are in fact modified. Investigations in this post-translational modification would provide new insights into the mechanisms of signalling of kisspeptins and the kisspeptin receptor and the structure of the kisspeptin receptor. In this chapter, two Cys residues (Cys^{338} and Cys^{340}) at C-terminus of the kisspeptin receptor are predicted as potential palmitoylated sites by bioinformatics analysis. Site-directed mutagenesis was conducted in order to examine their roles in the kisspeptin receptor signalling. Ser substitution was introduced to generate two single mutants (C338S and C340S) and a double mutant (C338S-C340S). A R331X mutant receptor, which contains a truncation of C-terminal tail, was additionally created as a negative control, since it has been shown that R331X mutation leads to a loss of function of the receptor (Seminara et al., 2003). The expression of the mutant receptors was evaluated and their KP-10-induced signalling was elucidated.

5.3 Results

5.3.1 Prediction of potential palmitoylation sites

In order to determine the likelihood of the human kisspeptin receptor being palmitoylated, the programs NBA-Palm (Xue et al., 2006) and CSS-Palm 4.0 (Ren et al., 2008) were used. Four Cys residues, Cys^{94}, Cys^{95}, Cys^{115} and Cys^{340}, were predicted as potential sites for palmitoylation by CSS-Palm program at a high stringency (Fig. 5.1a). Only one putative palmitoylation site, which is Cys^{340}, was identified by NBA-Palm program (Fig. 5.2b). The possibility of these residues being palmitoylated was further assessed by examining their locations on the kisspeptin receptor. Since the 3D structure of the kisspeptin receptor has not been determined, a prediction of the TMs of the receptor based on its primary sequence was conducted by using TMHMM version 2.0 (Krogh et al., 2001) (Fig. 5.2). The results showed that Cys^{94} and Cys^{95} are located in TM2, and so are buried in the lipid membrane (Fig. 5.2). Therefore, they are most unlikely to be *S*-palmitoylation sites. In addition, Cys^{115} is at the extracellular end of TM3 and so is unlikely to be a palmitoylation site. Instead, it has been predicted to form a disulfide bridge with Cys^{191} in the middle of ECL2 (Sigrist et al., 2013), which is conserved in all GPCRs (Di Pizio et al., 2016, Venkatakrishnan et al., 2013). Cys^{340}, which was predicted as a palmitoylation site by both programs, is located in the C-terminus of the kisspeptin receptor. The location provides high intracellular accessibility. In addition, it has been reported that the Cys residues positioned 10- to 14-amino-acid downstream of TM7 of GPCRs are highly possible to be palmitoylated (reviewed in (Escriba et al., 2007)). Hence, Cys^{340} is the most likely of the predicted palmitoylated site. Cys^{338}, which is

adjacent to Cys^{340}, was also hypothesized as a potential palmitoylation site due to its location.

a) ※ CSS-Palm Online Service

Results:4 .txt Download Visualize Return

ID	Position	Peptide	Score	Cutoff
Unnamed	94	TDVTFLLCCVPFTAL	7.674	1.906
Unnamed	95	DVTFLLCCVPFTALL	7.039	3.536
Unnamed	115	WVLGDFMCKFVNYIQ	10.146	3.536
Unnamed	340	AFRRVCPCAPRRPRR	5.744	3.536

b) ※ NBA-Palm : Prediction of Palmitoylation Site Implemented In Naive Bayesian Algorithm

Go back to NBA-Palm prediction page

Predicted Palmitoylation sites:

Download the TAB-deliminated data file from here.

ExampleProtein

Peptide	Position	Probability	CutOff
FRRVCPCAPRRPR	340	0.849	0.4

Download the TAB-deliminated data file from here.

Figure 5.1 Prediction of *S*-palmitoylation sites on the human kisspeptin receptor. The potential palmitoylated sites of the human kisspeptin receptor (GenBank accession number: AAK83235.1) were predicted by a) CSS-palm and b) NBA-palm program.

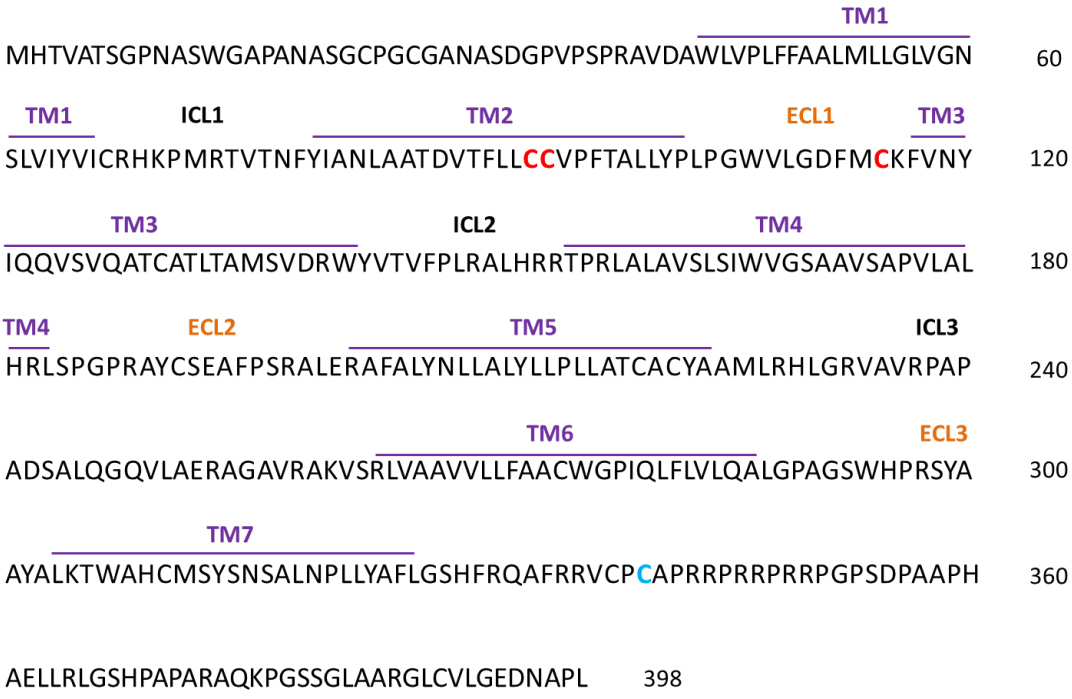


Figure 5.2 Prediction of TM helices in the human kisspeptin receptor. The seven TMs of the kisspeptin receptor (GenBank accession number: AAK83235.1) were predicted by TMHMM program. Residues in red are potential palmitoylation sites predicted by CSS-Palm, while the Cys residue in blue is the site predicted by both CSS-Palm and NBA-Palm programs.

5.3.2 Generation of the flag-tagged human kisspeptin receptor mutant constructs

In order to elucidate the roles of the two potential palmitoylated Cys residues (Cys^{338} and Cys^{340}) in the signalling of the kisspeptin receptor, several mutations were introduced into the C-terminal tail. Cys^{338} and Cys^{340} residues were substituted with Ser to generate two single mutants (C338S and 340S) and a double mutant (C338S-C340S). Ser was preferred to the conventional Ala substitutions, because it is more likely to be neutral in terms of structure and non-covalent interactions, since alcohols and thiols are more chemically similar. In addition, a mutant receptor with a truncated C-terminal tail at residue Arg^{331} was constructed (R331X; Fig. 5.3). For the generation of these mutants, the flag-tagged human kisspeptin receptor plasmid was used as template in the PCR site-directed mutagenesis.

Since the mutation sites for C338S and C340S mutants are located in the Pro-Arg-Arg repeats of the kisspeptin receptor, the designed primers for these two mutants possess very high CG content. In order to minimize the non-specific binding caused by these primers, a fragment PCR product of flag-kisspeptin receptor was produced initially, which was used as a template for the mutagenesis using common primers (Table 5.1). The primers used for each mutant are listed in Table 5.1 and the PCR products containing mutated residues were subcloned into flag-kisspeptin receptor between *AflII* and *NotI* sites. The mutated kisspeptin receptor plasmids were sent to Invitrogen (Thermo Fisher Scientific, USA) for sequencing.

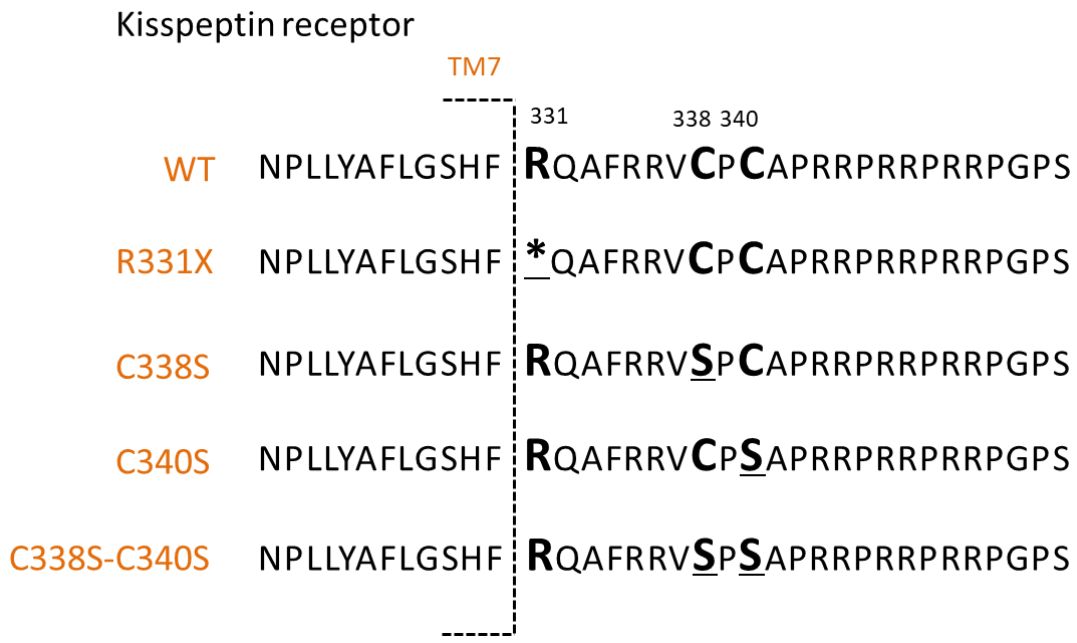


Figure 5.3 Partial protein sequences for wild type and mutated kisspeptin receptor. The amino acid sequences of mutated sites of four mutants are indicated and the mutated residues are enlarged and underlined. * stands for a stop codon (TGA).

Table 5.1 Primers for PCR site-directed mutagenesis. This table shows the sequences of primers used for the generation of four mutant receptors. The nucleotides encoding mutated residues are underlied and the mutated sites are labeled in bold.

Primer	Sequence (5' to 3')
Common Forward	TTCGCACTGTACAACCTGCT
Common Reverse	CCTCTACAAATGTGGTATGGCTG
R331X Forward	GCTCGCACTTCT**T**GACAGGC
R331X Reverse	GCCTGTC**A**GAAGTGCGAGC
C338S Forward	TTCCGCCGCGTC**A**GCCCCT
C338S Reverse	AGGGGC**T**GACGCGGCGGAA
C340S Forward	GCCGCGTCTGCCCC**A**GCGC
C340S Reverse	GCGC**T**GGGGCAGACGCGGC
C338S-C340S Forward	GCCGCGTC**A**GCCCC**A**GCGC
C338S-C340S Reverse	GCGC**T**GGGGC**T**GACGCGGC

5.3.2 Functional examination of the flag-tagged human kisspeptin receptor mutant constructs

Before testing whether these mutant receptors retain their biological functions, the total expression of each mutant was examined by western blot. The results of western blot were analyzed by quantifying the band intensity using Odyssey application software (version 3.0). The band for β-actin was selected as a loading control and the mean fold over the level of expression of wild-type (WT) receptor for each sample is calculated and plotted. MCF-7 cells were transiently transfected with WT and mutant plasmids and the results are shown in Fig. 5.4. The expression profile of these mutants showed significantly increased expression in R331X (1.65 ± 0.401 fold change; white) and C338S (1.90 ± 0.317 fold change; red). The expression of the C340S mutant (orange) remained a similar level as that of WT receptor. By contrast, the expression of C338S-C340S mutant was significantly decreased (0.54 ± 0.16 fold change; green).

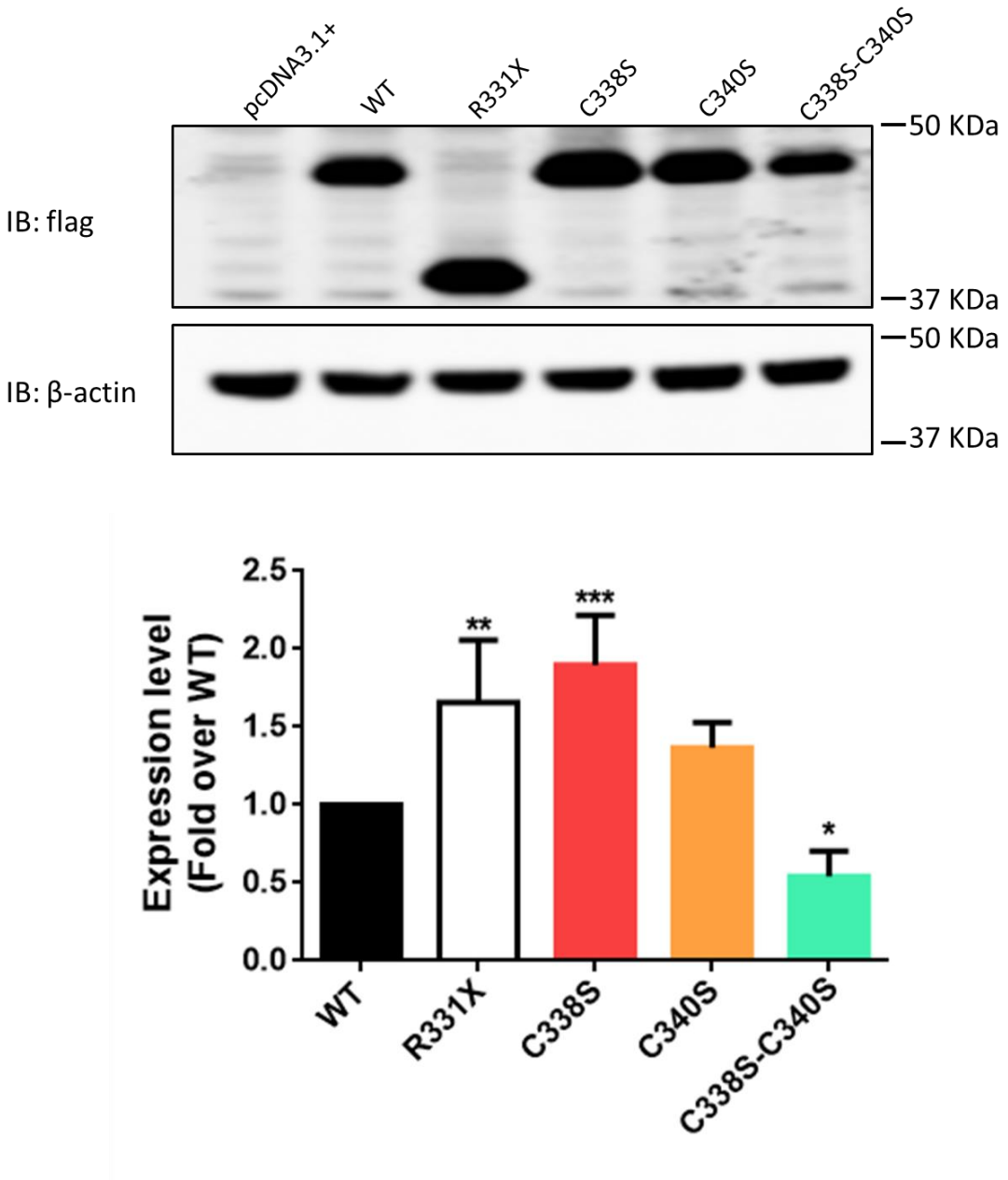


Figure 5.4 Total expressions of the mutant receptors. MCF-7 cells were transiently transfected with WT and the mutant receptors as indicated. Cells transfected with pcDNA3.1+ was used as negative control. After 48 hours culture, cells were lysed by SDS sample buffer and the expression of these mutants were measured by western blot. Representative blots are shown. Data from four independent experiments were quantified (using β-actin as a loading control) and the mean fold over the expression of WT ± SD is presented. *p<0.05* (*), *p<0.01* (**) and *p<0.005* (***) represent statistical significance from the expression level of WT group.

The biological functions of the mutants were initially determined by measuring the KP-10-induced dual phosphorylation of ERK1/2 by western blot in MCF-7 cells expressing WT or the mutant receptors. The mean fold over vehicle treated group (cells expressing pcDNA3.1+) of each sample is calculated and plotted (Fig. 5.5). Cells were stimulated with a final concentration of 100 nM KP-10 or vehicle (0.02% (v/v) propylene glycol) for 10 minutes, when the maximal phosphorylation of ERK1/2 was observed in MCF-7 cells (Section 3.3.2). Stimulation by 100 nM KP-10 caused a significant increase in the phosphorylation of ERK1/2 in cells expressing WT (2.02 ± 0.388-fold change; black), C338S (2.23 ± 0.364-fold change; red) and C340S (2.13 ± 0.245-fold change; orange) mutant (Fig. 5.5). No statistically significant increase in the level of the phosphorylation of ERK1/2 was detected in cells transfected with pcDNA3.1+ (blue), R331X (white) or C338S-C340S (green) mutant upon KP-10 stimulation (Fig. 5.5).

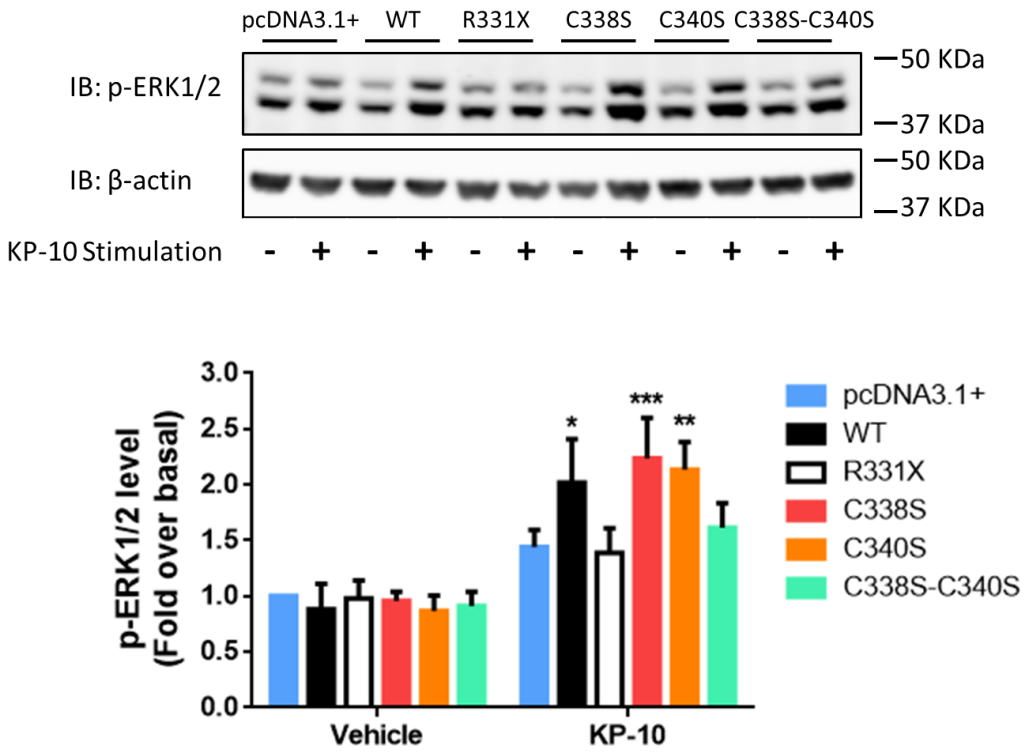


Figure 5.5 Immunoblots depicting the KP-10-induced phosphorylation of ERK1/2 using MCF-7 cells expressing WT or mutants. MCF-7 cells were transiently transfected with pCDNA3.1+ (blue), flag-WT (black), flag-R331X (white), flag-C338S (red), flag-C340S (orange) or flag-C338S-C340S (green).Cells were serum-starved for 20 hours prior to being treated with vehicle (-; 0.02% (v/v) propylene glycol) or 100 nM KP-10 (+) for 10 minutes. Representative blots are shown. Data from three independent experiments were quantified (using β-actin as a loading control) and the mean fold over control (vehicle treated cells expressing pcDNA3.1+) ± SD for the phosphorylation of ERK1/2 is presented. *p<0.05* (*), *p<0.01* (**) and *p<0.005* (***) represent statistical significance from respective pcDNA3.1+-expressing sample.

In addition to the measurement of the KP-10-induced phosphorylation of ERK1/2, the calcium responses to KP-10 stimulation were measured for the purpose of elucidating the biological functions of the mutant receptors. Calcium response to 100 nM KP-10 was measured in MCF-7 cells, which are transiently transfected with mutant receptors, by using Fluo-4 Direct™ calcium assay kits (Section 2.18). Cells were stimulated by vehicle (0.02% (v/v) propylene glycol; Fig. 5.6a) or 100 nM KP-10 (Fig. 5.6b). Two different kinetic patterns of calcium response were observed. Stimulation by KP-10 caused an immediate calcium flux in the cells expressing WT, C338S and C340S mutant receptors. By contrast, a delayed calcium response was found in the cells expressing pcDNA3.1+, R331X and C338S-C340S mutants. This delayed response may not be mediated by the kisspeptin receptor signalling pathway, as it was also observed in the empty vector control. Therefore, the percentage response is calculated by the maximum response between 20 to 30 seconds minus the baseline (average of first 20 seconds) divided by the baseline for each sample. The percentage response for each vehicle and KP-10 treated groups from four independent experiments is plotted in Fig. 5.7. Statistically significant responses were identified in the cells expressing WT (54.29% ± 16.88), C338S (47.26% ± 11.73) and C340S (42.96% ± 7.977) compared with that in vehicle treated samples.

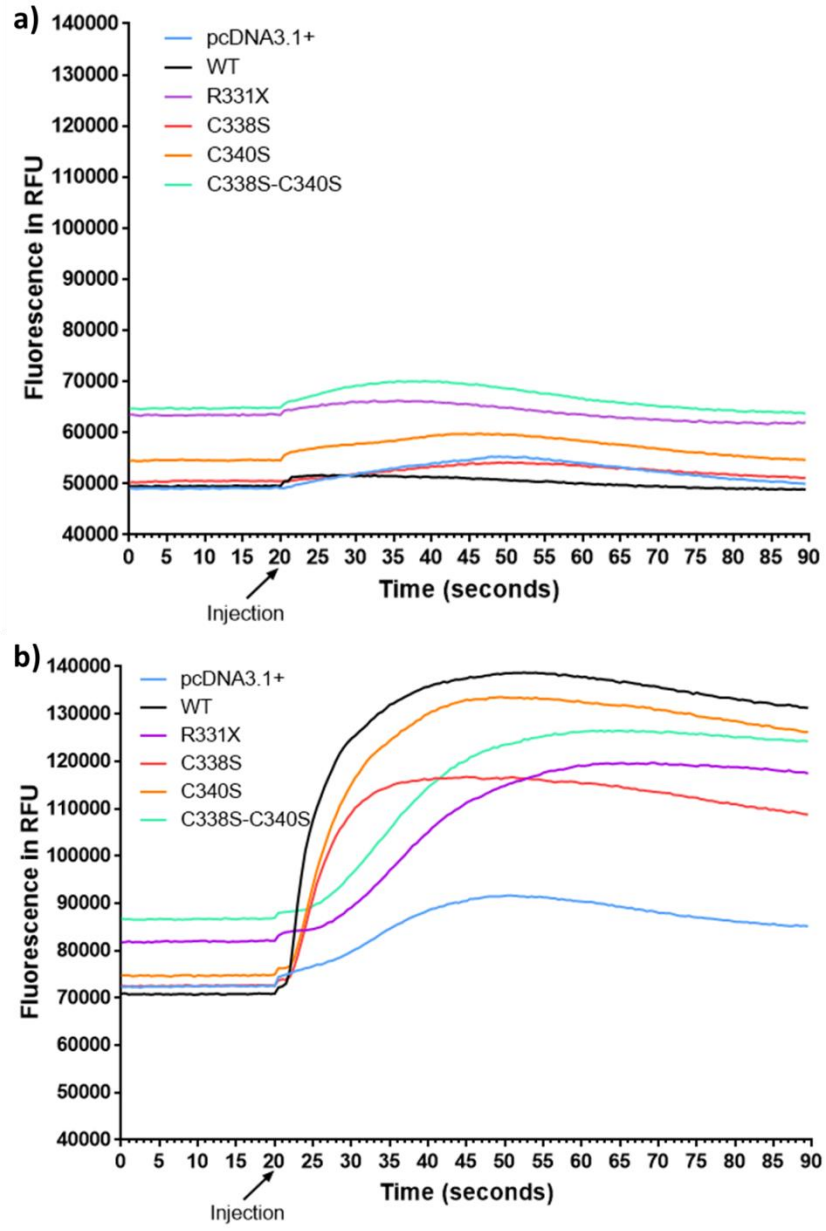


Figure 5.6 Calcium response to KP-10 in MCF-7 cells expressing WT and mutant receptors. MCF-7 cells transfected with pcDNA3.1+ (blue), WT (black), R331X (purple), C338S (red), C340 (orange) or C338S-C340S (green) were plated in a poly-D-Lysine coated 96-well plate. After 48 hours incubation, cells were assayed for calcium response using the Fluo-4 Direct™ Calcium Assay Kit. Cells were stimulated with vehicle (0.02% (v/v) propylene glycol; Figure 5.6a) or 100 nM KP-10 (Figure 5.6b) after 20-second reading of the baseline. Measurements are given in relative fluorescent units (RFU) every 0.5 second. Four independent expriments were conducted, and only one of them is plotted here.

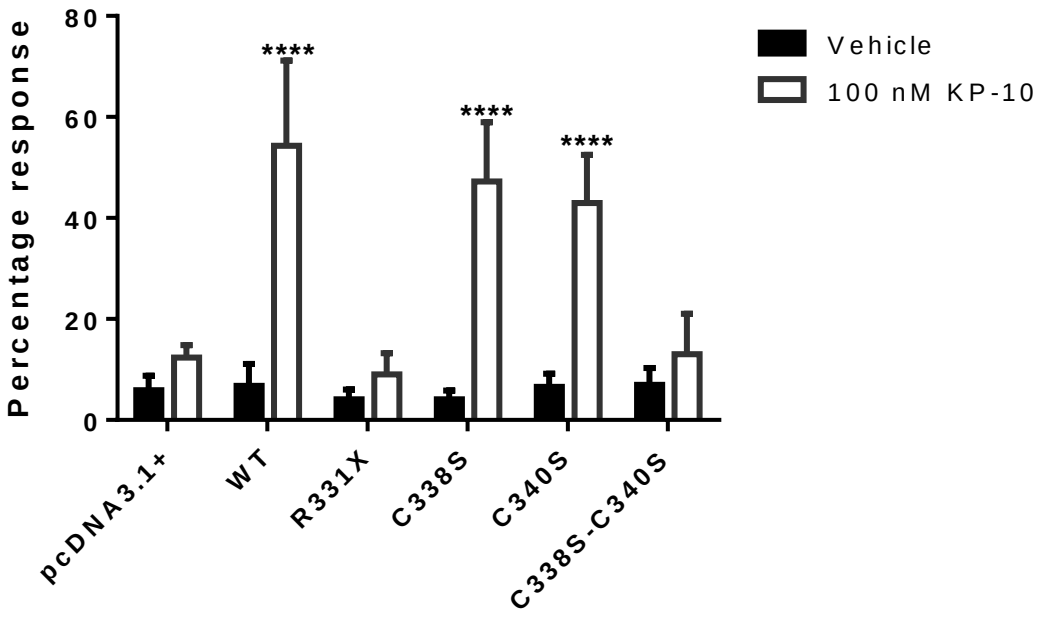


Figure 5.7 Analysis of calcium response in figure 5.4. Percentage response over respective basal level is calculated by the maximum response between 20.5 to 30.5 seconds minus the baseline (average of first 20 seconds) divided by the baseline. The mean percentage response ± SD from four independent experiments are presented. *p<0.001* (****) represents statistical significance from respective vehicle treated group.

In addition to above functional assays, the level of agonist-induced internalization of the receptor was further tested (Section 3.3.4). The levels of internalization of the receptor were observed after 10-minute stimulation and remained at a similar level in the course of up to 60-minute stimulation. A significant increase in internalized Cy5-KP18 level was detected in cells expressing WT (3.31 ± 0.525-fold change) and C338S (3.05 ± 0.563-fold change) compared with the baseline in the cells expressing pcDNA3.1+ (2.03 ± 0.105-fold change) after 30-minute incubation (Fig. 5.8). However, the internalization of R331X (2.15 ± 0.344-fold change), C340S (2.54 ± 0.625-fold change) and C338S-C340S (2.12 ± 0.382-fold change) mutant receptors remained at the baseline level. Similar results were found at the other tested incubation time (Fig.5.8).

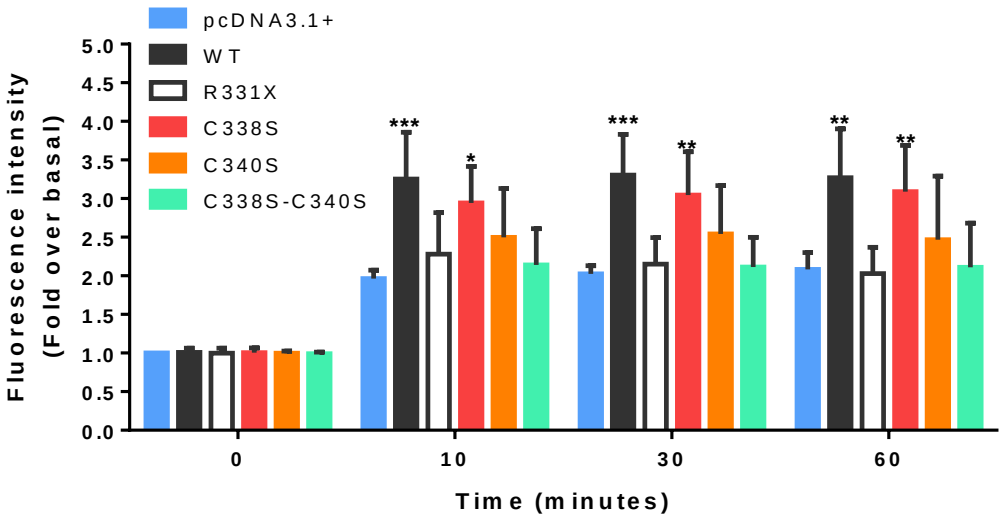


Figure 5.8 Cy5-KP18 binding properties of the kisspeptin receptor mutants. MCF-7 cells were transiently transfected with pcDNA3.1+, WT or mutant receptors as indicated and cultured for 48 hours. After that, cells were incubated with 50 nM Cy5-KP18 for different time as indicated. The baseline activity was measured in cells expressing pcDNA3.1+. Data from four independent experiments were quantified (compared with the baseline of cells expressing pcDNA3.1+) and the mean fold over the baseline of cells expressing pcDNA3.1+ ± SD for the level of internalized Cy5-KP18 is presented. *p<0.05 (*), p<0.01* (**) and *p<0.005* (***) represent statistical significance from respective pcDNA3.1+ expressing sample.

5.4 Discussion

In this chapter, site-directed mutagenesis studies were used to investigate the functional roles of potential palmitoylated Cys residues, Cys^{338} and Cys^{340}, of the kisspeptin receptor. Four mutant receptors were generated including two single mutants (C338S, C340S and R331X) and one double mutant (C338S-C340S). R331X has been shown to be a loss-of-function mutation (Seminara et al., 2003) and, therefore, was used as a negative control. The expression level of these mutants was initially accessed by western blot. A reduction in receptor expression was observed with the C338S-C340S mutation, suggesting that palmitoylation may be pivotal for the stabilization of the receptor. Palmitoylation has been shown to play an important role in maintaining the stability of several GPCRs. For example, deletion of the palmitoylated Cys residue of D_2R resulted in a reduced level of protein expression, while the mRNA level of the receptor retained (Ebersole et al., 2015). Similarly, the Ala substitution of all three palmitoylated Cys residues of CCR5 led to a decrease in the level of protein expression (Percherancier et al., 2001). On possible explanation could be that palmitoylation prolongs the half-life of the receptor, as the triple-Cys to Ala mutant of CCR5 exhibited shortened half-life (3-fold) compared with the WT CCR5 (Percherancier et al., 2001). In contrast, an increase in the expression of C338S mutants was seen. The reasons of the observation remain to be investigated.

Palmitoylation has been shown to be able to regulate GPCRs signalling *via* affecting their G protein coupling (Qanbar and Bouvier, 2003). Mutation of the palmitoylation site of several GPCRs, such as μ-OR (Zheng et al., 2012), NTR1 (Heakal et al., 2014),

and muscarinic acetylcholine receptor m2 (Hayashi and Haga, 1997), resulted in a defective interaction between the receptor and the cognate G proteins. In addition to the coupling to G proteins, the association between some GPCRs and other non-G protein interacting proteins was also reported to be regulated by palmitoylation. For example, mutation on the palmitoylation site of protease-activated receptor 2 affected localization of both β-arrestin-1 and β-arrestin-2 to the inner surface of membrane by eliminating the plasma membrane coupling of β-arrestin-1 and delaying the surface relocation of β-arrestin-2 (Adams et al., 2011). Therefore, the effects of the Ser substitutions on $G_{q/11}$- or β-arrestin-mediated signalling of the kisspeptin receptor were measured by detecting agonist-induced calcium flux and phosphorylation of ERK1/2. The single mutation on neither Cys^{338} nor Cys^{340} was unable to affect the maximum responses of the KP-10-induced phosphorylation of ERK1/2 and calcium flux. By contrast, the activation of these two signalling pathways was completely abolished in the double C338S-C340S mutant, as well as in the R331X deletion mutant. These results show that Cys^{338} and Cys^{340} are involved in the receptor signalling and palmitoylation on either Cys^{338} or Cys^{340} may be required for proper $G_{q/11}$- and β-arrestin-dependent signalling of kisspeptins and the kisspeptin receptor.

Involvement of palmitoylation in receptor internalization and endocytosis has been reported in several GPCRs such as protease-activated receptor 2 (Adams et al., 2011), β_2AR (Adachi et al., 2016) and dopamine D_3 receptor (Zhang et al., 2016). So the effects of these mutations on agonist-induced internalization of the kisspeptin

receptor were investigated by detecting the co-internalized level of Cy5-KP18. Mutation of Cys^{338} did not affect the internalization of the receptor. In contrast, Cy5-KP18-induced internalization of the kisspeptin receptor was undetectable in the C340S and in the C338-C340S mutant. GRK and β-arrestin have been reported to mediate the internalization of the kisspeptin receptor through the interaction with the ICL2 and C-terminal tail of the receptor (Pampillo et al., 2009). Therefore, palmitoylation on Cys^{340} may be required for folding of the C-terminal tail of the kisspeptin receptor, which allows the phosphorylation of the receptor and mediates the receptor/β-arrestin interaction. Indeed, for some GPCRs, such as thyrotropin-releasing hormone receptor (Gehret et al., 2010), mutations on the palmitoylation sites can inhibit the GRK-mediated phosphorylation of the receptor, which is required for receptor downstream signalling and internalization.

Taken together, the Ser substitution of Cys^{338} had little effect on receptor expression and functions. By contrast, the replacement of Cys^{340} to Ser had little effect on expression and the downstream signalling evens measured, but it did affect internalization. Mutation of both Cys^{338} and Cys^{340} (C338S-C340S) decreased the total expression of the receptor and abolished receptor signalling and internalization. It has been shown that the kisspeptin receptor regulates the activity of ERK1/2 by both $G_{q/11}$- or β-arrestin-2-dependent manners (Szereszewski et al., 2010). The mutation on Cys^{340} may affect the configuration of the C-terminal tail of the kisspeptin receptor, which only affect the binding of the receptor to β-arrestins, but not $G_{q/11}$. As consequences, the mutant receptor could activate $G_{q/11}$-dependent signalling,

such as calcium and EKR1/2 response, but not β-arrestin-mediated signalling pathways, such as the internalization of the receptor. These data may indicate that Cys^{340} is a primary palmitoylation site. When is Cys^{340} mutated, an alternative palmitoylation may occur on Cys^{338}, which would then rescue at least some aspects of the kisspeptin receptor signalling. Alternatively, Cys^{338} may be normally palmitoylated, but this has effects on signalling functions not measured here, and does not impact on receptor internalization. Although, it has been reported that palmitoylation is pivotal for the cell surface translocation of many GPCRs, such as β_2AR (Adachi et al., 2016), CCR5 (Percherancier et al., 2001), D_2R (Ebersole et al., 2015) and protease-activated receptor 2 (Adams et al., 2011). The failure of the measurement of internalization and signalling functions of the C338-C340S mutant may result from its low expression level to cell surface. Under this consideration, effects of the Cys residues on cell surface expression of the kisspeptin receptor will be investigated in the future. To determine whether the Cys residues of the kisspeptin receptor are in fact palmitoylated in the further studies, two main approaches could be used to detect and quantify protein palmitoylation instead of the incorporation assay using radioactive labelled palmitate in the future studies. One of them is based on acyl-biotinyl exchange chemistry (Drisdel and Green, 2004). Briefly, cell lysates are treated with *N*-ethylmaleimide to block free thiol groups. After that, the palmitoyl group is removed from palmitoylated Cys residues by hydroxylamine treatment and the newly generated free thiol groups are ready for a variety of isotopic or chemical probes. The other approach is based on metabolic incorporation of the palmitic acid analogue, 17-octadecynoic acid (17-ODYA), followed by bio-orthogonal click chemistry (Martin and Cravatt, 2009, Martin, 2013).

Chapter 6

Conclusion

6.1 Conclusion

Kisspeptins and the kisspeptin receptor play a pivotal role in the puberty onset by exciting GnRH neurons, which mediates the secretion of GnRH. GnRH is the central regulator of mammalian reproductive system (Ronnekleiv and Kelly, 2013). Natural occurring loss-of-function mutations on the kisspeptin receptor are associated with IHH disease (Seminara et al., 2003, de Roux et al., 2003, Semple et al., 2005, Tenenbaum-Rakover et al., 2007, Wacker et al., 2008, Teles et al., 2008) and the gain-of-function mutation on the receptor is linked to idiopathic central precocious puberty (Section 1.3.1) (Teles et al., 2008). Another important role of kisspeptins and the kisspeptin receptor is involved in the suppression of cancer metastasis. Kisspeptin has been demonstrated to inhibit cell growth and migration in a variety of cancers (Section 1.3.2) (Ohtaki et al., 2001, Masui et al., 2004, Stathatos et al., 2005, Jiang et al., 2005, Yoshioka et al., 2008, Kang et al., 2011, Takeda et al., 2012, Tan et al., 2014).

Due to the importance in the regulation of puberty and suppression of cancer metastasis, the kisspeptin receptor may be a good target for the treatment of cancer metastasis and IHH disease. However, kisspeptins are metabolic unstable and rapidly metabolized in mouse serum (Asami et al., 2012a, Asami et al., 2012b). Development of kisspeptin analogues with enhanced stability and potency may provide better therapeutic efficacy and improve the understanding of long-term signalling of the kisspeptin receptor in *in vitro* and *in vivo* studies. Several studies have designed and synthesized several kisspeptin analogues with increased metabolic stability and at least similar potency compared with kisspeptins (Section 1.2.3.2) (Asami et al.,

2012b, Asami et al., 2012a, Tomita et al., 2008, Nishizawa et al., 2016, Scott et al., 2013). In this thesis, a novel kisspeptin lead possessing the kisspeptin receptor-agonistic activity is developed based on the last five residues of KP-10. The peptide termed compound B contains a phosphinic acid moiety (-PO_2-CH_2-), which replaces the peptide bond between Gly^{51} and Leu^{52} of KP-10, and has inhibition on MMP-2. Since phosphinic peptides represent high metabolic stability *in vivo* and the containing phosphoryl group is a weak zinc-chelating group (Matziari et al., 2007), compound B may form a stable complex with MMP-2 and be resistant to MMP-2-mediated digestion. However, the potency of compound B to activate the kisspeptin receptor and inhibit MMP-2 activity remains low, but may serve as a lead compound to further modification. For the further studies, the structure of compound B will be determined by NMR studies.

The kisspeptin receptor is a $G_{q/11}$ coupled receptor (Muir et al., 2001, Kotani et al., 2001) and mediates conventional $G_{q/11}$-dependent signalling pathways (Section 1.4.1). Other kisspeptin receptor-interacting proteins identified are β-arrestin1, β-arrestin2 (Pampillo et al., 2009) and PP2A (Evans et al., 2008). The kisspeptin receptor couples to these proteins to transduce downstream signalling (Section 1.4.2). However, it remains to be explored if there are more GIPs that regulate the functions of the kisspeptin receptor. Three Pro-Arg-Arg repeats within the C-terminal tail of the kisspeptin receptor are predicted as SH3-binding motifs by the NEW Scansite 3 software, which may mediate the interaction of the receptor with SH3 domain-containing proteins (Obenauer, 2003). The data presented revealed two novel SH3 domain-containing proteins, p85α and c-SRC, that could directly interact

with the kisspeptin receptor. The results imply that the kisspeptin receptor may couple to p85α and c-SRC to activate PI3K/AKT and c-SRC-dependent ERK1/2 signalling respectively. Since metastasis is a progress that occurs over long time, the early signalling of the kisspeptin receptor to PI3K/AKT and c-SRC presented here may not be involved in the suppression of cancer metastasis. Alternatively, the signalling pathways measured here might be involved in the regulation of the development of puberty, as both PI3K/AKT pathway (Novaira et al., 2009, Luque et al., 2011) and c-SRC (Zhang et al., 2013) have been shown to play a key role in kisspeptin-induced excitation of GnRH neurons (Section 1.4.4). Interestingly, kisspeptins and the kisspeptin receptor can suppress the phosphorylation of AKT induced by other GPCRs, such as CXCR4 (Navenot et al., 2005), and receptor tyrosine kinase, including EGFR and insulin receptor (Navenot et al., 2009). Consistently, data presented here show a weak negative regulation of the kisspeptin receptor on insulin-induced phosphorylation of AKT. Thus, the downregulation of a subset of receptor tyrosine kinases may be involved in the suppression activity of kisspeptins and the kisspeptin receptor on cancer metastasis. In the future, to fully understand the mechanisms underlying the suppression activity of kisspeptins and the kisspeptin receptor on cancer metastasis, a longer stimulation of kisspeptins is necessary. In addition, the potential sites of the kisspeptin receptor that mediate the interaction of the receptor with p85α and c-SRC will be identified by mutagenesis. The effects of mutant receptors on the kisspeptin receptor signalling and expression will be measured.

Moreover, palmitoylation is one of post-translational modification on GPCRs and has been demonstrated to be involved in receptor signalling, trafficking and stability

(Section 1.2.2) (Goddard and Watts, 2012, Norskov-Lauritsen and Brauner-Osborne, 2015, Zhang and Kim, 2017). Cys^{338} and Cys^{340}, which are about 10-amino-acid distant from the C-terminal end of TM7 of the kisspeptin receptor, are predicted to be two potential palmitoylation sites by bioinformatics analysis. The roles of Cys^{338} and Cys^{340} are uncharacterized. In this thesis, the Cys residues were studied by site-direct mutagenesis to generate two single (C338S and C340S) and one double (C338S-C340S) mutants. Only the C338S-C340S mutant reduced receptor expression to 54% of the level of the expression of WT receptors, and completely abolished the kisspeptin receptor signalling measured here. Either the C338S-C340S or the C340S mutant impaired receptor internalization, whereas the C338S mutant had little effect on receptor expression and function studied here. The data indicate that Cys^{338} and Cys^{340} are essential for the kisspeptin receptor signalling. Cys^{340} might be a primary palmitoylation site and Cys^{338} may substitute Cys^{340} for palmitoylation when Cys^{340} is mutated. Alternatively, Cys^{338} may be normally palmitoylated, but this has effects on signalling functions not measured here. Palmitoylation has been reported to assist the tethering of H8 of some GPCRs to the cell membrane, which is a conserved structure of GPCRs and is important for receptor functions (Qanbar and Bouvier, 2003, Escriba et al., 2007). The kisspeptin receptor contains the conserved sequence [F(R/K)XX(F/L)XXX(L/F)], which is conserved in the H8 region of GPCRs (Section 1.2.1). Hence, the kisspeptin receptor may also form H8 structure at the C-terminus and Cys^{338} and Cys^{340} may be palmitoylated to anchor and stabilize the structure of H8. However, whether these two Cys residues of the kisspeptin receptor are palmitoylated remains to be elucidated in the future. In addition, since palmitoylation is pivotal for the cell surface translocation of many GPCRs

(Percherancier et al., 2001, Ebersole et al., 2015, Adachi et al., 2016), the failure of the measurement of internalization and signalling functions of the mutant receptors may result from their low expression level on cell surface. Hence, the effects of the Cys residues on cell surface expression of the kisspeptin receptor will be investigated in the further studies.

In summary, the results presented within the thesis have provided some details in the signalling pathways of the kisspeptin receptor. The kisspeptin receptor has been shown to interact with p85α and c-SRC to probably activate PI3K/AKT and c-SRC-dependent signalling pathways, which may underlie the mechanisms of functions of kisspeptins and the kisspeptin receptor in reproductive development and cancer suppression. In addition, Cys^{340} and Cys^{338} of the kisspeptin receptor may be palmitoylated, which the Cys^{340} might be a primary palmitoylation site, to assist the tethering of the H8 with the inner surface of cell membrane and thus regulation the kisspeptin receptor functions. Furthermore, a novel phosphinic peptide lead possessing the kisspeptin receptor-agonistic activity and suppression activity on MMP-2 has designed and assessed. The novel kisspeptin analogue may serve as a lead for design of more potent and metabolic stable kisspeptin analogues for potential therapeutic application.

Chapter 7

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7.1 References

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