The Role of $G_{\alpha i}$ in the Gonadotropin- Releasing Hormone (GnRH) Receptor Inhibition of cell proliferation

By

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Contents

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List of Abbreviations

PTX	Pertussis toxin
GDP	Guanine diphosphate
GTP	Guanine tri-phosphate
GPCR	G protein coupled receptors
cAMP	cyclic adenosine monophosphate
PLC	phospholipase C
DAG	Diaglycerol
PKC	Protein kinase C
PIP_2	phosphatyidylinositol bisphophate
AC	adenylyl cyclase
GnRH	Gonadotropin releasing hormone
GnRHR	Gonadotropin releasing hormone receptor
PKA	Protein kinase A
SDS	Sodium dodecyl sulfate
SDS-PAGE	Sodium dodecyl sulfate- polyacrylamide gel electrophoresis
MTT	Methylthiazolyldiphenyl- tetrazolium bromide
MW	Molecular weight
DMSO	Dimethyl sulfoxide
TM	Transmembrane
HEK293	Human embryonic kidney cells 293
DMEM	Dulbecco's modified eagle medium
HEPES	N-2-Hydroxyethylpiperazine-N-2'ethansulphonic acid
RIPA	radioimmunoprecipitation lysis buffer

Abstract

The activation of Gonadotropin- releasing hormone receptor (GnRHR) by the GnRH ligand has been shown to mediate antiproliferative effects in extra-pituitary cells and in reproductive cancer cell lines. The GnRHR couples to Gαq in pituitary gonadotropes. However, the GnRHR expressed in reproductive cancer cell lines is thought to couple to Gαi. Recent evidence also suggests that the antiproliferative effects may be mediated via Gαq in these cells. Therefore our study involved determining the role of Gαi in the antiproliferative effects mediated by the GnRHR. We used a Gαq/i chimera that is able to interact with Gq- coupling GPCRs but signals via the Gi pathway. We created stable cell lines expressing the rat gonadotropin- releasing hormone receptor (rGnRHR) only and the rGnRHR and Gαq/i in HEK293 cells. The signalling and proliferation profiles of these cells were compared in response to treatment with GnRH. The rGnRHR and Gαq/i chimera were shown to be stably expressed in HEK293 cells. We were able to show coupling of rGnRHR with Gαq/i but weak or non significant coupling with Gαi. The expression of Gαq/i increased the inositol phosphate production in cells expressing rGnRHR and Gαq/i compared to cells with receptor only; however there was no significant change in the potency of GnRH observed. Expression of Gαq/i also did not affect the kinetics of ERK activation. The antiproliferative effects of GnRH were increased in cells expressing the rGnRHR and Gαq/i relative to cells expressing the GnRHR only. In conclusion, these results suggest that the Gi pathway could play a role in mediating the antiproliferative effects of GnRH.

1. Introduction

1.1 G protein- coupled receptors (GPCRs)

G protein-coupled receptors (GPCRs) are the largest family of mammalian cell surface receptors (Dorsam and Gutkind 2007) (Tuteja 2009). In humans, there are over 800 genes that encode for GPCRs (Karnik, Gogonea et al. 2003) (Bai 2004). They play important roles in various biological and physiological processes such as development, vision and immune response. As a result they are the target for most drugs (Tuteja 2009) (Vaunquelin and Liefde 2005) (Mustafi and Palczewski 2009). They boast a variety of endogenous and exogenous ligands; including hormones, neurotransmitters and photons (Hermans 2003). GPCRs are characterised by seven transmembrane α helices (TM); N- and C- terminal tails and extracellular and intracellular loops (ECL and ICL) of varying length as shown in figure 1.1(Yin, Gavi et al. 2004) (Hermans 2003) (Millar 2003). The N-terminus and the pocket formed by the TM alpha helices contain the ligand binding sites (Hermans 2003). The ICL and C-terminal tail are involved in the mediation of intracellular actions through pathways involving Guanosine triphosphate binding proteins (G- proteins) and other cellular proteins (Kristiansen 2004). Each transmembrane helice contains a characteristics residue: Asn (1.50); Asp (2.50); Arg (3.50); Trp (4.50); Pro (5.50); Pro (6.50); Pro (7.50), indicated in Fig. 1.1 (Kristiansen 2004).

GPCRs are grouped differently depending on the classification method used. They have previously been classified by the nature of their ligand; the conservation of amino acid sequence and by the clustering of genes from different species. The classification of GPCRs expressed in humans is based on conservation of structural motifs within the TM helices and results in five families, the GRAFS classification system (Karnik, Gogonea et al. 2003).

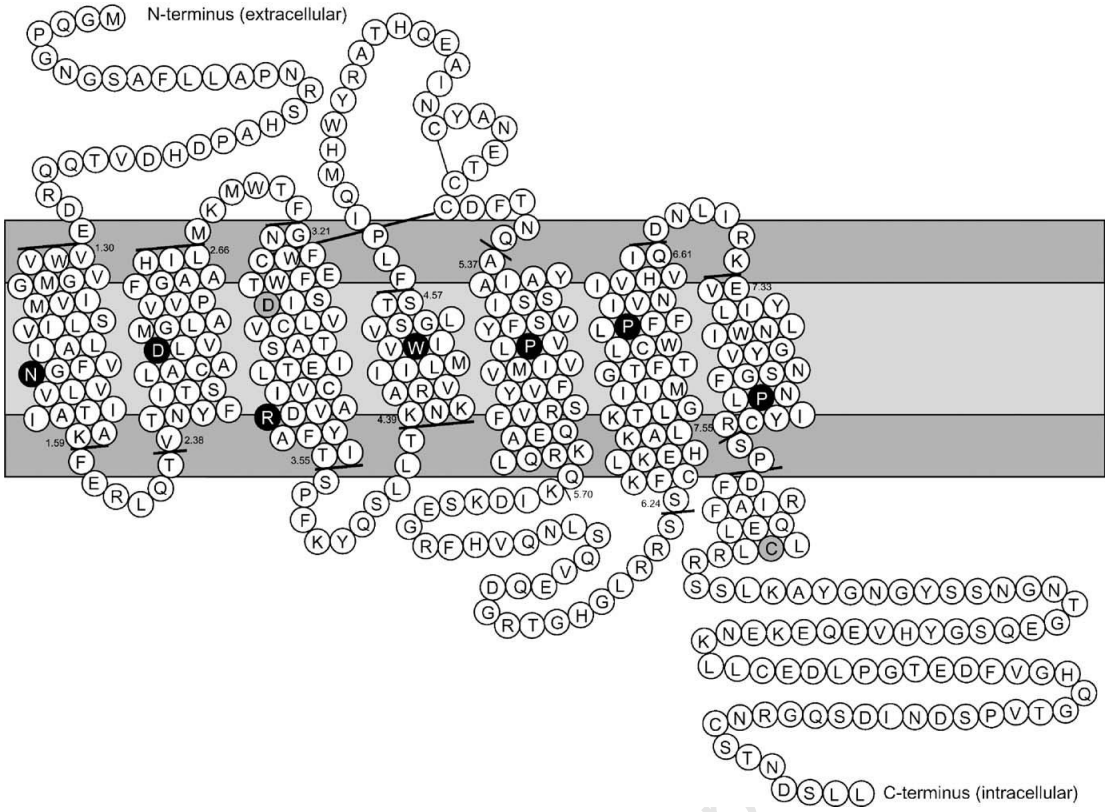


Figure 1.1: Representation of a GPCR structure (the human α2 adrenergic receptor is shown as an example). GPCRs are characterised by N- and C- terminus tails, a seven transmembrane α helices (TM) separated by 3 extracellular (ECL) and intracellular loops (ICL). The boundaries of the helices are indicated by the black horizontal short lines. The characteristic residue in each TM, Asn(1.50); Asp(2.50); Arg(3.50); Trp(4.50); Pro(5.50); Pro(6.50); Pro(7.50) are indicated in white on black circles. The figure was taken from (Kristiansen 2004).

The five families are: Glutamate, Rhodopsin, Adhesion, Frizzled/taste2 and Secretin. I shall focus on the Rhodopsin family as the GnRH receptor belongs to this family.

The Rhodopsin family contains 701 members including 241 non-olfactory receptors. The N-terminal residues of this family typically contain less than 100 residues. It is further subdivided into 4 groups with 13 branches.

The first group is called the α group and is further broken up into 5 branches. The prostanoid receptor cluster has 15 members. The cumine receptor cluster has 39 members. The opsin

receptor cluster has 9 members. The melatonin receptor cluster has 3 members. The melanocortin-endoglin-cannabinoid-adenosine receptor cluster has 22 members.

The γ group contains 3 main branches; the somatostatin-opioid-galanin cluster which has 15 members. The melanin-concentrating hormone has 2 members. The chemokine receptor has 42 members.

The σ group has 4 branches: The MAS-related cluster contains 8 members. The glycoprotein receptor cluster has 8 members. The purine receptor cluster has 42 members, while the olfactory receptor cluster has 460 members.

The β group is not subdivided into branches but has 36 receptors that bind peptides. The GnRH receptor belongs to this group (Fredriksson et al 2003). Our study will focus on this G-protein coupled receptor.

1.2 Guanosine triphosphate binding proteins (G- proteins)

G -proteins are so named because they bind guanosine triphosphate (GTP). There are two types of G- proteins; small GTP binding proteins and heterotrimeric G proteins. Both of these play important roles in the transmission of extracellular signals (Matozaki, Nakanishi et al. 2000). However, in this introduction, I shall only focus on heterotrimeric G proteins.

1.2.1 Heterotrimeric G- proteins

Heterotrimeric G- proteins are responsible for transducing signals from activated GPCRs to effector systems which alter the concentration of intracellular secondary messengers (Milligan and Kostenis 2006) (Fields. 1997). G- proteins bind the nucleotide GTP when activated (Offermanns 2003). They are composed of differently sized α, β and γ subunits. The α subunit binds and hydrolyses the GTP, while the β and γ form a dimer. Multiple isoforms of these have been isolated in mammalians: 23 α, 5β, 12 γ (see Table 1) (Hermans 2003). The α and combined βγ subunits are involved in receptor binding and regulation of effectors. The α subunit is also involved in regulation of the signal duration through its ability to hydrolyse GTP (Offermanns 2003). G proteins have been classified into four families: $G_{\alpha s}$, $G_{\alpha i}$, $G_{\alpha q}$, $G_{\alpha_{12}}$; according to the sequence homology of the α subunit. Each G protein family interacts with specific primary effector molecules as shown in table 1. The activated βγ subunit can either act on the same effector that the α subunit is interacting with or it may act on a different effector. The signalling resulting from the βγ interactions may act in a synergistic or antagonistic manner with the α subunit signalling (Hermans 2003).

1.2.1.1 The $G\alpha_S$ family

The proteins in this family are ubiquitously expressed and couple to the activation of adenylyl cyclase resulting in an increase in cAMP (Wettschureck and Offermans 2005). There are two members in this family: Gs and Golf. The Gs has four splice variants; two short [GsS] and two long [GsL]. They are structurally related and functionally interchangeable (Offermanns 2003). There is also another long splice variant, GsXL, which contains a long N-terminal portion and is expressed in neuroendocrine cells only (Offermanns 2003). Golf is expressed in the olfactory epithelium and in the central nervous system.

1.2.1.2 The $G\alpha_{i/o}$ family

This family is made up of 9 members: Gi_1, Gi_2, Gi_3, Goa, Gob Gz, Ggust and Gt-r and Gt-c. The Gi_{1-3} are widely expressed at high levels and mediate the inhibition of adenylate cyclase. The high structural similarities between the three Gi isoforms suggest overlapping functions. Members of the Gαi/o family, with the exception of Gz, are substrates to pertussis toxin (PTX). This toxin ADP- ribosylates the C-terminal residues of the α subunit thus inactivating the G protein. Activation of Gi subunits leads to an increase in free βγ subunits. Therefore the activation of Gαi/o leads to the initiation of βγ signalling processes (Ivanina, Varon et al. 2004). The Go is widely expressed in the central nervous system. Its downstream effects are said to be mediated by the βγ subunit. It has two isoforms; Goa and Gob. Gz is less widely expressed in neuronal cell and in platelet cells (Ho and Wong 2001). It shares similar functions with Gi but also has specific interactions with p21- activated kinases and certain RGS (Ho and Wong 2001). The remaining members, Gt and Ggust are involved in sensory functions (Wettschureck and Offermans 2005). Gt has two isoforms; Gt-r and Gt-c which are mainly expressed in retinal cells and taste cells. Ggust is expressed in taste and brush cells (Wettschureck and Offermans 2005).

1.2.1.3 The G $\alpha_{q/11}$ family

The Gq/11α family is made up of 4 members. G_q and G_{11} are ubiquitously expressed in the central nervous system and share more than 80% sequence homology (wettschureck, Moers et al. 2004; Wettschureck and Offermans 2005). Due to the high sequence homology, G_q is interchangeable with G_{11}.The other members of the family are expressed in a more restricted manner (Wettschureck and Offermans 2005). G_{14} is expressed in kidneys, lungs and spleen while the $G_{15/16}$ are expressed in hematopoietic cells only (Offermanns 2003). Members of the $G\alpha_{q/11}$ family couple to β isoforms of phospholipase C (PLC) and catalyses the hydrolysis

of phosphatidyl inositol bisphophate (PIP_2) resulting in the generation of inositol trisphosphate (IP_3) and diaglycerol (DAG) which causes the release of intracellular stores of calcium and activation of protein kinase C (PKC) (Wettschureck and Offermans 2005) (Mizuno and Itoh 2009).

1.2.1.4 The Gα $_{12}$ family

The two members of the G α12 family, G_{12} and G_{13}, are often activated by receptors which couple to $G_{q/11\alpha}$. They are ubiquitously expressed. There is very little information with regards to the signalling initiated by these proteins due to a lack of inhibitors. However, studies have shown that $G_{12/13\alpha}$ can activate phospholipase A_2, Na^+/H^+ exchanger and c-jun N-terminal kinase (JNK) (Wettschureck and Offermanns 2005) and small G protein Rho pathways (Kostenis et al 2005) (Hsu and Luo 2007).

1.2.1.5 The β and γ families

There are 5 β and 12 γ subunit isoforms (see Table 1). The first four β subunits are highly homologous 36 kDa proteins, sharing 80-90 % identity. The β5 subunit is only 50 % identical to the others and has a molecular weight of 40 kDa (McIntire 2009).While the first four β subunits are widely distributed, the fifth is expressed mainly in the central nervous system (Offermanns 2003).

The 12 γ subunit isoforms are all between 7 and 8.5 kDa and are much more divergent than the β isoforms. Given the large number of each β and γ isoforms, raises the question regarding specificity in the pairing between the β and γ. Evidence suggests a limited and

sometimes specific pairing. For example, β_2 dimerizes with γ_2 in vitro but not γ_1, indicating specificity in Gβγ coupling. Also the β5 subunits are able to interact with more γ subunits compared to the β1- β4 isoforms (Milligan and Kostenis 2006).

βγ subunits have been shown to couple to various effectors including; K^+ selective channel, PLCβ2 and PLCβ3, adenylyl cyclase and others (Cabrera- Vera T M 2003).

It is evident that unique protein-protein interactions within the G protein subunits (alpha, beta and gamma) as well as their interactions with effectors continue to be discovered. This increases the complexity of G protein signalling.

Table 1: Classification of heterotrimeric G protein subunit. Primary effector molecules are shown. Modified from Hermans 2003

Subunit	Family	Main subtypes	Primary Effector
α	**αs**	**Gαs; Gαolf**	**Adenylate cyclase ↑**
	αi/o	**Gαi1-3**	**Adenylate cyclase ↓**
		GαoA-B	**K channels ↑**
		Gαtr-c	
		Gαgust	**Ca channels ↓**
		Gαz	**Phosphodiestarase ↑**
	αq/11	**Gq**	**Phospholipase C ↑**
		Gα11	
		Gα14-16	
	α12	**Gα12-13**	**Rho pathway**
β	**β1-5**	**Different assemblies of β and γ subunits**	**Adenylate cyclase ↑/ ↓**
			Phospholipases ↑
			Phosphatidylinositol 3-kinase ↑
γ	**γ1- 12**		**PKC ↑**
			PKD ↑
			GPCR kinases ↑
			Ca; K; and Na channels

1.3 Activation cycle of GPCR and G protein

The activation of GPCR follows the cubic ternary complex where a receptor fluctuates between inactive and active states (ligand bound) (Krumins and Barber 1997; Kinzer-Ursem and Linderman 2007) (Kobilka and Deupi 2007). The receptor can be in the active or inactive state and it moves between them at equilibrium. The binding of ligand can either stabilise the receptor in the inactive (inverse agonist) or active (agonist) state and results in the activation of G proteins (Nickolls and Strange 2004) (Vaunquelin and Liefde 2005; Gilchrist 2007).

In the inactive state, the α subunit binds GDP and is associated with the βγ subunit, as shown in figure 1.2. Binding of a agonist to a GPCR catalyses the exchange of GDP for GTP by the α subunit (Herrmann, Heck et al. 2004), thus leading to the dissociation of α from βγ or to rearrangement of the α-βγ complex (Vilardaga, Bunemann et al. 209; Wang, Golebiewska et al. 2009). Both α-GTP and the free βγ are free to act upon effector molecules such as adenylyl cyclase and the proton exchange transporter. The α subunit is able to hydrolyse GTP to GDP, which results in the reassociation of α-GDP and βγ subunits. The inactivation of the cycle may be regulated by proteins called regulators of G protein signalling (RGS) which act upon the α subunit (Blummer, Smrcka et al. 2007; Mizuno and Itoh 2009) (Yanamadala, Negoro et al. 2009) to increase hydrolytic activity.

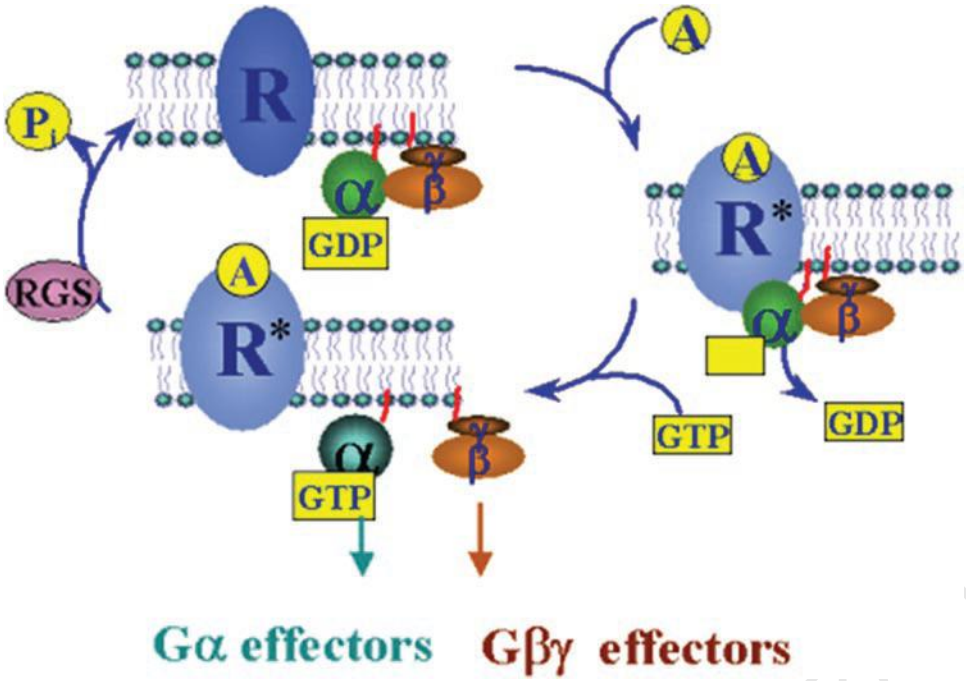


Figure 1.2: The G protein activation cycle. Agonist (A) binding to the receptor (R) causes a conformational change that catalyses the release of GDP and binding of GTP by Gα of heterotrimeric G protein. Disassociation of Gα and Gβγ allows effector activation The Gα has intrinsic GTP hydrolysis that inactivates Gα. GTP hydrolysis and Inactivation may be catalysed by regulators of G protein signalling proteins (RGS). The figure was taken from Cabrera-Vera et al 2003.

1.4 Activation of MAPK by GPCR

The proteins that link the extracellular signals that bind to GPCRs and the nucleus are serine/ threonine kinases called the Mitogenic- Activated Protein Kinase (MAPK) (Gutkind 1998) (Fukuhara, Marinissen et al. 2000). There are four MAPK pathways in mammals: extracellular signal- regulated kinase (ERK); Jun N-terminal kinase (JNK); p38MAPK; and big MAPK or ERK5 as shown in figure 1.3. They are activated by a wide variety of stimulus such as growth hormones and cytokines; as well diverse stresses such as oxidative stress and UV irradiation (Fukuhara, Marinissen et al. 2000). Activated MAPK translocate into the nucleus and trigger transcription of genes involved in a variety of cellular processes (Kraus et al 2001) (Naor, Bernard et al. 2000). The activated MAPK may also affect cytoskeletal

processes in the cell (Davidson et al 2004).

MAPK form cascades that contain up to five tiers of protein kinases that sequentially activate

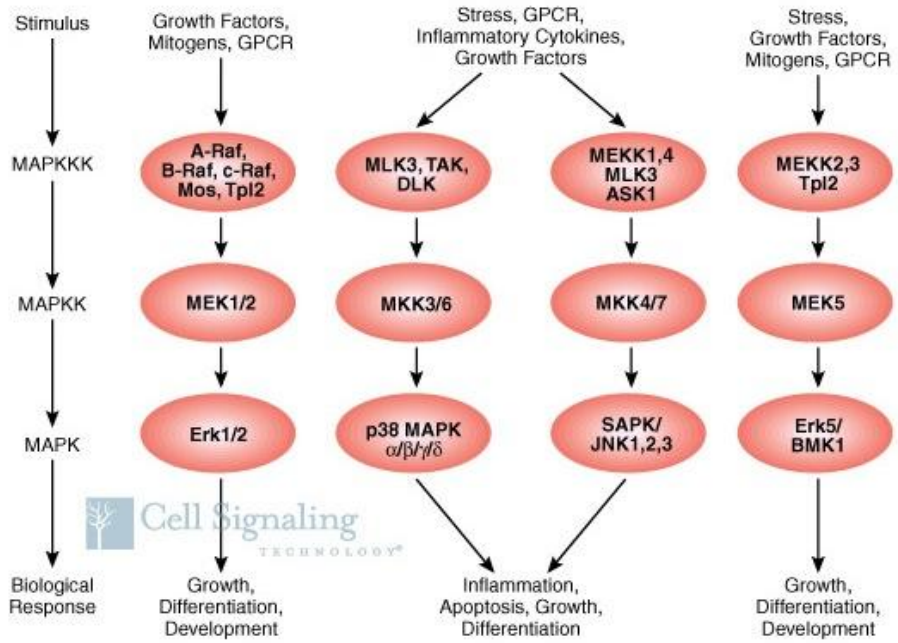


Figure 1.3: The MAPK activation cascade. Various stimuli act via GPCR and other receptors to activate the MAPK cascade which culminate in a biological response. There are four families of MAPK: ERK ½; p38 MAPK; JNK and ERK5. Taken from www.cellsignal.com/reference/pathway/MAPK_Cascades.html

one another by phosphorylation (Naor, Bernard et al. 2000). GPCRs signal through the various G proteins and thus the pathway leading to MAPK activation may be dependent on the G protein subtype (Hawes et al 1995).

1.6 The GnRH/ GnRH receptor system

1.6.1 The GnRH ligand

Twenty three different structural isoforms of GnRH have been elucidated in vertebrates (Millar, Lu et al. 2004). These structural isoforms can be grouped into three main types: GnRH I, GnRH II and GnRH III. They are expressed in a wide range of tissues in which they have diverse functions such as neuroendocrine; paracrine/autocrine roles and neurotransmitter roles in the central nervous system (Chen, Jeung et al. 1999)(Millar, Lu et al. 2004).

The type I gonadotropin- releasing hormone (GnRH I) (pGlu-His-Trp-Ser-Tyr-Gly-leu-Arg-pro-Gly.NH_2) is the central regulator of the reproductive hormonal cascade in mammals (Millar 2005). It is synthesized in the hypothalamus and released into portal circulation. It binds to high affinity GnRH receptors on the gonadotropes of the pituitary. This initiates the synthesis and release of the gonadotrophins, luteinising hormone (LH) and follicle-stimulating hormone (FSH) (Millar 2005) (Harrison, Wierman et al. 2004). The type II GnRH (GnRH II) was first located in the midbrain and is structurally conserved in all vertebrate species (Miyamoto, Hasegawa et al. 1984) (Ramakrishnappa, Rajamahendran et al. 2005), whereas the type I is heterogeneous and different species have different sequences (Sealfon, Weinstein et al. 1997) (Millar, Lu et al. 2004; Millar 2005). GnRH II (pGlu-His-Trp-Ser-His-Gly-Trp-Tyr-Pro-Gly.NH2) shares 70% homology with GnRH I and is ubiquitously expressed in human tissue, compared to GnRH I (White, Eisen et al. 1998; Millar 2003). GnRH II has been implicated in the regulation of M currents in the sympathetic ganglion and in co-ordinating energetic state of females with mating behaviour of marmoset

monkeys and musk shrews (Barnett, Bunnell et al. 2006) (Temple, Millar et al. 2003) (Schneider and Rissman 2008) during food restrictions. A third type of GnRH (pGlu-His-Trp-Ser-Tyr-Gly-**Trp-Leu**-Pro-Gly-Gly-NH2) has only been isolated in a few vertebrates such as teleost fish including salmon (Okubo, Ishii et al. 2003) (Powell, Zohar et al. 1994). It shares 80% structural similarities with GnRH I. Although GnRH I and GnRH III share high sequence similarity, they have different functions. GnRH III regulates and co-ordinates sensory inputs, and the reproductive requirements of the organism (White, Kasten et al. 1995) (Somoza, Miranda et al. 2002).

In conclusion, the three variants of GnRH may have different structures and function but they are involved, one way or the other in mammalian reproduction.

1.6.2 The GnRH receptor

Just as there are three types of GnRH ligands, there are three types of the GnRH receptor isolated in vertebrates; the type I, type II and type III GnRH receptor (Troskie, Illing et al. 1998; Wang, Bogerd et al. 2001). The type I GnRH receptor (GnRHRI) differs among GPCR, mainly in lacking a cytoplasmic tail as shown in figure 1.4 (Millar, Lu et al. 2004). The tail has been shown to be important in the desensitisation and internalisation of the receptor (Millar, Lowe et al. 2001; McArdle, Frankiln et al. 2002; Neill 2002). The type II GnRHR is expressed more widely in the brain, and in diverse tissues (Millar *et al*, 2001) Unlike the type I GnRHR, it contains a C- terminal tail and has a higher affinity for GnRH II (Cheng and Leung 2005) (Millar, Lowe et al. 2001). In humans, the gene for the type II receptor encodes for a premature stop codon and thus the gene is thought to be a pseudogene (Stewart, Katz et al. 2009) (Neill, Musgrove et al. 2004). Since humans express both GnRH I and GnRH II, it is thought that both GnRH types signals through the type I GnRH receptor only (Marelli,

Moretti et al. 2009). However there is evidence that a functional GnRH type II receptor or a splice variant, may exist in humans (Grundker, Gunthert et al. 2002) (Grundker, Schlotawa et al. 2004). A third type of GnRHR has been isolated in non- mammalians (Seong, Wang et al. 2003) (Oh, Wan et al. 2003). The type III GnRHR is expressed in the pituitary and regions of the brain (Wang et al 2001). It was highly sensitive to GnRH III and GnRH II (Wang, Bogerd et al. 2001) (Seong, Wang et al. 2003).

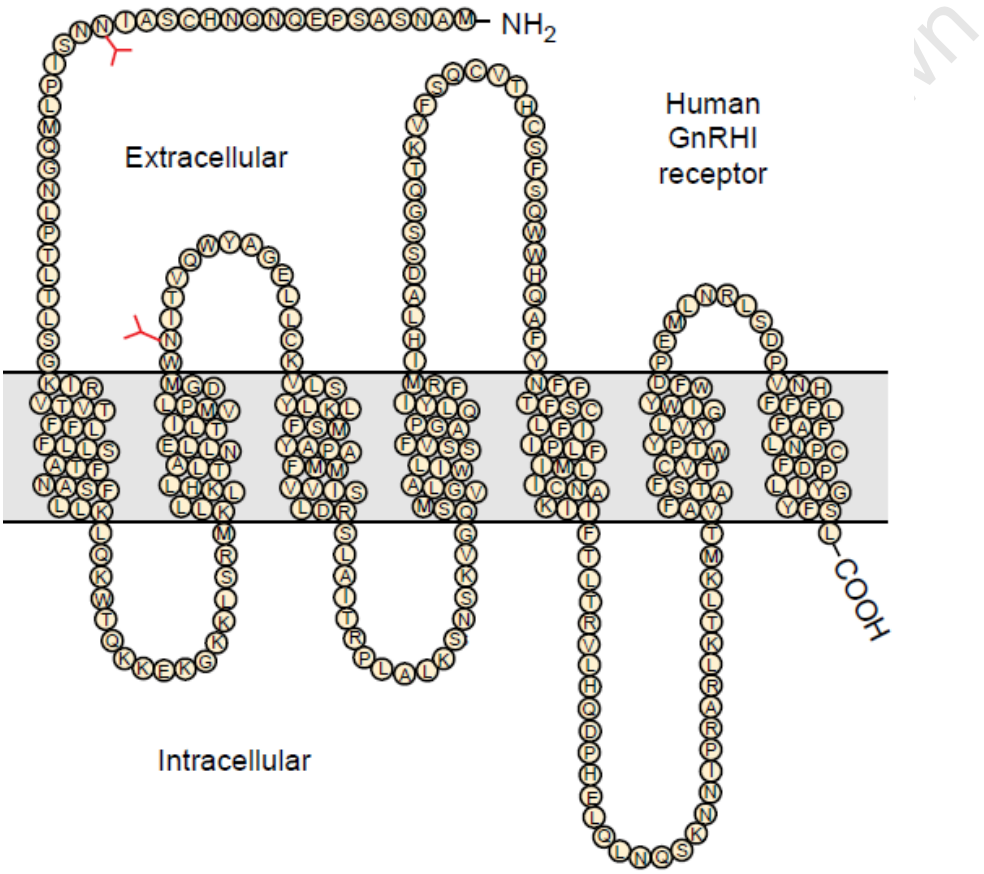


Figure 1.4: Diagram of a 2D structure of the human GnRH receptor. The receptor differs from other GPCR in lacking a C-terminal tail. Glycosylation sites are shown as red Y-shaped symbol. Taken from Neill et al 2004

1.7 The physiological role of GnRH

GnRH is the central regulator of the reproductive hormone cascade (Counis, Lavarreire et al. 2005; Millar 2005). GnRH is synthesised and secreted by GnRH neurons in the hypothalamus. It is released into the hypophyseal portal system in a pulsatile manner and travels to the pituitary gonadotropes. Upon binding to cognate receptors, it causes the synthesis and release of LH and FSH (Mortimer, McNeilly et al. 1974). These are responsible for gonadal steroidogenesis (Hawes and Conn 1993) (Stanislaus, Pinter et al. 1998) (Kraus, Naor et al. 2006).

1.7.1 GnRH Signalling in Pituitary Gonadotropes

The signalling that occurs following GnRH binding to receptors in the pituitary has been studied in gonadotropin derived cell lines. The multiple effects of GnRHR in pituitary cells is thought to be mediated by the activation of Gαq/11 only (Grosse, Schmid et al. 2000) (Cornea, Janovick et al. 1998) (Stanislaus, Janovick et al. 1998) (Hawes and Conn 1993). However, activation of Gαs and Gαq in cell lines derived from pituitary gonadotropes has been suggested (Liu, Usui et al. 2002). Activation of Gq/11 subsequently activates Phospholipase C (PLC) β which catalyses the formation of inositol trisphosphate (IP_3) and early diaglycerol (DAG) thus leading to the accumulation of intracellular calcium and activation of conventional PKC isoforms (McArdle and Conn, 1986). After a lag phase, Gαq/11 will activate Phospholipase A_2 (PLA_2) and phospholipase D (PLD) which generate late DAG and arachidonic acid (AA). The late DAG and AA activate novel PKC isoforms (Dobkin-Bekman et al 2006).

PKC activation leads to the activation of ERK (Mulvaney, Zhang et al. 1999), JNK (Naor, Bernard et al. 2000) and p38 MAPK. The above mentioned pathways culminate in gonadotropin (LH and FSH) synthesis (mediated by MAPK) and release (mediated by calcium) (Stanislaus, Pinter et al. 1998) (Harris, Bonfil et al. 2002; Harris, Chuderland et al. 2003).

Due to the physiological actions of GnRH, it may be used to regulate the activity of systems that may need LH and FSH secretion for functioning, such as hormone dependent cancers (Kraus, Naor et al. 2006). GnRH and its analogs are used in the treatment of hormone dependent cancers and in assisted reproduction (Casper 1991) (Ramakrishnappa, Rajamahendran et al. 2005) (Kraus, Naor et al. 2006). In the treatment of hormone dependent cancers, GnRH and its analogs block the synthesis of LH and FSH, thus decreasing the production of sex steroids by the gonads, testosterone in males and estrogen in females (Huirne and Lambalk 2001)(Engel, Winzen et al. 2005) (Harrison, Wierman et al. 2004).

1.8 Expression of GnRH and GnRH Receptors in Peripheral Tissues

In addition to the GnRH and GnRHR expressed in the brain, there has been increasing evidence that GnRH and GnRH receptors are expressed in peripheral tissues such as liver, heart, skeletal muscle, placenta, kidney (Kakar and Jennes 1995) (Chen, Jeung et al. 1999) and in reproductive tissues such as the endometrium, breast and prostate (Harrison, Wierman et al. 2004; Maudsley, Davidson et al. 2004). In addition to this, GnRH and GnRH receptors have also been demonstrated to be expressed, using RT- PCR and southern blot approaches,

in reproductive tissue tumours and their cell lines (Chatzaki, Bax et al. 1996) (Limonta, Moretti et al. 1999) (Segal-Abramson, Kitroser et al. 1992) (Grundker, Schulz et al. 2000) (Ramakrishnappa, Rajamahendran et al. 2005) and in human melanoma cells (Moretti, Marelli et al. 2003).

The GnRH system has been hypothesized to have an autocrine/ paracrine role in these cells, eliciting a variety of responses depending on the tissue involved. In endometrial stromal cultures from first trimester decidual tissues (Harrison, Wierman et al. 2004) and in ovarian cancer cell lines (Cheung, Leung et al. 2006) GnRH I increased the mRNA levels of MMP 2 and 9. These results suggest that GnRH plays a role in promoting cellular invasiveness. Studies performed in rat ovaries have indicated that GnRH is involved in steroidogenesis and in the transcription of several genes involved in follicular maturation and ovulation (Metallinou, Asimakopoulos et al. 2007).

Analysis of the GnRHR sequence in extra-pituitary tissues, reveal that it is identical to the GnRHR sequence found in the pituitary. Radioligand studies performed in endometrial cancer cell lines reveal that peripheral receptors contain high affinity binding sites (Emons, Schroder et al. 1993) similar to receptors found in pituitary gonadotropes.

Studies performed in ovarian and endometrium carcinomas have demonstrated that tumors express higher levels of GnRHR than normal tissues (Wilkinson, Kucukmetin et al. 2008) (Ramakrishnappa, Rajamahendran et al. 2005). However, levels of the GnRHR in the prostate (Harrison, Wierman et al. 2004) and in OVCAR-3, an ovarian cell line (Kang, Cheng et al. 2000) were 10- fold less compared to those of the pituitary αT3-1 cell line (Harrison, Wierman et al. 2004; Ramakrishnappa, Rajamahendran et al. 2005). Indicating that although the levels of GnRHR in tumors are high, they are considerably small compared to the levels expressed by gonadotropes.

In conclusion, studies indicate that the GnRH receptor expressed in peripheral tissues is identical to the receptor expressed in pituitary gonadotropes. Therefore, the differences observed in function could be due to changes in cellular location.

1.9 Antiproliferative effects of GnRH mediated through the type I GnRHR

Studies on tumor cell lines have indicated that GnRH analogs also have direct antiproliferative effects on androgen dependent and androgen independent tumor cell lines (Harrison, Wierman et al. 2004) (Limonta, Moretti et al. 1999) (Kraus, Naor et al. 2006). Transfection of MCF-7, a breast cancer cell line with mammalian GnRHR inhibited the growth of these cells (Everest, Hislop et al. 2001; Finch, Green et al. 2004). Cell proliferation was measured using thymidine incorporation. It was observed that treatment with GnRH agonist resulted in a decrease in proliferation. The potency of the inhibition was dependent on the number of receptors on the surface of the cell. They also observed that the type II GnRHR was less efficient at inhibition due to higher internalisation rates (Finch, Green et al. 2004). Continous treatment of the human benign prostate hyperplasia (BPH-1), expressing endogenous GnRHR, with GnRH I for 5 days produced a dose-dependent antiproliferative effect as evidenced by cell number (Maudsley, Davidson et al. 2004). The antiproliferative effects of GnRH in androgen- dependent LNCaP and androgen-independent DU 145 prostate cancer cell lines were found to be mediated by the type I receptor (Limonta, Moretti et al. 1999) (Kraus, Naor et al. 2006).

In HEK293 cells transfected with type I GnRHR and in LβT2, a gonadotrope- derived cell line; continous treatment with GnRH reduced the proliferation of cells as evidenced by

thymidine incorporation (Miles, Hanyaloglu et al. 2004) and cell number (Maudsley, Davidson et al. 2004). Pre-incubation of the cells with an antagonist blocked this GnRH mediated growth inhibition; suggesting that it is mediated via the GnRHR (Miles, Hanyaloglu et al. 2004). In ovarian cancer cell lines, both GnRH agonist (Kim, Chio et al. 2006) (Grundker, Volker et al. 2001) and antagonist (Tang, Yano et al. 2002) have been shown to inhibit cell proliferation. However, the effects of antagonist are stronger than those of agonists (Yano, Pinski et al. 1994) indicating that the agonist/antagonist dichotomy may not apply in this system. The antiproliferative effects observed in the various cell lines mentioned above indicate that the antiproliferative effects may not depend on the cell context (Miles, Hanyaloglu et al. 2004). Interestingly, GnRH II has enhanced antiproliferative effects in cancers of the reproductive system, compared to those mediated by GnRH I and its superagonistic analogs (Harrison, Wierman et al. 2004) (Emons, Grundker et al. 2003). These effects were thought to be mediated through the type II GnRH receptor (Grundker, Schlotawa et al. 2004). RT-PCR and southern blot analysis have suggested that the type II GnRHR is expressed in these cells (Grundker, Gunthert et al. 2002). Also, silencing of the type I GnRHR did not abrogate the antiproliferative effects (Marelli, Moretti et al. 2009). However in prostate cancer cell lines, silencing of the type I GnRHR completely counter-acted the effects of GnRH II (Marelli, Moretti et al. 2009). In conclusion, there is uncertainty on the type of GnRH receptor that mediates the antiproliferative effects of GnRH II on cancer cells.

1.10 The G-protein mediating the antiproliferative effects of GnRHR

In the pituitary, the GnRH receptor has been shown to couple to the Gq/PLC pathway (Grosse et al 2000) as mentioned above. The signalling of the GnRHR culminates in the activation of ERK in a PKC dependent manner in various cell lines (Kim et al 2006) (Davidson et al. 2004) (White et al 2008) (Park et al 2009) (Wu et al 2009) thus indicating receptor coupling to Gq. A study performed by Grosse et al in COS-7 cells transfected with the GnRHR and a Gsi_5 chimera indicated that GnRHR could not couple to Gi but elicited IP (Arora, Krsmanovic et al. 1998) and calcium production, thus indicating Gq signalling (Silver and Sower 2006). However multiple G proteins have also been shown couple to couple to the GnRHR in pituitary cells (Hawes, Barnes et al. 1993) (Liu, Usui et al. 2002) and in other cellular context (Knollman and Conn 2008) (Krsmanovic, Mores et al. 2003) (Ulloa-Aguirre, Stanislaus et al. 1998). The discrepancies in results could be due to the different experimental conditions and assays used to detect G protein binding.

The antiproliferative effects of the GnRHR have been shown to be mediated through Gq (Kim et al 2006) (White et al 2008). In contrast, increasing data suggest that the antiproliferative effects of GnRH in human reproductive cancers and in peripheral cells are mediated by the Gi protein (Imai, Horibe et al. 1997) (Limonta et al 1999) (Grundker et al 2001) (Maudsley et al 2004) (Park et al 2009). The methods used to show Gi coupling are, however indirect methods and more direct evidence is needed. The researches employed methods such as cross linking studies using disuccinimidyl suberate (grundker et al 2001); a decrease in forskolin induced cAMP (Limonta et al 2003; Maudsley et al 2004) and treatment with pertussis toxin (Limonta et al 1999). These studies indicated that the GnRH elicited ERK activation was mediated via a Gi/o and Gβγ dependent (Grundker et al 2001) (Kimura

et al 1999) (Kraus, Benard et al. 2003) activation of MAPK through the epidermal growth receptor (EGFR) pathway. Therefore these data indicate that there is contradictory data concerning the G protein that mediates the antiproliferative effects of the GnRH receptor.

1.11 Hypothesis and Aim

In order to clarify the question of the G protein that mediates the antiproliferative effects of the GnRHR receptor, we used a G protein chimera. This would be a more direct approach in determining the G protein that mediates the antiproliferative effects of G proteins.

G- protein chimeras were initially designed to be part of GPCR- ligand high-throughput screening assays (Hsu and Luo 2007). The purpose was to design universal chimera that would couple maximum amount of GPCRs to a common end point, as many orphan Gi coupling- GPCR ligands are difficult to isolate (Kostenis et al 2005). The initial chimeras shared a common design in that 3 to 8 C-terminal residues of the reporter Gα protein are substituted with the residues of an alternate Gα protein (Conklin, Farfel et al. 1993). The extreme N-and C-termini are important in GPCR- G protein specificity (Blahos, Mary et al. 1998) (Kostenis, Waelbroeck et al. 2005). GPCR- G protein coupling specificity is changed but signalling occurs via the reporter Gα pathway. A number of chimeras have been designed with the Gq backbone such as the G_{qi5} (White et al 2008). The last 5 C-terminal Gαq residues of were substituted for Gαi2 residues. This chimera and others like it would facilitate Gi/o coupled- GPCR signalling through a PLC output system. A similar chimera with a Gs backbone was called Gsi5 (Grosse et al 2000). The C-terminal 5 amino acids of Gαi2 were inserted into Gαs. The resulting chimera however, signalled through an adenylate cyclase output system.

The chimera utilized here is different from the Gqi5 in that it binds Gq-coupled GPCRs and signals via the Gi pathway. This chimera would better elucidate the role of Gi in growth inhibition. If Gi mediates antiproliferation then introducing the chimera into cells should increase the growth inhibition caused by GnRH.

The chimera we would be using has the first 5 N-terminal and last 35 C-terminal amino acid sequences of Gαi replaced with those of Gαq (Slessareva and Graber 2003). It has been previously shown that the proximal sequences of the N-terminus and C- terminus are important for receptor- G protein binding (Blahos, Mary et al. 1998) (Slessareva, Ma et al. 2003) (Kostenis, Waelbroeck et al. 2005). The study by Slessareva and Graber (2003) showed coupling to Gq-coupled GPCRs, the M1 muscarinic receptor using an *in vitro* method. They used the affinity shift assay which uses reconstituted membranes expressing the receptor of interest with exogenously supplied G proteins.

1.12 Objectives

- The establishment of a model system to examine whether Gi mediates the antiproliferative effects of GnRHR. This will be done by the generation of a HEK293 stable cell line co-expressing the GnRHR and Gαq/i chimera and as control a HEK293 stable cell line expressing the GnRHR only.
- To characterise the two types of cell lines with regards to receptor expression and signalling.
- To compare the signalling and antiproliferative properties of GnRHR in cells co-expressing the GnRHR and the Gαq/i chimera relative to control cells.

2. Materials and Methods

2.1 Chemicals Reagents and DNA constructs

Dulbecco's minimum essential medium (DMEM), Trypsin and Hygromycin B were purchased from Gibco (Invitrogen). Fetal Calf Serum (FCS), Penicillin-Streptomycin and Med- 199 were purchased from Highveld Biological (WhiteScience). G418 was purchased from Sigma. Fugene HD and protease inhibitors (EDTA- free) were purchased from Roche.

The Bradford Assay kit was purchased from Bio-Rad. The Spectra multicolour broad range protein molecular weight marker was obtained from Fermentas. The PVDF Hybond P membrane, supersignal west pico chemiluminescent substrate and the hyperfilm MP were all purchased from Amersham Biosciences. GnRH I was obtained from Bachem. [His^5-D-Tyr^6] GnRH was sysnthesised using reverse solid-phase synthesis and was purified using reverse phase HPLC. The radiochemicals (Myo-[2-^{3}H(N)] inositol and Iodine-125) were purchased from Perkin Elmer. Scintillation fluid was from Zinsser Analytical. The QAE sephadex A25, sephadex G25 and 1x8-200 DOWEX-1 were obtained from Sigma. Forskolin was also purchased from Sigma. The Dual luciferase reporter assay kit was from Promega. The microfluoro white plate was from Thermo. All other typical reagents were purchased from Sigma.

The rabbit polyclonal anti- Gαq/11 (C-19), $G\alpha i_1$ (I-20) and β- actin primary antibodies were all purchased from Santa Cruz. The polyclonal goat anti- rabbit conjugated HRP secondary antibody was also purchased from Santa Cruz. The rabbit polyclonal phospho and total p44/ 42 MAPK antibodies were purchased from Cell Signalling Technology.

The cDNAs encoding Gαq, Gαi and Gαq/i were cloned into pcDNA3.1 Neo (+) [Invitrogen]. The cDNA encoding the rat GnRH receptor was cloned into the pMEP4 vector [Invitrogen]. The Gαq/i chimera cDNA, consists of Gq in which the first six N terminal residues and last

35 C-terminal residues of Gi were exchanged for those of Gαq and is a kind gift from Dr Stefen Graber. The cAMP response element firefly (pCRE-Luc) vector was from Invitrogene. The renilla luciferase (pCMV renilla) vector was a gift from Dr Sharon Prince and was purchased from Clontech.

2.2 Cell culture

Human embryonic kidney (HEK293) cells were maintained in DMEM containing 10% FCS and 1% Penicillin/streptomycin (diluted from a stock solution of 500 IU/ml penicillin and 500 µg/ml streptomycin). The cells were grown at 37°C in a 5% CO_2 incubator. SCL60 cells are HEK293 cells stably expressing the GnRH receptor (pcDNA3.1) (Miles et al 2004; White et al 2008) and HEK293 stably expressing Gαq/i (clone 6A) were maintained in DMEM supplemented with 400 µg/ml G418. The HR6 cell line stably expressing the GnRH receptor (pMEP4) was maintained in DMEM supplemented with 200 µg/ml Hygromycin B. The HEK cell clones stably expressing the GnRHR and Gαq/i (clones 6AR2, 6AR3, 6AR4, 6AR5 and 6AR11) were maintained in DMEM supplemented with 400µg/ml G418 and 200 µg/ml Hygromycin B.

2.3 DNA Transfections

2.3.1 Fugene- HD reagent transient Transfections

Fugene- HD was used to transfect cells with DNA constructs. Cells were seeded at a density of $1x10^5$ cells per well in 12 well plates. The following day, the cells in each well were transfected by mixing 6ul of fugene with 2ug of DNA in 100ul serum free DMEM. The solution was left to equilibrate for 15 minutes before adding to cells in a dropwise fashion. The cells were then grown for 48 hours, after which they were assayed.

2.3.2 Stable Transfections

HEK cells were plated in a 6 well plate at a density of $5x10^5$ cells per well. The following day, cells were transfected with 6µl of fugene and 2µg of DNA per well. Forty eight hours later, antibiotics (200 µg/ml Hygromycin B and/or 400 µg/ml G 418) were added to the growth medium for selection. The growth medium was changed every 3 days to select for cells expressing the gene of interest. About 3 weeks later, discrete colonies were picked; propagated and then characterized using the indicated assays.

2.4 Western Blotting

2.4.1 Preparation of Cellular Extracts

Confluent cell monolayers ($1x10^6$ cells) in a 6 well plates were washed twice with ice cold 1x phosphate buffered saline (PBS) on ice. They were lysed in 70 µl RIPA buffer containing 1x complete protease inhibitor for 15 min. Lysates were clarified by centrifugation at 12 000 rpm for 15 min at 4°C. The supernatant was stored at -80°C until further use. The protein

concentrations were measured using the Bradford Assay Kit. Briefly, the 2mg/ml bovine serum albumin that was supplied was diluted with RIPA buffer to give final concentrations ranging from 0- 1 ug/ul. An aliquot of 10ul of each concentration as well as the protein samples were added sequentially in a 96 well plate. Reagent A and reagent B were mixed at a ratio of 50:1 and 200ul of the mixture was added per well. The plate was then incubated at 37°C for 30 min. The colour change was measured at 595nm using an ELISA plate reader, the Anthos 2001 spectrophotometer (Anthos Labtec Instruments) and the accompanying WinRead software (V.2.3). The background was subtracted by using absorbance values obtained from wells with RIPA buffer only. The standard curve was constructed using the absorbance values normalized for background. Cellular extracts containing 15µg of protein were used for SDS-PAGE. Each extract was mixed with 5X loading dye to give a final concentration of 1X loading dye. This mixture was boiled at 95 °C for 5 min before loading.

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

The Sodium dodecyl sulfate- polyacrylamide gel electrophoresis (SDS-PAGE) was composed of a 5% stacking gel and a 10% resolving gel. A 1.5 mm 10 well comb was used to load samples. Equal volumes of cellular extracts were loaded per well. 5µl of the protein molecular weight (MW) marker was loaded in the first lane. The proteins were resolved, according to size, through the gel at 100 volts.

2.4.3 Immunoblotting

Following SDS-PAGE the proteins were electroblotted onto PVDF-Hybond P membrane for the transfer of protein from the gel to the membrane. The membrane was activated with 100% methanol before equilibration with 1x transfer buffer (containing 20% methanol). The transfer was performed in the presence of 1x transfer buffer at 100 volts for at least an hour. A cooling system was used to keep the transfer cool. Following transfer, membranes were washed in transfer buffer before blocking with 5% (w/v) fat free milk in PBS containing 0.1% tween (PBS-T) for an hour before incubating with primary antibody (1:1000, diluted in 5% milk) overnight at 4°C with shaking. The antibody solution was removed, the blot drained before excess antibody was washed with 1x PBS-T. The membranes were first washed twice for 10 min followed by two short 5 min washes. The membrane was then incubated with a horseradish peroxidase (HRP) conjugated goat anti- rabbit secondary antibody at a dilution of 1:1000 in 5% milk for 1 hour. Following washing of excess antibody as described above, proteins were detected using a supersignal west pico chemiluminescent substrate kit which detects HRP on immunoblots. The chemiluminescence produced was captured on film. To quantify protein expression, the films were scanned using an Alpha-Inotech Chemi-Imager. The FluorChem 5500 programme was used to analyse the scanned X-ray films. The data was plotted using Microsoft excel.

2.4.4 Stripping of Nitrocellulose membrane

In order to probe the membrane with a different antibody, the membrane was washed with dH_2O for 5 min before stripping with 0.2 M NaOH for 5 minutes at RT. This was followed by

another wash with dH_2O for 5 minutes. The membrane was then blocked with 5% milk and subjected to probing with a different antibody following procedure described in section 2.4.3.

2.5 GnRH Radioligand Binding Assays

2.5.1 Radiolabelling

[His^5- D- Tyr^6] GnRH was radiolabelled with I^{125} using a variation of the Chloramine T method described previously by (Flanagan, 1998 #5). Five micrograms of peptide in 20ul 0.5M phosphate buffer (pH7.4) was reacted with 1mCi $Na^{125}I$ and10ul chloromine T (3mg/ml in phosphate buffer) for 20 sec. The reaction was terminated by the addition of 50ul sodium metabisulfate (1.2 mg/ml in phosphate buffer). The completed reaction mixture was loaded onto a sephadex G25/C25 size exclusion column. Phosphate buffer was used to elute the labelled GnRH agonist. The fractions were collected and a 1ml aliquote was counted for 1 minute using a gamma counter (Berthold LB2111). Fractions with the highest counts were further aliqouted and stored at -70° C.

2.5.2 Competition Binding Assay

Cells expressing the GnRH receptor were seeded (about $1x10^5$ cells per well in a 12 well plate) to reach 60- 80% confluency the following day. A minimum of 50 000 cpm of I^{125}-[His^5-D- Tyr^6] GnRH was mixed with increasing concentrations (10^{-9}-10^{-6} M) of unlabelled GnRH I in HEPES - DMEM. Five hundred microliters of the HEPES-DMEM/peptide solution was added to the cell monolayers in the appropriate wells. The cells were then incubated at 4ºC for 4 hours and were washed twice with cold 1x PBS to remove unbound

ligand. The cells were then solubilized with the addition of 1M NaOH at RT. An aliquot of 1ml was removed for counting. The bound radioligand was measured with a Berthold LB2111 gamma counter for 1 minute.

Receptor specific binding (SP) is the difference between Total Binding (TB) and non-specific binding (NSB). Total binding is binding in the absence of competitive ligand, while non-specific binding is binding in the presence of saturating conditions (10^{-6}M) of unlabelled ligand. SP = TB – NSB.

2.6 Inositol Phosphates (IP) Assay

Cells expressing the GnRH receptor were seeded at a density of $2.5x10^5$cells per well in 12 well plates. The following day, wells were washed with med199 (2% FCS) before labelling with 0.5ml med 199 containing 2 μCi/ ml [^{3}H] Inositol. After another 20 hours, the cells were incubated with Buffer I for 15 min to allow for LiCl to be taken up. Subsequently the cells were stimulated with increasing concentrations of GnRH agonist from zero to 1μM for 60 min at 37ºC. After that time, 1ml 10mM formic acid was added to each well for at least 30 minutes at 4^{o}C plate in order to extracted the inositol phosphates. Isolation of inositol phosphate from cell extract was performed on 1x8-200 DOWEX-1 ion exchange columns. Columns were first, washed with 3ml 3M ammonium formate with 0.1M formic acid followed by 10ml distilled water. Cell extracts were then loaded on to the charged columns,

column were then washed with 10ml dH_2O, followed by 5ml 5mM myo-inositol with 0.1M formic acid. Total inositol phosphates were eluted with 3ml 1M-ammonium formate with 0.1M formic acid into scintillation vials containing 16ml of scintillation fluid and the radioactivity was measured with a liquid scintillation analyzer (PackardTri-Carb 2100TR) for 1 min.

2.7 Methylthiazolyldiphenyl- tetrazolium bromide (MTT) assay for cell proliferation

Cell proliferation was measured using the Methylthiazolyldiphenyl- tetrazolium bromide (MTT) assay. Cells were plated at a density of $2x10^4$ cells per well in 12 well plates. Following 24 hours, duplicate wells were treated with 1µM GnRH I for up to 3 days. Following treatment, the cells were incubated with 0.5ml DMEM containing 5% MTT solution (5mg/ml in 1xPBS) for 2 hours. An equal volume of Dimethyl sulfoxide (DMSO) was added to extract the formazan dye. An aliquot of 200µl was used for measurement in a 96 well plate at 595 nm.

2.8 Luciferase Assays

Cells were seeded at a density of $10X10^5$ per well in a 12 well plate. The following day cells were transfected with appropriate plasmids as described in section 3.1. Forty eight hours later, the cells were stimulated with 12mM forskolin with or without 1µM GnRH I for 5 hours. Cell lysates were prepared by the addition of 1X Passive Lysis Buffer (PLB) and frozen at -20ºC until further use. The luciferase reporter activities were measured by mixing 50µl of Luciferase Assay Reagent (LAR) II with 10µl of the lysate in a 96 well microfluoro

white plate. The reading was taken for 10 seconds using a Lumat LB9501 luminometer (Berthold). The activity of the renilla luciferase was measured to control for transfection efficiency. 50µl of Stop & Glo Reagent was added to terminate the previous reaction and also serve as a substrate for renilla luciferase.

2.9 Data Analysis

Each experiment was performed in duplicate and repeated at least two times, unless otherwise stated. Data analysis was performed using GraphPad Prism5 (GraphPad, San Diego, CA). Emax and EC_{50} values were calculated using the nonlinear regression (four parameters) analysis parameter. Statistical analysis was performed using two-way ANOVA with bonferroni post tests, accepting $P < 0.05$ as statistically significant. Data in figures are represented as mean ± SEM unless otherwise stated.

3. Results

3.1 Testing for coupling between the GnRHR and Gaq/i

Prior to establishing model cell lines to test the role of Gαq/i on the antiproliferative effect of the GnRHR we first needed to test whether the GnRHR can couple to the Gαq/i. The Gαq/i chimera signals like Gαi and therefore it can lead to a reduction in cAMP levels. We decided to test whether the GnRHR can interact with Gαq/i by employing a luciferase based assay in which the luciferase gene is under the control of a promoter harbouring a cAMP response element and as such will be regulated by the levels of cAMP in the cell. Therefore, the level of the luciferase enzyme would be dependent on the level of cAMP and could be used as an index for activation Gαq/i by the GnRH receptor. Parental HEK293 cells were transfected with a plasmid encoding firefly luciferase as well a plasmid encoding renilla luciferase, in order to control for transfection efficiency. The firefly luciferase plasmid would be under the regulation of cAMP levels that can be affected by the activation of the GnRHR. The assumption is that if the GnRHR activates Gαq/i it will lead to a reduction in levels of cAMP and therefore lead to a reduction in the level and activity of the luciferase enzyme. The assay was performed in the presence of forskolin which increases cAMP levels and therefore makes the reduction of cAMP due to Gαq/i easier to detect.

Cells in 12 well plates were transfected using fugene-HD with DNA expression constructs encoding for luciferase, renilla, rGnRHR and either Gαq/i, or Gαi or an empty vector. Forty eight hours after transfection, cell monolayers were treated with 10^{-6}M GnRH I in the presence of 12micromolar forskolin for 5 hours before harvesting and testing for luciferase activity.

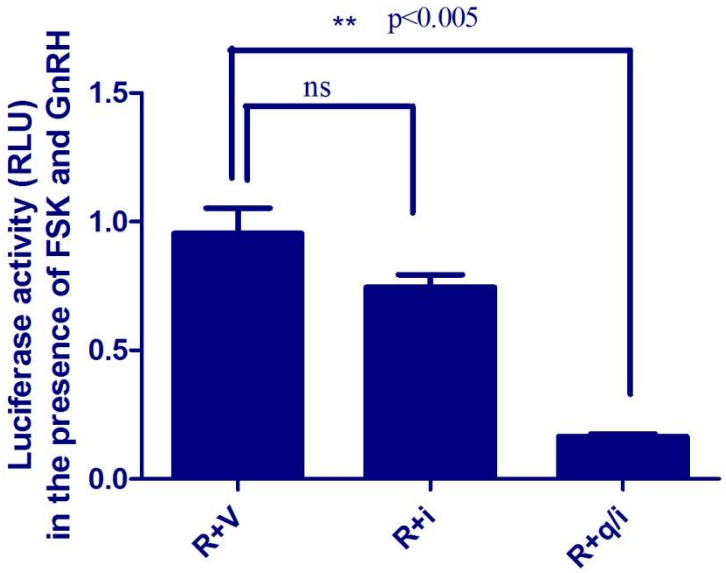


Figure 3.1: Determining the GnRHR interaction with Gαq/i employing a luciferase reporter assay. Cells were seeded in a 12 well plate before transfecting firefly luciferase; renilla luciferase; rGnRHR cDNA with empty vector (indicated by R+V) or Gαi (R+i) or Gαq/i (R+ q/i) cDNA. 48hrs following transfection cells were stimulated with forskolin (FSK) in the presence of 10^{-6}M GnRH I for 5 hours before harvesting. The figure shows the representation of 3 similar experiments performed in duplicate. ns, represents statistical insignificance.

The results in figure 3.1 demonstrate that cells transfected with the rGnRH receptor together with empty vector had high levels of luciferase activity due to the forskolin induced luciferase activity. GnRH stimulation of cells over-expressing Gαi and the GnRHR did not significantly inhibit the forskolin induced luciferase activity. These results demonstrate that the GnRHR does not activate Gαi or activates Gαi very weakly. However stimulation of cells co-transfected with the GnRHR and Gαq/i significantly decreased forskolin induced luciferase activity, indicating efficient coupling of the GnRH receptor to the Gαq/i chimera.

In conclusion our results indicate that Gαq/i is able to couple to the GnRH receptor which is a Gq- coupled GPCR.

3.2 Generation of a HEK293 stable cell line expressing Gαq/i

The finding that the GnRHR can couple to the Gαq/i chimera, which signals as Gαi and reduces cAMP levels, was a clear indication that there is merit in establishing a cell model system to test the role of Gαi in GnRH receptor mediated inhibition of cell proliferation. We decided to create model cell lines that co-express the GnRH receptor and the Gαq/i chimera and as control, cells that express the GnRH receptor only. We chose HEK293 cells as our parental cell line. The strategy was to initially create a stable cell line expressing Gαq/i chimera and subsequently to transfect the GnRHR into cells expressing the Gαq/i only and into parental HEK293 cells as control. Since, the Gαq/i chimera was cloned into a neomycin pcDNA3.1 expression vector; we first performed a neomycin toxicity test on HEK293 cells. HEK 293 cells were seeded in 12 well plates and were treated daily with G418 at concentrations ranging from 50µg/ml to 800µg/ml. This toxicity test indicated that 400µg/ml of G418 was required in order to kill all untransfected HEK293 cells after 1 week of treatment.

In order to generate cell lines stably expressing Gαq/i, we transfected HEK293 cells that were seeded a day earlier in a six well plate. Selection with 400ug/ml G418 was initiated 48 hrs following transfection. Media including G418 was changed every 3 days and after 3 weeks, colonies were noticeable. We initially picked and expanded 4 colonies for screening for Gαq/i expression using Western blotting with an antibody that recognises the C-terminus of Gαq. The cell clones were named: 6A, 7C, 7D and 7F. The expression of Gαq/i chimera from the clones' [lanes 6A, 7C, 7D, 7F] cell extracts were compared to extracts prepared from HEK293 cells transfected with empty vector (lane V) and from cells transiently transfected with Gαq/i [lane Gq/i(t)]. These extracts were used as negative and positive control, respectively.

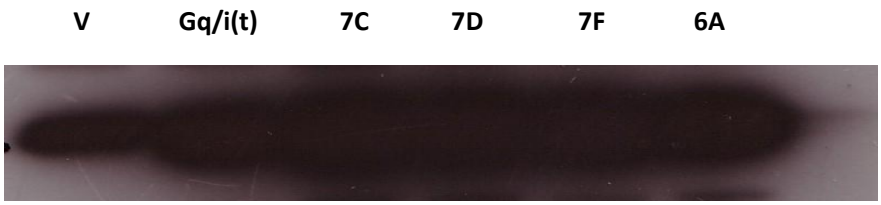


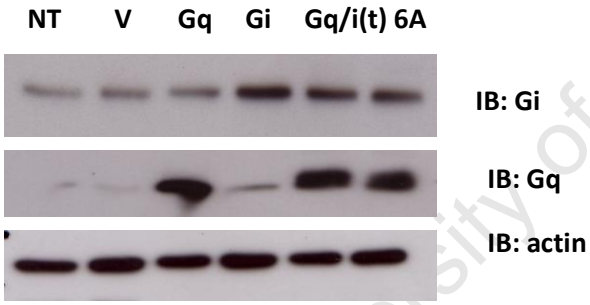
Figure 3.2: Detection of Gαq/i in HEK cells stably transfected with Gαq/i. Cell monolayers were lysed, and 15µg of cellular extract was resolved through SDS PAGE and proteins transferred to nitrocellulose membranes and immunoprobed with antibody against Gq. The level of Gαq/i expression in the cell clones (indicated by lane 6A, 7C, 7D and 7F) was compared with non- transfected (lane NT) cells and HEK cells transiently transfected with Gq/i (indicated as Gq/i(t)) that served as negative and positive controls, respectively. The blot is a representative of 2 independent experiments.

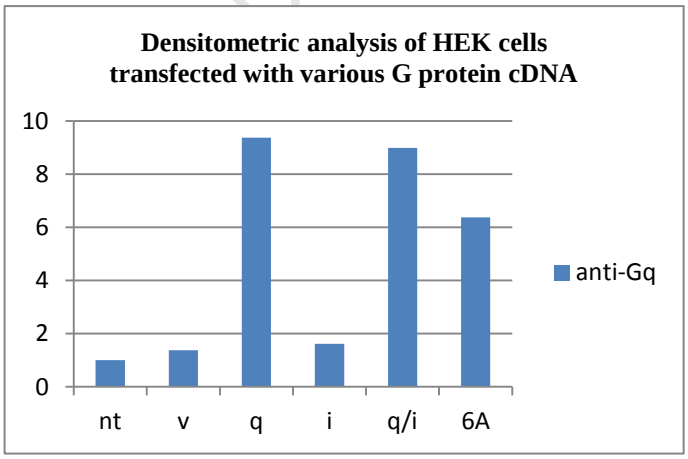
The results in figure 3.2 show that there is endogenous Gαq in HEK293 cells transfected with empty vector (lane V). However transient expression of Gαq/i increased the area and intensity of the band size in lane Gq/i(t) thus indicating the specificity of the Gq antibody to detect expression of Gαq/i. The area and intensity of the band size in lanes 7C, 7D, 7F and 6A also increased in comparison to that of basal level found in lane V. In order to see the differences more clearly it may be necessary to load less protein. Nevertheless,these results demonstrate that Gαq/i was successfully stably transfected into HEK293 cells to generate clones, 7C, 7D, 7F and 6A. However, the results need to be further confirmed by blotting with an antibody that recognises Gαi.

In order to confirm expression of Gαq/i in the stably transfected clones, we repeated the Western Blot probing with a $G\alpha i_1$ (internal) antibody and the anti- Gαq (C- terminal) used

above. Since the above clones (7C, 7D, 7F and 6A) expressed similar levels of Gαq/i, we analysed only clone 6A as a representative of the 4 isolated clones (Fig 3.2).

HEK293 cells plated in a 6 well plate were transiently transfected with an empty pcDNA3.1 vector and Gαq, Gαi, Gαq/i cDNA that served as negative and positive controls, respectively. The cellular extracts were resolved in 10% SDS-PAGE, transferred to nitrocellulose paper and probed with antibodies that recognise Gαq, Gαi and β-actin. The protein levels of Gαi and Gαq found in extracts made from the transient transfections were compared to the cellular extract of the stable cell line, 6A. The results shown in Figure 3.3 are the representative of two independent experiments that gave similar results.





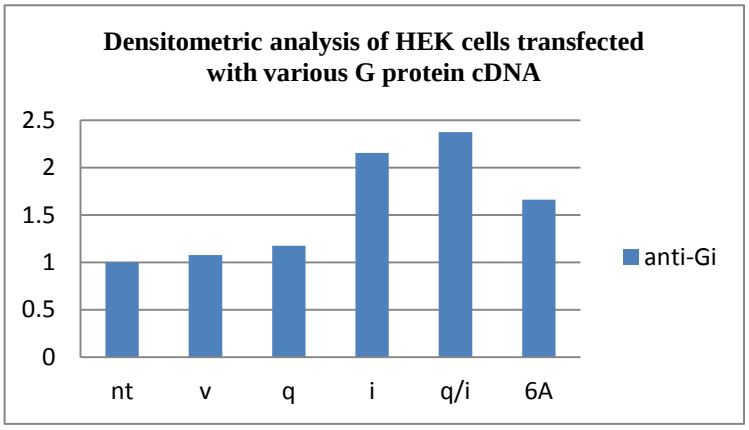


Figure 3.3: Immunoblot confirming expression of Gαq/i in HEK cells. HEK cells were transfected with various G protein and vector cDNA. Detection of Gαq/i was achieved using ani- Gαi (internal) and anti Gαq (C- terminal) antibodies. β-actin was used as a loading control. The levels of Gq/i in the cell line stably expressing Gαq/i (6A) were compared to HEK cells transfected with empty vector, Gαq, Gαi and Gαq/i [indicated as V, Gq, Gi and Gq/i(t), respectively]. Denstometric analysis: X-ray films were scanned and analysed using the Fluorchem programme to determine the relative level of Gαq and Gαi in HEK293 cells. The results are representative of 2 independent experiments

Immunoblotting for Gαi revealed that there was endogenous levels of Gi in the cells, as can be seen by the faint bands in the lanes containing non-transfected HEK293 cells (NT) and in cells transfected with empty vector and Gαq (lanes V and Gq, respectively). These levels were increased by 2 fold when exogenous Gαi was transfected (determined by densitometric analysis of films). The levels of Gαq/i were 2 fold that of endogenous Gi when Gαq/i was transiently transfected (lane Gq/i(t)) in HEK293 cells as well as in HEK293 cells stably expressing Gαq/i (6A lane). Similary, immunoblotting against Gαq, showed endogenous levels of Gαq in untransfected HEK293 cells and in cells transiently transfected with empty

vector and Gαi. The levels of Gq increased by 9 fold when exogenous Gαq were transiently transfected into HEK293 cells. The levels of Gαq/i were 9 fold to those of endogenous levels of Gαq when Gαq/i was transiently transfected into HEK293 cells. The 6A clone expressed Gαq/i levels that were 6 fold higher than endogenous levels of Gαq.

These results indicate that the Gαq and Gαi antibodies can be used to detect Gαq/i. The observation that both the Gαq and Gαi antibodies recognised the same band in the stable cell line 6A, confirms that the cell clone, 6A, stably expresses the Gαq/i chimera.

3.3 Generation of HEK293 stable cell lines expressing the Gαq/i and the GnRH receptor and as control HEK293 cells stably expressing the GnRH receptor only

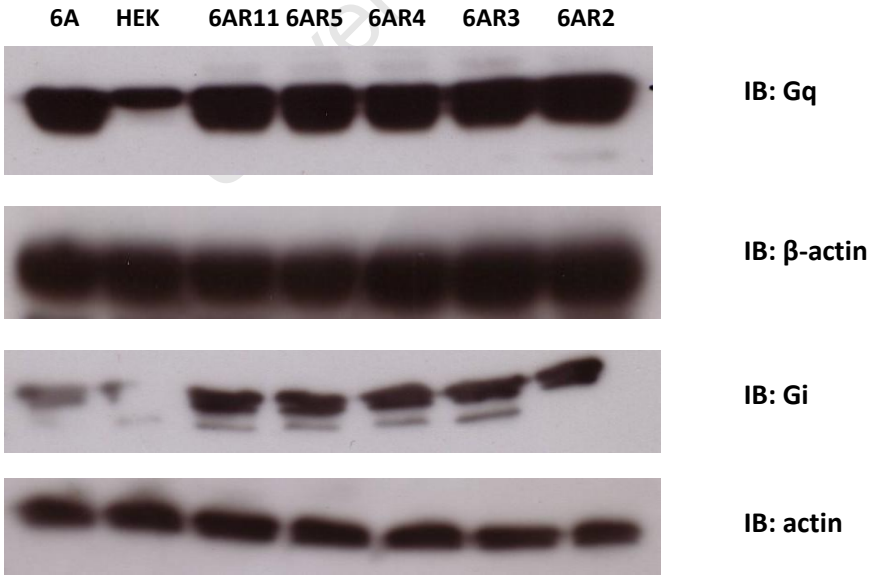
Since we have successfully created a HEK293 cell line stably expressing Gαq/i, 6A; our next step in creating our model cell lines was to transfect the GnRHR into 6A. Since these cells are resistant to G418, so we decided to transfect the GnRHR cDNA which is encoded by a hygromycin expression vector. For control cells we transfected this DNA expression construct into wild type HEK293 cells. We initially performed a hygromycin toxicity test on parental HEK293 cells. HEK293 cells were seeded in a 12 well plate and were treated daily with hygromycin at concentrations ranging from 50µg/ml to 800µg/ml. This toxicity test indicated that 200µg/ml of hygromycin was required in order to kill all untransfected HEK293 cells after 1 week of treatment.

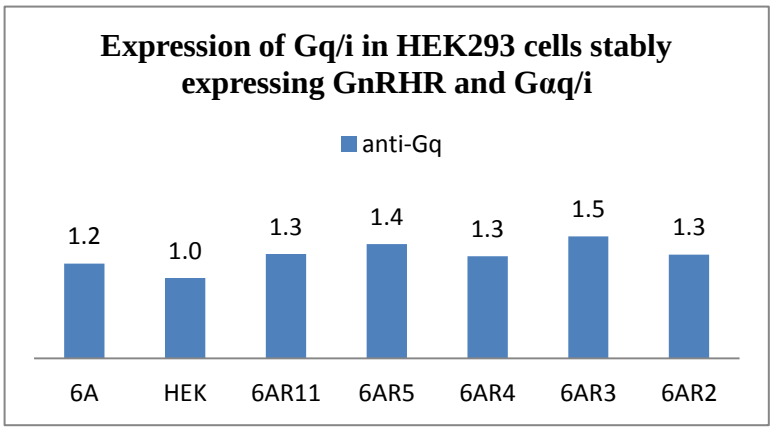
The GnRH receptor cDNA was transfected into 6A and into HEK 293 cells using fugene-HD. We obtained about 20 colonies from the 6A cell line. We initially picked five clones for screening and named them: 6AR2, 6AR3, 6AR4, 6AR5 and 6AR11. However during our

screening we continued with only 6AR2, 6AR3 and 6AR4. Transfection of the GnRHR into wild type HEK293 cells generated only one clone that we named HR6. The expression of the GnRH receptor in the isolated clones was screened employing radioligand binding.

3.3.1 Expression of Gαq/i in HEK293 cells stably co-expressing the GnRHR and Gαq/i

To confirm the expression of Gαq/i in the clones generated from transfecting the GnRHR into the 6A cell line (6AR2, 6AR3, 6AR4, 6AR5 and 6AR11), we performed Western blotting. Cells were grown in 6 well plates, lysed and resolved through 10% SDS-PAGE. The expression of Gαq/i was determined using Gi_1 (internal) and Gq (C-terminal) antibodies. Parental HEK 293 cells and the cell line stably expressing Gαq/i only (6A) were used as a negative and positive control, respectively.





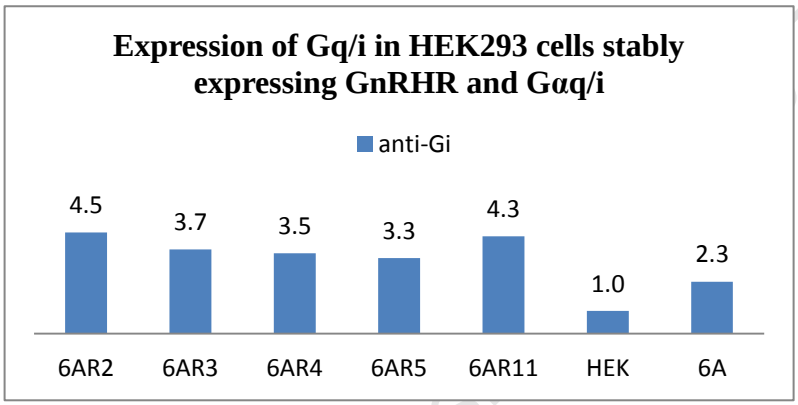


Figure 3.4: Expression of Gαq/i in HEK cells stably transfected with the GnRH receptor and Gαq/i cDNA. Cells were seeded onto 6 well plates and lysed with RIPA buffer. 15µg of protein extracts were resolved through a 10% SDS-PAGE and electroblotted to a nitrocellulose membrane. Expression of Gq/i was detected using an immunoblot (IB) against Gi and Gq. β-actin was used as a loading control. The expression of Gq/i in cell clones 6AR2, AR3, AR4, AR5, AR11 (lanes indicated by clone name) were compared to those expressed by untransfected HEK cells (lane HEK) and cells stably transfected with Gq/i (lane 6A). The blot is a representative of 2 independent experiments. Densitometric analysis: x-ray films were scanned and analysed using the fluorchem programme. The number in each graph represents the level of Gaq and Gai in each cel clone.

The results in figure 3.4 demonstrate that when probed with antibody against Gαi the 6A parental clone and the clones stably transfected with the GnRHR cDNA (6AR2, 6AR3,

6AR4, 6AR5, 6AR11) displayed an increase in band intensity (densitometric analysis) compared to the levels found in HEK cells, thus indicating expression of Gαq/i in cells expressing GnRHR and Gαq/i. The loading was similar across all lanes, as judged from the similar intensity of β-actin and therefore, indicating that the increase in the intensity of the Gαq/i is specific to the transfected cells. Probing for Gq similarly demonstrated an increase in Gαq/i levels in the cell clones (6AR2, 6AR3, 6AR4, 6AR5 and 6AR11) co-expressing GnRHR and Gq/i, compared to endogenous levels found in HEK cells.

In summary, these results demonstrate that Gαq/i is expressed in the cells co-expressing the GnRHR and Gαq/i.

3.3.2 Expression of GnRH Receptor in HEK293 co-expressing the GnRHR and Gαq/i and in HEK cells stably expressing the GnRH receptor only

The next step in our screening programme was to determine the expression level of the GnRHR in HEK293 cells expressing the GnRHR only (HR6) and those co-expressing GnRHR and Gαq/i (6AR2, 6AR3, and 6AR4). This was carried out by conducting whole cell binding assays which measures relative receptor binding sites on the cell surface. Briefly, cells were incubated with labelled I^{125}[His^5-D-Tyr^6] GnRH in the absence or presence of 10^{-6}M unlabelled competitive peptide (GnRH I). The receptor levels of the above mentioned cell clones were compared to those of SCL60; an established cell line that has been shown to express very high levels of the GnRH receptor (Miles et al 2003) (White et al 2008) (Morgan et al 2008) The results are shown in figure 3.5.

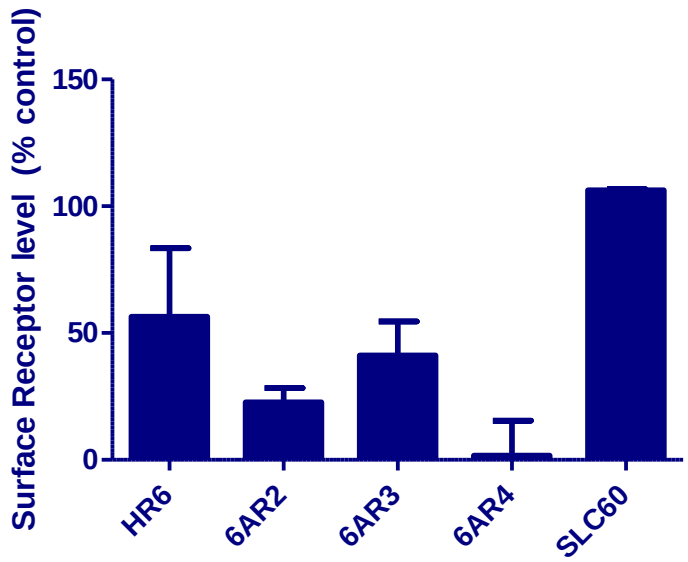


Figure 3.5: GnRHR expression levels in HEK cells stably expressing the GnRHR only, clone HR6 and in stable cell lines expressing Gαq/i together with the GnRHR, clones 6AR2, 6AR3, and 6AR4. Cells were incubated with labelled ligand in the absence or presence of increasing concentrations (10^{-9}-10^{-6} M) of competitive unlabelled ligand. The level of receptors was calculated using specific binding (SP). The equation used was SP =Total binding (TB) – non-specific binding (NSB). The SCL60 cell line was used as control, its level of specific binding was the highest and was defined as 100% and the specific binding of all other cell clones was given in % relative to the binding of SCL60 cell. The results show the averages (mean ± SEM) of 3 independent experiments.

The HR6 cell line, expressing the GnRH receptor only, had approximately 50 % of the receptor level expressed in SCL60 cells. The expression of the receptor in 6AR3 cell line displayed comparable levels to that of the HR6 cell clone. Therefore we will be able to make comparisons between the HR6 and 6AR3 cell clones and thus determine the effects of Gαq/i. The expression of the GnRH receptor in the 6AR2 cell line was 50% of the levels found in the HR6 and 6AR3 cell lines. While, the 6AR4 cell line expressed only 20% of the GnRH receptor levels of HR6 and 6AR3 cell lines.

In summary, our results demonstrate that we have succeeded in establishing a HEK293 cell line that stably express the GnRHR only (clone HR6) and those that co-express the GnRHR and Gαq/i (clones 6AR2, 6AR3, and 6AR4).

3.4 Comparison of GnRH-R induced Inositol Phosphate production in stable cell lines co-expressing the GnRH-R and Gαq/i and in cells expressing the GnRH receptor only

The cells lines stably co-expressing GnRH receptor and Gαq/i (clones 6AR2, 6AR3 and 6AR4) were assessed for their ability to produce inositol phosphate in response to GnRH stimulation. The HR6 and SCL60 cell lines that express the GnRH receptor only, were used as controls. Briefly, cells in 12 well plates were pre-incubated with inositol-free medium containing tritiated myo- inositol for 24 hours before stimulation with increasing concentrations of GnRH I in buffer containing LiCl. Total inositol phosphates were extracted, purified and counted.

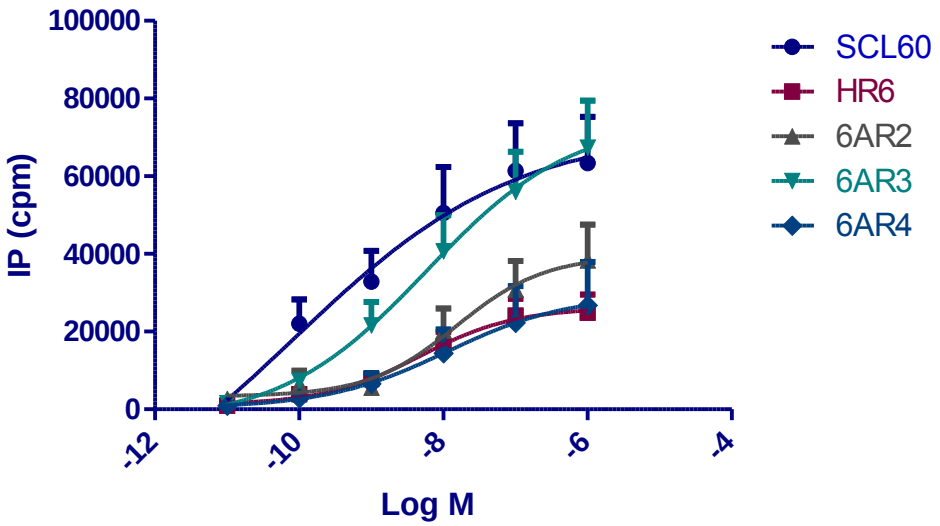


Figure 3.6: Comparison of Inositol phosphate production of cells co-expressing the GnRHR and Gαq/i (6AR2, 6AR3, 6AR4) to cells expressing the GnRHR only (HR6 and SCL60). Cell monolayers were treated with increasing concentrations of GnRH I in the presence of LiCl to prevent the degradation of inositol phosphates (IPs). The IPs were isolated from the cell extract using anion exchange column. The figure shows the averages of 5 independent experiments performed in duplicate.

The results in figure 3.6 indicate that the total inositol phosphates produced by the cell clones co-expressing GnRHR and Gαq/i (6AR2, 6AR3 and 6AR4) were higher than the IPs produced by the cell clone expressing the GnRHR only, clone HR6 . The SCL60 cell line which expresses the highest receptor levels produced the highest inositol phosphates when treated with concentrations of GnRH lower than 1micromolar. However at 1micromolar (Emax) the IPs produced by the SCL60 cell line were equal to those produced by clone 6AR3. This suggests that at high GnRH concentrations, the Gi pathway increases the production of IPs instead of decreasing them, as expected from the competitive binding of Gαq/i and subsequent Gαi signalling. Amongst the cell clones co-expressing the GnRHR and Gαq/i, the 6AR3 produced the highest inositol phosphates. This clone expresses similar GnRH receptor level as found in HR6. The inositol phosphates produced by the 6AR2 cell

clone were roughly half the levels produced by 6AR3. This was expected as the 6AR2 cell clone has half the receptor levels found in the 6AR3 and HR6 cell clones. These results indicate that receptor level affects the level of IPs produced when the cellular backgrounds are similar. The 6AR4 cell clone produced IPs comparable to those of HR6. These levels were the lowest of all clones co-expressing the receptor and Gαq/i, which is consistent with the low levels of GnRHR expressed in this clone.

Table 3.1: Comparison of the EC_{50} values derived from the dose response curve. The values were calculated using GraphPad Prism.

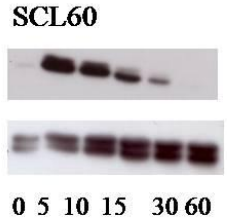
Cell Line	EC_{50} (nM)
SCL60	0.08
HR6	4.7
6AR2	15.2
6AR3	5.8
6AR4	11.3

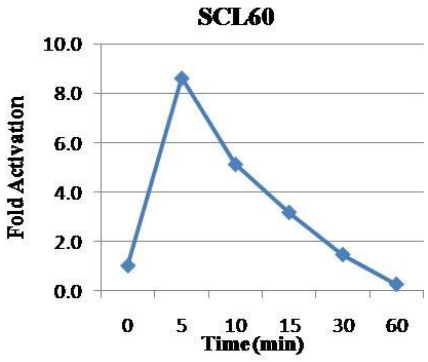
The potency of GnRH in each cell clone was determined using GraphPad Prism. There was no change in the potency of GnRH in cell lines co-expressing the GnRHR and Gαq/i relative to cells expressing GnRHR only (Table 3.1). The potency of GnRH in the HR6 cell clone was similar to that of 6AR3. The potency of GnRH in 6AR2 and 6AR4 was decreased by 2-3 fold and the change was thus considered insignificant.

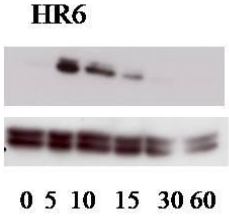
In conclusion, our results indicate that Gαq/i increases the production of inositol phosphates produced by the cell lines co-expressing GnRHR and Gαq/i but had no effect on the potency of GnRH.

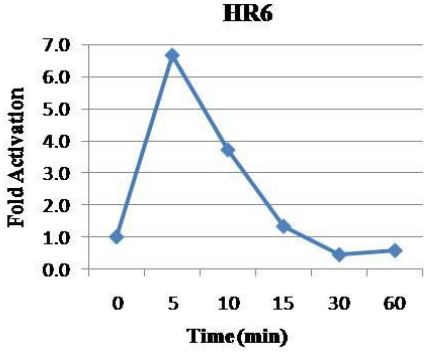
3.5 Comparison of GnRH-R induced ERK activation in stable cell lines co-expressing the GnRH-R and Gαq/i and in cells expressing the GnRH receptor only

Growth inhibition mediated by the GnRHR has been previously shown to act via ERK activation (Kim, Chio et al. 2006; White, Coetsee et al. 2008). In view of that we decided to determine the effect of Gαq/i on ERK activation; cell monolayer in 6 well plates were treated with 1µM GnRH I for 5, 10, 15, 30 and 60 minutes. Untreated cells were used as a negative control.









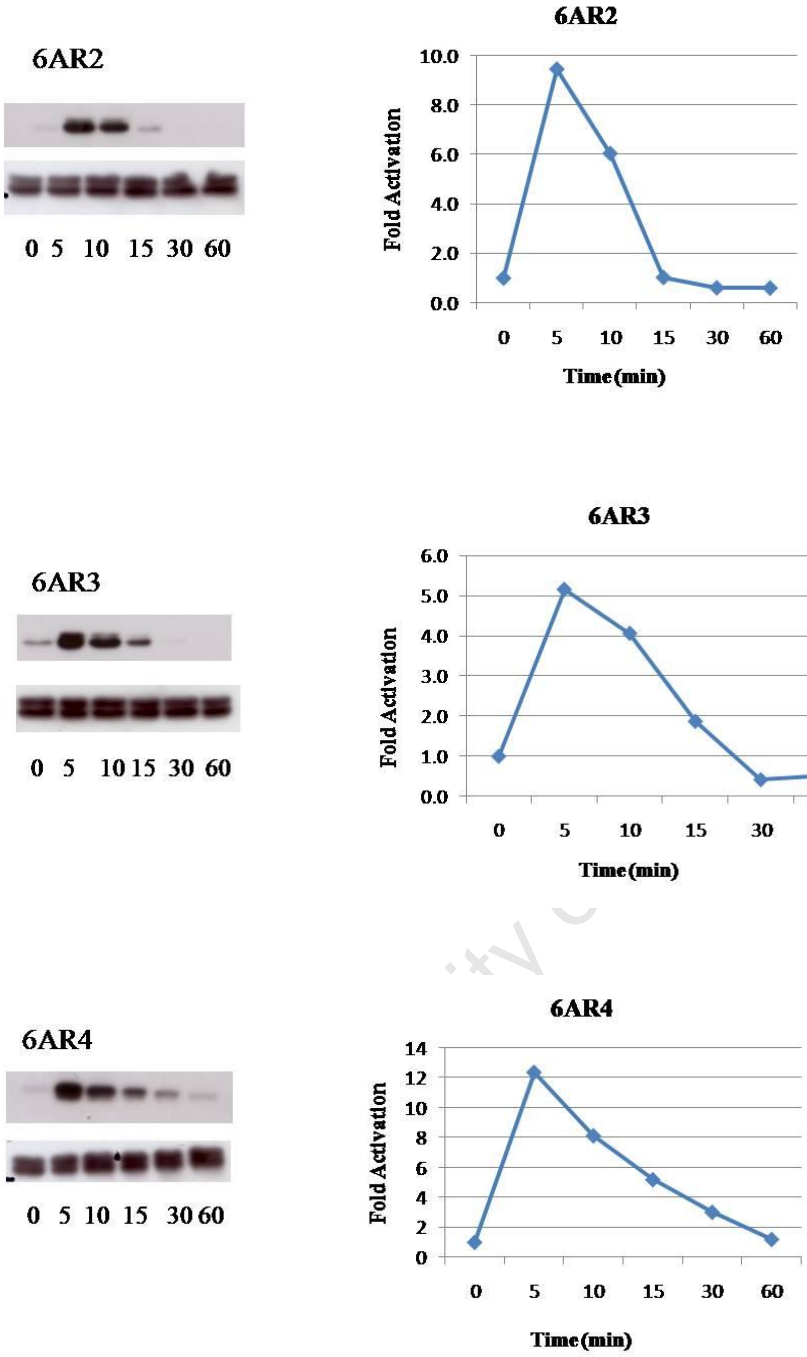


Figure 3.7: Comparison of ERK activation in HEK293 cells stably expressing the GnRHR (SCL60 and HR6) and in HEK293 cells co-expressing the GnRHR and Gq/i (6AR2- 6AR4 cell lines). Cell monolayers were incubated with medium containing 10^{-6}M GnRH I for the indicated times. Nitrocellulose membranes containing resolved proteins were probed with phospho ERK antisera (top gel). Total ERK (bottom gel) was used as a loading control. The films were scanned and analysed using the flourchem programme. The change in the levels of pERK were levelled against levels of tERK. The figure is a representative of 2 experiments.

Figure 3.7 shows that in untreated cells, there was little or no ERK 1/2 activation. However, when treated with GnRH I, ERK1/2 was rapidly phosphorylated within 5 min stimulation and was dephosphorylated after 60 minutes stimulation. Maximum activation occurred at 5 minutes stimulation. As seen from the graphs constructed from densitometric scanning (Fig 3.7, lower panel) of the Western blots probed with anti phospho ERK and total ERK, the kinetics of ERK1/2 phosphorylation were similar in cell lines expressing the GnRHR only (SCL60 and HR6) and in cell lines expressing the GnRHR together with the Gαq/i chimera (6AR2, 6AR3 and 6AR4). However the extent to which ERK was activated, as seen from the fold activation, differed in the cell lines studied. There was generally higher induction in cells expressing lower receptor levels.

In conclusion our results indicate that expression of Gαq/i did not affect the kinetics but seemed to affect the extent to which GnRHR activated ERK.

3.6 Comparison of cell growth in HEK293 cells expressing GnRHR only and in those co-expressing GnRHR and Gαq/i

The effect of Gαq/i on GnRHR mediated growth inhibition was determined using an MTT assay. This was done on the model cells co-expressing the GnRHR and the Gαq/i (6AR2, 6AR3 and 6AR4) and on cells expressing the GnRHR only (HR6 and SCL60). These cell lines were seeded in 12 well plates and treated daily with GnRH I (10^{-6} M) for 3 days. The control cells were treated with vehicle only. Following three days of treatment, the medium was removed and cells were incubated with DMEM containing 5% MTT for 2 hours.

Subsequently, DMSO was added to each well to extract the formazan dye. An aliquot was taken for absorbance at 595 nm.

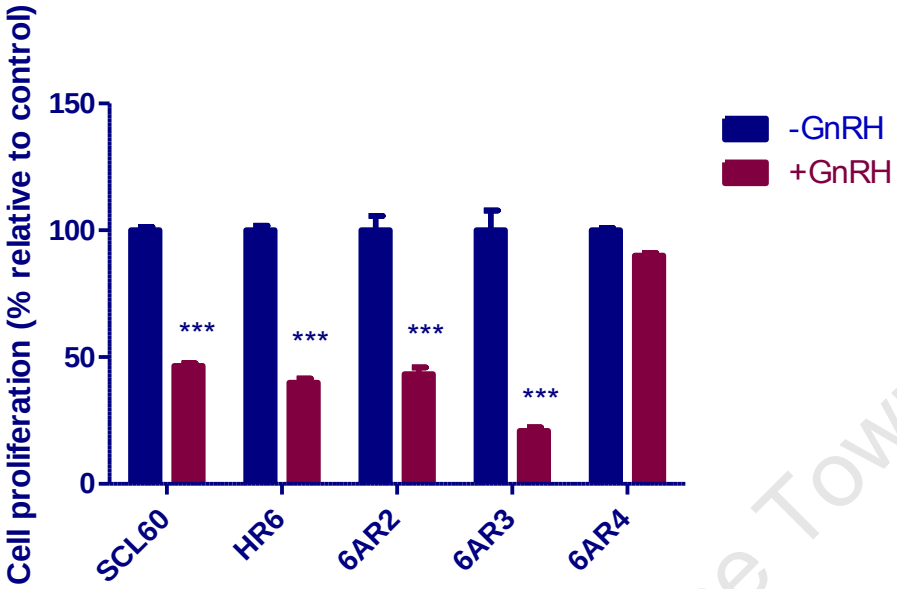


*Figure 3.8: Growth curves of cells treated with 10^{-6} M GnRH I daily for 3 days. Cells were seeded onto 12 well plates and treated with GnRH I, while the control cells were treated with vehicle only and their growth was taken as 100%. Harvesting was performed by incubating the cells with DMEM/ 5% MTT for 2 hours. DMSO was subsequently added to extract the dye. An aliquot was read at 595 nm. The figure shows the averages of three independent experiments performed in duplicate. ***, $p<0.001$, representing statistical significance from untreated cells.*

The results in figure 3.8 indicate that treatment of SCL60 cells, our positive control, with GnRH for 3 days resulted in significant inhibition of proliferation, indicating that the assay employed could detect changes in cell proliferation. The proliferation of the HR6 cell clone was significantly inhibited by 60% when treated with GnRH. Interestingly, treatment of the 6AR3 cell clone which expresses similar receptor level as HR6 with GnRH resulted in 80% inhibition of cell proliferation. The proliferation of the 6AR2 cell clone, which expresses half the receptor levels found in the HR6 and 6AR3 cell clones, was also significantly inhibited by

60%. These results indicate that expression of Gαq/i sensitises the cells to the antiproliferative effects of GnRH. The proliferation of the 6AR4 cell clone, which expressed the lowest receptor levels, was not significantly inhibited by GnRH.

Our results indicate that over expression of Gαq/i, which increases the Gi pathway increases the GnRH receptor mediated growth inhibition. This growth inhibition was dependent on the surface receptor level.

4. Discussion

The antiproliferative effects of the GnRH receptor on cancers of the reproductive system ((Imai, Takagi et al. 1996) (Tang et al 2002) (Park et al 2009) (Finch et al 2004) (Emons et al 1993) (Emons et al 2000) (Marelli et al 2009) (Maudsley, Davidson et al. 2004) and other cell lines (Kraus et al 2001) (Moretti et al 2003) (Miles et al 2004) have been documented. These effects were suggested to be mediated by Gαi (Grundker et al 2001) (Kraus et al 2001) (Maudsley et al 2004) (Kimura et al 1999) and not via Gαq which is the G protein known to be activated by the GnRHR. However, other studies have shown that the GnRHR only couples to Gαq (Grosse et al 2000) and that the GnRHR antiproliferative effects are mediated via Gαq (White et al 2008).

We decided to shed light on the role of Gαi by employing a G protein chimera (Gαq/i) that binds Gq-coupling GPCRs but signals through the Gαi pathway. If Gαi mediates the antiproliferative effects of the GnRHR, we would expect an increase in growth inhibition when cells are treated with GnRH. In order to study the effects of Gαq/i, we created two types of cell lines using HEK293 cells: one cell line expressed the GnRHR only while the other cell line expressed GnRHR and Gαq/i.

Critical to this experimental approach is whether the GnRHR couples to Gαq/i. This was done by employing a luciferase reporter assay. The assay would detect changes in cAMP levels that are affected by the Gαi pathway. If the GnRHR couples to Gαq/i, it would lead to a decrease in luciferase activity. Our results showed that the GnRHR couples to Gαq/i. This indicates that the approach of using G protein chimeras can help us answer our experimental question. Our results support the study performed by Slassereva and Graber (2003) in which they showed coupling of Gαq/i to the M1 muscarinic receptor, using *in vitro* methods. Our study however shows novel results regarding interaction of Gαq/i with another Gq- coupled

GPCR in intact cells. Employing the luciferase assay, we did not observe coupling between Gαi and the GnRHR. This could be due to a lack of interaction or weak coupling of the GnRHR with Gαi that our assay could not pick up. Therefore, assays that are more sensitive are needed to detect weak receptor- G protein interactions. The lack of interaction between the GnRHR and Gαi suggests that Gαi does not mediate the antiproliferative effects of GnRHR. This would be in agreement with the results observed by White et al (2008) in which they also observed lack of interaction between the GnRHR and Gi using the GTPyS assay in SCL60 cells. They also showed the importance of Gq in growth inhibition when treatment of cells lacking Gq (MEF cells) with GnRH, had no effect on growth. However the results may indicate weak interaction of Gαi with the GnRHR suggesting that the Gq and Gi pathway may act in a synergistic manner to inhibit cell proliferation. The GnRHR is known to mediate the diverse effects of GnRHR in pituitary gonadotropes via crosstalk of different signalling pathways (Naor, Harris et al. 1998) (Naor, Jabbour et al. 2007) (Siso-Nadal, Fox et al. 2009).

Having established that Gαq/i couples to the GnRHR we decided to transfect HEK293 cells with Gαq/i as our first step in creating stable cell lines expressing GnRHR and Gαq/i. This resulted in a few colonies expressing similar levels of Gαq/i so we picked one that we were going to continue our experiments with, the 6A cell clone.

We transfected the 6A cell clone with the GnRHR cDNA and isolated 5 colonies that we named 6AR2, 6AR3, 6AR4, 6AR5 and 6AR11. These cell clones expressed different receptor levels that could be used to determine effect of receptor level on the properties to be investigated. As control, we transfected the GnRHR cDNA to HEK293 cells and isolated only one colony that we named HR6 that had similar receptor levels as the 6AR3 cell clone.

Since Gαq/i signals via the Gi pathway, we would expect it to have a negative effect on the inositol phosphate production induced by the GnRHR due to competitive binding of Gαq/i with Gq to the GnRHR and subsequent Gi signalling. However, in cells co-expressing the GnRHR and Gαq/i there was an increase in inositol phosphates produced compared to those expressing the GnRHR only. These paradoxical results could be explained by the decrease in the inhibitory regulation of PLCβ. Signalling of Gi causes a decrease in cAMP, thus decreasing PKA activity (Stanislaus, Arora et al. 1996). It has been shown that PKA regulates PLCβ activity (Barnes and Conn 1993) (Ali, Richardson et al. 1999) (Xia, Bao et al. 2001). Decreased PKA activity would lead to sustained PLCβ activity and a decrease in the desensitisation of other signalling molecules regulated by PKA. The increased PLCβ activity will lead to increased IP levels. In our luciferase assay results, we observed that Gαq/i lowers luciferase activity, indicating a decrease in cAMP levels and thus supporting our desensitisation hypothesis.

Numerous studies (Pinter, Janovick et al. 1999) (Everest, Hislop et al. 2001) (Finch, Green et al. 2004) (Morgan, Stewart et al. 2008) have revealed that IP production was a function of receptor level. Comparison of IP production in cells co-expressing GnRHR and Gq/i, revealed that generally, IP levels were determined by receptor levels. The 6AR3 cell clone produced the highest IPs while the 6AR2 cell line which expresses half the receptor levels produced approximately half the IPs. The 6AR4 cell clone which has the lowest receptor levels produced the lowest amount of IPs that were equal to those produced by the HR6 cell clone. The IPs produced by the 6AR3 cell line were lower than those of SCL60 cell line until at 10^{-6}M. This indicates that the desensitisation effect is at its highest at high ligand concentrations. Although there was a change in the maximum amount of IPs produced in cells co-expressing GnRHR and Gαq/i, there was no significant change in the potency of GnRH when compared to cells expressing GnRHR only. We observed differences of 2-3 fold

in the potency of GnRH in the 6AR2 and 6AR4 cell clones that were due to a number of factors. There is competition between the Gq and Gi pathways that should lead to a decrease in potency. The decrease in desensitization of PLCβ may also affect the potency of GnRH. Receptor desensitisation may also be a factor even though the type I GnRHR is known not to desensitise due to a lack of C-terminal tail (McArdle, Frankiln et al. 2002) (Finch, Green et al. 2004). Therefore, these factors play a role in determining the potency of GnRH in a given cell. In literature, a difference of at least 10 fold is considered to be significant (Everest, Hislop et al. 2001) (Miles, Hanyaloglu et al. 2004) (Joseph, Morgan et al. 2009). That is why we considered the 2-3 fold difference in potency to be insignificant.

The ERK pathway has been shown in numerous studies (Kimura et al 1999) (Kim et al 2006) (White et al 2008) to be critical in the growth inhibition of cells expressing the GnRHR. Inhibition of ERK abolished the inhibition of cellular growth (Kim et al 2006; White et al 2008) (Morgan et al 2008). Our results confirm that ERK is indeed rapidly phosphorylated within 5 minutes and dephosphorylated by 60 minutes. These results are in agreement with the published kinetics of ERK activation in HEK cells stably expressing GnRHR (Davidson et al 2004) (Caunt et al 2006) and in other cellular contexts (Kraus et al 2003). However, there was no difference in the kinetics of ERK activation between cells expressing receptor only and in cells expressing receptor and Gαq/i. Activation of ERK by GnRHR is cell context dependent. In αT3-1 gonadotrophic cells ERK activation is PKC dependent (Sundaresan, Colin et al. 1996). In the human ovarian cancer cell line Caov-3, ERK activation was shown to be dependent on Gi using pertussis toxin (Kimura, Ohmichi et al. 1999). Incubation with pertussis toxin blocked GnRH induced ERK activation. In HEK293 cells, ERK activation has been shown to be activated via the Ras pathway involving the monomeic G protein, Rac, in focal adhesion complexes (Davidson et al 2004) (Dobkin-Bekman et al 2006) (Caunt et al 2006). The Gi signalling pathway would hypothetically not affect ERK activation in HEK293

cells as activation of ERK is dependent on the structural integrity of the cell (Davidson et al 2004) and was shown to be dependent on PLC and PKC (White et al 2008). The Gi pathway usually activates ERK in a protein tyrosine kinase/Ras/Raf pathway that is independent of PKC (Hawes et al 1995). We observed differences in the extent to which ERK was activated in each cell clone. There was higher induction of activated ERK in cells with lower receptor levels. However, the differences observed may be due to variation in the basal levels of ERK in the blots of each cell clone (Fig. 3.7). The lower induction level in the 6AR3 clone is due to the higher basal levels of pERK in these cells. Also, our densitometric analysis was from scanned x-ray films and not membranes, thus we would expect for there to be more error accumulated in analysis. There were also differences in exposure time and development that would affect the results of the densitometric scan. Therefore, we cannot use our results to determine the correlation between receptor level and induction of activated ERK. However, research done on virus immortalised prostate cells showed that receptor levels affected the modulation of ERK activation (Morgan et al 2010). The higher the receptor, the faster ERK was activated. However it should be noted that pERK levels can be transiently decreased in certain cell types.

GnRH treatment of HEK293 cells stably transfected with GnRHR results in growth inhibition (Miles et al 2004) (Maudsley et al 2004). Our results also show that continuous treatment of HEK cells stably expressing the GnRHR, for 3 days with GnRH I, resulted in growth suppression. Interestingly the antiproliferative effects of GnRH increased in cells expressing the GnRHR and Gαq/i. This is seen by comparing the effects of GnRH on the HR6 and 6AR3 cell clones which express similar receptor levels. The expression of GnRHR and Gαq/i in the 6AR3 cell line increased the antiproliferative effects of GnRH compared to receptor only in HR6. These results suggest that the Gαi pathway mediates the growth inhibition of GnRHR in HEK293 cells stably expressing GnRHR. However, we are not sure if this pathway is

activated physiologically in cells as we did not observe Gαi coupling to the GnRHR in luciferase assays. However, the luciferase assay is an indirect method and may not be sensitive enough to detect weak interactions. In cells co-expressing the GnRHR and Gαq/i there was also a positive correlation between receptor levels and growth inhibition, thus supporting previous studies (Everest, Hislop et al. 2001) (Finch, Green et al. 2004; Morgan, Stewart et al. 2008). There was also a positive correlation between IP levels and the degree of growth inhibition. The study by Morgan et al (2008) also observed this correlation. This could be due to receptor level affecting both IP and growth inhibition. Nevertheless, the augmentation of PLCβ activity caused by Gi signalling of Gαq/i highlights the crosstalk between the Gq and Gi pathway (Figure 4.1). They also support the hypothesis that the Gq and Gi pathway act in a synergistic manner to mediate growth inhibition. In our cellular system, by expressing Gαq/i we increased the Gi signalling, which lead to decreased PKA activity and thus positively affecting the activity of PLC, thereby increasing the growth inhibitory effects (Figure 4.1). The study by White and others (2009) concluded that ERK was required for inhibition but here we show that Gq/i increases inhibition although ERK activation was not different in cells expressing receptor and Gq/i than in cells expressing GnRHR only.

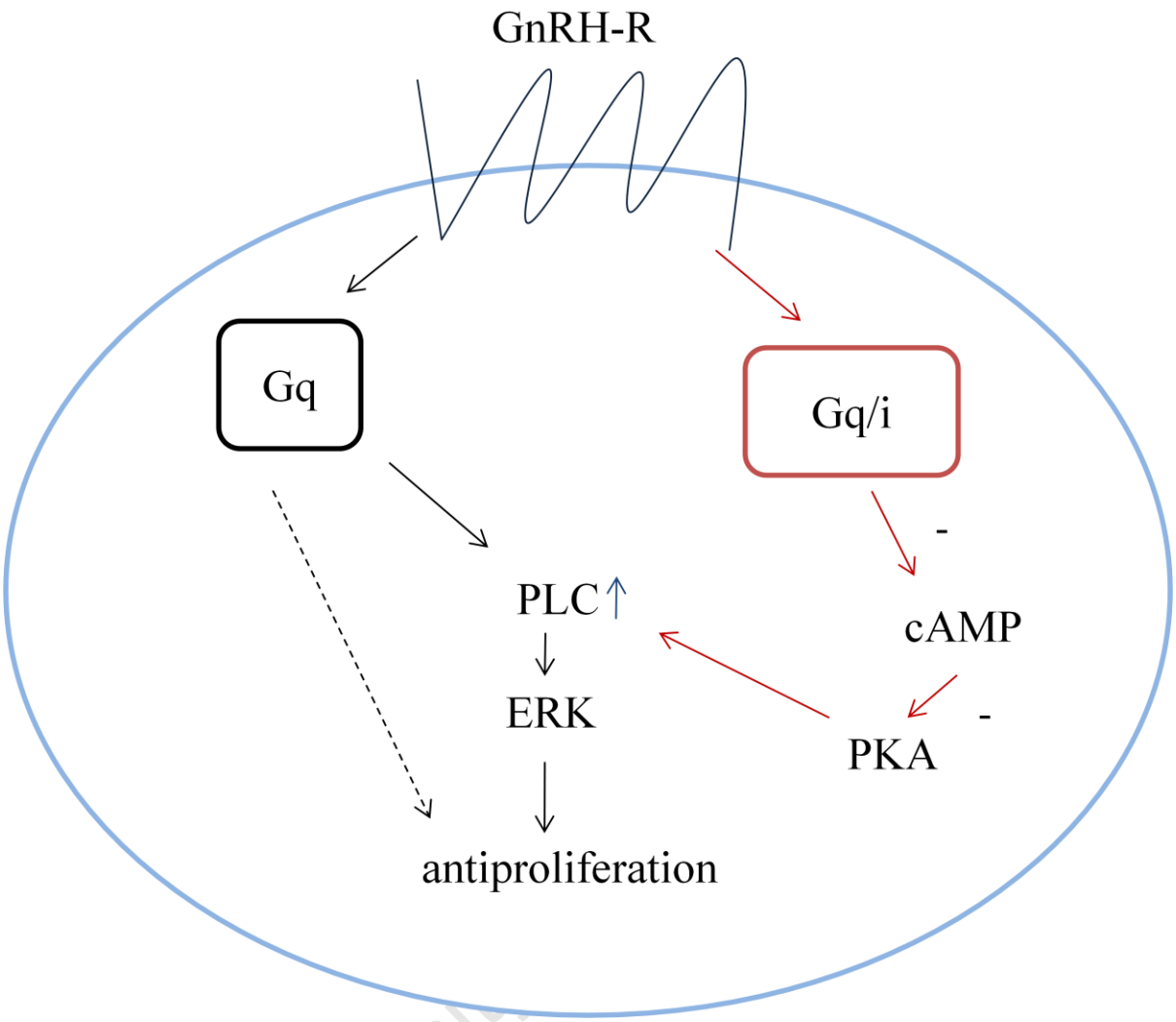


Figure 4.1: Model describing the signaling mediated by Gq and Gq/i that leads to inhibition of cell proliferation by the GnRHR .The Gq/PLC pathway which leads to the activation of ERK is required for the antiproliferative effects of the GnRHR. Gq may also activate other unknown pathways that lead to growth inhibition (dashed line). The Gi pathway activated in cells expressing Gq/i decreases cAMP levels. This negatively affects the levels of PKA, which result in increased levels of PLC activity. The enhancement in PLC activity leads to enhanced antiproliferation.

In conclusion, our results demonstrate that the expression of Gαq/i may increase growth inhibition. This indicates that Gi may play a role in mediating the antiproliferative effects of GnRH. Our results suggest that the blocking of PLC desensitization by Gi can augment Gq signalling, thus indicating that Gi may help in inhibition. Studies by White et al 2009 show the involvement of Gq in cells expressing Gq only (MEF cells). Therefore, our results demonstrate that Gi may play a role in inhibition of cell proliferation. This is consistent with

papers that show that Gi is important in growth inhibition mediated by GnRHR. However, it is possible that in certain cell types Gi does not play a role.

Future work

- In order to validate that an increase in PLCβ activity may cause enhancement of the antiproliferative activity of GnRH; the toxin *Pasteurella multocida* (PMT) may be used to PLCβ signalling (Preuss et al 2009) in cells expressing the GnRHR only. The proliferation of these cells in the presence of PMT may be compared to those expressing the GnRHR and Gi/q.
- To further validate the role of PLCB in antiproliferation a PLCB inhibitor may be used in cells expressing GnRHR and Gi/q

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Appendices

Appendix I

Western Blot Solutions

RIPA (Lysis) Buffer

25mM Tris – HCl pH 7.6

150 mM NaCl

1% NP-40

1% sodium deoxycholate

0.1% SDS

1X complete protease inhibitor

10% Resolving Gel

30 % Acrylamide

1.5 M Tris - HCl pH 6.8

10 % SDS

10 % Ammonium Persulfate

0.04 % TEMED

Distilled Water

A total of 10 ml is required per gel

5% Stacking Gel

30 % Acrylamide

1.5 M Tris - HCl pH 6.8

10 % SDS

10 % Ammonium persulfate

0.1 % TEMED

Distilled Water

A total of 5ml is required per gel

10X SDS-PAGE Buffer (2L)

Tris - HCL	60g
Glycine	288g
SDS	20g
Distilled Water	

10X Transfer Buffer (2L)

Tris – HCL	48g
Glycine	224g
Distilled Water	

1X Transfer Buffer (2L)

10 X Transfer Buffer	200 ml
Methanol	400 ml
Distilled Water	1400 ml

5X SDS Sample Buffer (20ml)

1M Tris-HCl pH 6.8	
Glycerol	10ml
2-b mercapthenol	4ml

Bromophenol	pinch
SDS	2g

10X Phosphate Buffered Saline (PBS, 1L)

NaCl	80g
KCl	2g
KH_2PO_4	2.4g
Na_2HPO_4	14.2g

Distilled Water

pH to 7.4 with HCl

1X PBS- 0.1% Tween (1L)

10X PBS	100 ml
Tween	1 ml

Distilled Water

1X PBS (1L)

10X PBS	100 ml

Distilled Water

Inositol Phosphate Solutions

Buffer I (1L)

40 mM NaCl

4 mM KCl

20 mM HEPES

0.1 % BSA

8.3 mM Glucose

0.4 % Phenol Red

1 mM $CaCl_2$ (+ H_2O)

1 mM $MgCl_2$

10 mM LiCl

pH 7.4