THE KISSPEPTIN AND GPR54 LIGAND-RECEPTOR PAIR IN AUTOCRINE AND ENDOCRINE SIGNALLING IN CANCER

by

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ABSTRACT

Kisspeptins and their receptor, GPR54, mediate sex hormone release through stimulation of the hypothalamic-pituitary-gonadal axis and have been implicated as metastasis suppressors. Expression of kisspeptin and GPR54 has been associated with less invasive cancers as determined by RNA expression, and a multitude of *in vitro* studies has consistently shown that overexpression of either ligand or receptor in malignant cell lines results in a less invasive phenotype. We hypothesized that expression of GPR54/kisspeptin in epithelial malignancies is predictive of disease outcome and altering endogenous GPR54 signalling in malignant breast and ovarian epithelial cells could alter their metastatic properties.

We have determined by immunohistochemistry that kisspeptin and GPR54 are independent favourable prognostic markers for ovarian carcinoma and are specific for the clear cell cancer subtype; the least characterized of the subtypes. Additionally, loss of GPR54 is associated with poor prognosis in node positive breast cancer patients and is also lost in prostate cancer and testicular germ cell nonseminomas as compared to more benign disease. Moreover, secreted kisspeptin is elevated above physiological levels in the plasma of women with gynaecological cancers, including ovarian cancer.

We evaluated GPR54 expression across a panel of breast and ovarian cancer cell lines to create an *in vitro* model system with which to knockdown GPR54 expression using RNA interference. However, we discovered that endogenous GPR54 was internalized rather than localized to the plasma

membrane of these cancer cell lines. Consequently, internal GPR54 was unable to signal through its canonical $G\alpha_q$ pathway.

To discover novel genes involved in kisspeptin-GPR54 signalling, we assessed gene expression differences between the *Gpr54* and *Kiss1* knockout mice as compared to wildtype mice. Our novel candidate list provides insight into physiological signalling in the hypothalamus that can then be applied to epithelial anti-metastatic signalling. Our results also support the sex hormone negative feedback effect on kisspeptin expression as reported in the current literature.

In summary, we have confirmed kisspeptin and GPR54 as favourable prognostic markers, are the first to report the intracellular localization of GPR54 in endogenously expressing cancer cell lines, and we have introduced a list of novel genes involved in signalling.

TABLE OF CONTENTS

LIST OF TABLES

LIST OF FIGURES

LIST OF ABBREVIATIONS

Ab	Antibody
ABI	Applied Biosystems
ANOVA	Analysis of variation
Ar	Androgen receptor
ATP	Adenine triphosphate
BACs	Bacterial artificial chromosome
BCA	Bicinchoninic acid
BPH	Benign prostatic hyperplasia
BSA	Bovine serum albumin
cAMP	Cyclic adenine monophosphate
cDNA	Complementary DNA
CHO	Chinese hamster ovary
CI	Confidence interval
CK	Cytokeratin
Cy-5	Cyanines-5
DAG	Diacyl glycerol
DAPI	4,6-diamidino-2-phenylindole
DDT	Dithiothreitol
DMEM	Dulbelco's modified Eagle's medium
DMSO	Dimethyl sulfoxide
dNTP	Deoxy ribonucleotide triphosphate
DSS	Disease specific survival
ECL	Enhanced chemiluminescence
EDTA	Ethylenediaminetetraacetic acid
EGF	Epidermal growth factor
EGTA	Ethylene glycol tetraacetic acid
EIA	Enzyme immunoassay
ER	Estrogen receptor
Erbb2	Epidermal growth factor receptor 2

ERK	Extracellular signal-regulated kinase
FBS	Fetal bovine serum
FISH	Fluorescent in situ hybridization
FITC	Fluorescein isothiocyanate
FSH	Follicular stimulating hormone
GDP	Guanine diphosphate
GFP	Green fluorescent protein
GnRH	Gonadotropin releasing hormone
GPR	G-protein coupled receptor (GPCR)
GPR54	G-protein coupled receptor 54/ hOT7T175/ AXOR12/KiSS1R
GTN	Gestational trophoblastic neoplasia
GTP	Guanine triphosphate
H&E	Hematoxylin and eosin
hCG	Human choriogonadotropin
HCl	Hydrochloric acid
HF	Hank's balanced buffer with 2% FBS
HRP	Horseradish peroxidase
IBMX	Isobutylmethylxanthine
IF	Immunofluorescence
IHC	Immunohistochemistry
IOSE	Immortalized ovarian surface epithelial
IP3	Inositol 1,4,5-triphosphate
IR	Immunoreactivity/Infared
Kp	Kisspeptin/metastin/KiSS1
L2K	Lipofectamine 2000
LDH	Lactate dehydrogenase
LH	Lutenizing hormone
MAPK	Mitogen activated protein kinase
min	Minute
MMP	Matrix metalloproteinase
NaDOC	Sodium deoxycholate

NMDA	N-methyl-D-aspartate acid
OS	Overall survival
PBS	Phosphate buffered saline
PCR	Polymerase chain reaction
PI	Propidium iodide
PIP2	Phoshpatidylinositol 4,5-bisphosphate
PKC	Protein kinase C
PLC	Phoshpolipase C
PR	Progesterone receptor
PVDF	Polyvinylidene fluoride
QPCR	Quantitative/real-time polymerase chain reaction
RFU	Relative fluorescence units
RIA	Radioimmunoassay
RIPA	Radioimmunoprecipitation assay
RT	Room temperature
SDS	Sodium dodecyl sulphate
shRNA	Short hairpin
siRNA	Short interfering RNA
TBS	Tris buffered saline
TBST	Tris buffered saline with Tween
TMA	Tissue microarray
WB	Western blot
WHO	World Health Organization

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To my family,

You have all encouraged me to succeed, and with your love and support I have achieved above and beyond.

In memoriam:

My mother, Sharon Diane Prentice, 1949-2002

Love you and miss you everyday

CO-AUTHORSHIP STATEMENT

All tissue microarrays described were already pre-existing and the original publications are referenced.

Two pathologists performed all the immunohistochemical analysis: Dr. Ashish Rajput for Chapter 3 and Dr. Martin Köbel for Chapters 2 and 4, both from the Genetic Patholgy Evaluation Centre, Vancouver, BC.

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All study designs were collaborations between the candidate, Dr. David Huntsman, and Dr. Samuel Aparicio. Chapter 3 also had Dr. Peter Leung contribute to the study design and Chapter 6 had Dr. William College as a study design contributor.

Excluding what has been noted above, the candidate performed all the experiments, wrote all drafts of the manuscripts, performed all revisions of the manuscripts, and created all the figures and tables for the manuscripts. The candidate assisted and conferred with the pathologists, technician and statisticians for all the immunohistochemical projects as well as conferred with Steven McKinney for the Affymetrix and real-time PCR analysis presented in Chapter 6.

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1 LITERATURE REVIEW, HYPOTHESES AND SPECIFIC AIMS

Since the discovery of the anti-metastatic *KiSS1* gene and its encoded kisspeptins over 10 years ago, there has been a flurry of publications attempting to describe the mechanism responsible for the anti-invasive phenotype associated with kisspeptin overexpression. The kisspeptin story became more complex upon the discovery of its G-protein coupled receptor GPR54 being mutated in idiopathic hypogonadotropic hypogonadism. Subsequent studies demonstrated the ligand-receptor pair as key players in regulating the hypothalamic-pituitary-gonadal axis in many species including humans. The focus of my research has been to assess if kisspeptin and GPR54 as anti-metastatic agents could be used as predictive markers for disease outcome. Furthermore my research examined endogenous GPR54 expression in breast and ovarian cells to create a knockdown cell line model to further study the autocrine signalling effects of kisspeptin and GPR54 in an epithelial environment.

1.1 Epithelial Kisspeptin

Though the kisspeptin encoding gene *KiSS1* is now known to be located on chromosome 1 [1], kisspeptin (Kp) was first discovered through microcell-mediated transfer of chromosome 6 [2]. Highly metastatic melanoma tumours have been characterized as missing genomic material from chromosome arm 6q and subsequent replacement of chromosome 6 in a melanoma cell line inhibited

metastasis [3, 4]. By comparing gene expression differences between the metastatic parental cell line and the chromosome 6 carrying line, *KiSS1* was revealed as a metastasis suppressor gene candidate [2, 5]. Since then, a regulator for *KiSS1* expression located on chromosome 6 has yet to be determined although there is evidence CRSP3 (DRIP130), a co-factor for the Sp-1 transcription factor, may regulate *KiSS1* expression through regulation of TXNIP which is also located on chromosome 1 [6].

The *KiSS1* gene locus encodes for the kisspeptins, of which four of varying lengths are currently known; kisspeptin-54, kisspeptin-14, kisspeptin-13, and kisspeptin-10, the largest of which (Kp-54) is also known as metastin due to its anti-invasive properties [2, 7, 8]. Prior to processing, the 145 amino acid KiSS1 pro-peptide contains a secretory sequence, two dibasic cleavage sites, and an amidation site [7]. The putative first cleavage site is of unknown significance as a kisspeptin-64 has yet to be observed, however the second dibasic cleavage creates the N-terminus of kisspeptin-54 (Figure 1.1 A, B). These processing sites are conserved in rodents, although the 52aa kisspeptin in mice has only 46% similarity to human Kp-54 [9] (Figure 1.1 C). Both the secretory sequence and the amidation site are necessary for a functional kisspeptin as when either are mutated or omitted Kp is inactive [8, 10]. The enzymes required for this kisspeptin processing have yet to be elucidated, however the matrix metalloproteinases 2 and 9 (MMP2, MMP9) have been shown to cleave and inactivate metastin and Kp-10 at Gly118-Leu119 [11] (Figure 1.1 B).

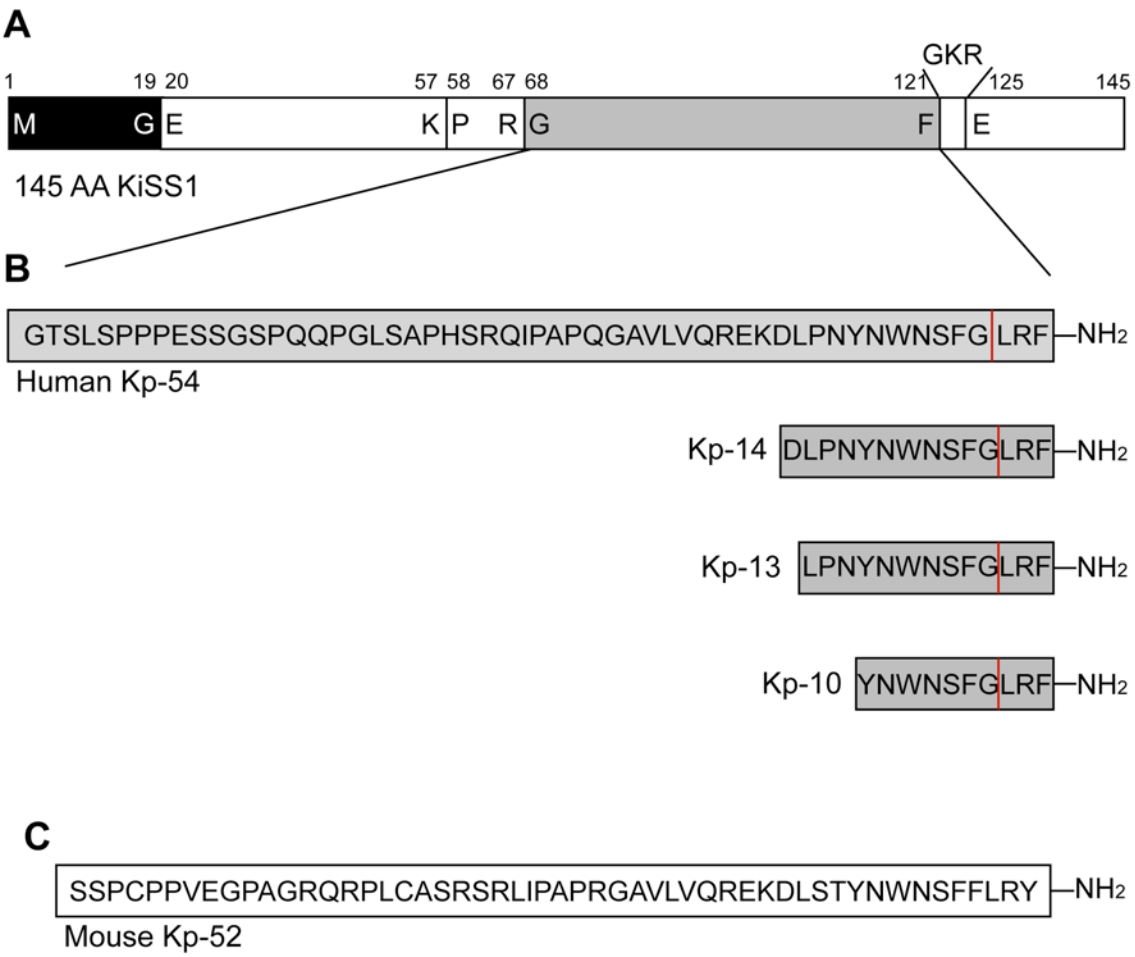

Figure 1.1 The kisspeptins

A) The full length pro-peptide for kisspeptin is 145 amino acids (aa) in length, has a 19 aa secretory sequence (black) and three cleavage sites (K57-P), (R67-G), and (R124-E). Full length human Kp-54 is shaded in gray, and the N-terminal becomes amidated losing three amino acids during processing (B). B) The four known human kisspeptins can be cleaved by MMPs at G118-L (red line) and become deactivated. C) The mouse Kp-52 sequence is shown for comparison (Modified from review articles [4, 19]).

Although it was determined that Kp can be inactivated by MMP2 and MMP9 [11], both activity [12] and expression [13] of MMP2 was decreased after Kp stimulation. Furthermore, the *KiSS1* overexpressing fibrosarcoma HT-1080 cells demonstrated a decrease in MMP9 activity and expression. Inverse correlation between Kp and MMP expression may be relevant to the anti-invasive ability of Kp as MMP activity has been credited with creating a metastatically favourable environment.

There has been some debate among research groups as to whether there are differences in affinity for GPR54 among the Kp peptides. Using competitive binding assays and calcium release asays, it has been claimed that there is no difference among kisspeptins [7], while others have claimed that Kp-10 has the highest affinity for GPR54 [8, 9, 14], and one group has claimed Kp-10 to be the only Kp to elicit a response from endogenous GPR54 in trophoblast cells [12]. However, endogenous Kp-10 was purified from the placenta by only two of these research groups [12, 14] whereas endogenous Kp-54 was always detected. Finally, it has been claimed that Kp-54 has the highest potency *in vivo* [15, 16] and it is not cleaved into smaller products in human plasma [17, 18]. Since the 145 amino acid pro-peptide kisspeptin sequence has no cleavage sites specific for the smaller peptides, this argues that the smaller peptides may be degraded products of Kp-54.

Throughout this dissertation the kisspeptins will be referred to collectively as kisspeptin or Kp unless specification of which kisspeptin is merited. Similarly,

the cognate receptor for Kp, GPR54, has had a variety of aliases (AXOR12; HOT7T175; KiSS1R) but will be referred to as GPR54 throughout the text.

1.1.1 Establishing kisspeptin as anti-metastatic

Originally discovered to be lost in a highly metastatic melanoma cell line, kisspeptin was initially termed metastin after *in vitro* and *in vivo* studies demonstrated that overexpression of *KiSS1* led to a less invasive phenotype [2]. Since this key discovery paper subsequent *in vitro* and *in vivo* studies conducted in a variety of cell lines have had similar conclusions. The first of these cell lines was a highly metastatic melanoma cell line, C8161, which demonstrated decreased invasiveness upon *KiSS1* overexpression as measured by reduced metastases when injected into athymic nude mice as compared to parental controls [2]. This same group went on to transfect a highly invasive breast cancer cell line, MDA-MB-435, and had a similar outcome with reduced metastases and decreased tumour burden [20]. These original *KiSS1* overexpressing studies report specifically as Kp being anti-metastatic and not anti-tumourigenic [2, 20].

GPR54, a G-protein coupled receptor of the calcium release $G\alpha_q$ family of G-proteins [7, 8, 14] (Figure 1.2) located on chromosome 19p13.3 [21], was discovered as the cognate receptor for Kp simultaneously by three groups [7, 8, 14]. Ohtaki et al [8] had similar findings as Lee et al. [2, 20] by stimulating *GPR54* overexpressing cells with Kp. Using CHO and mouse melanoma B16-BL6 cells, there was a decrease in *in vitro* invasion and migration as well as decreased *in vivo* metastases, however proliferation was not specifically assessed at this time.

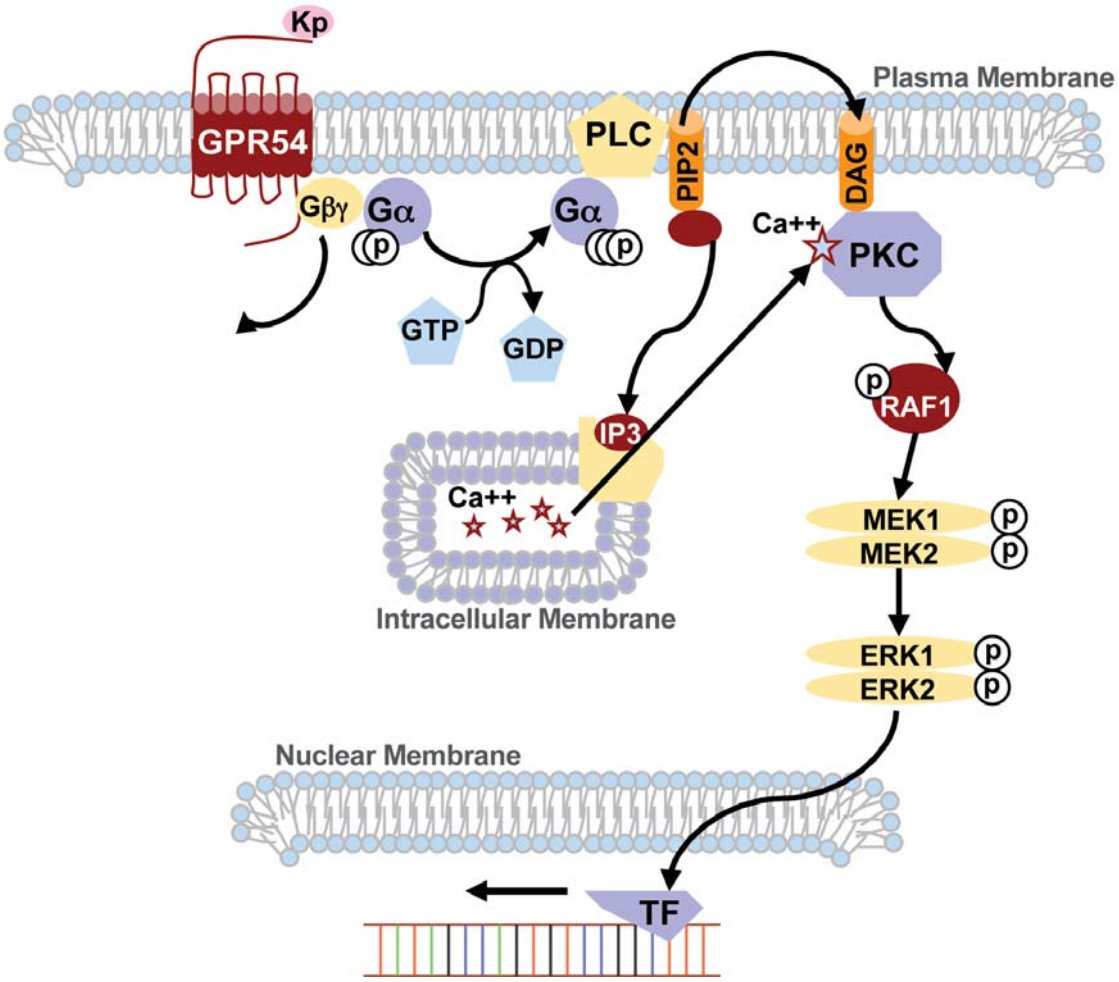

Figure 1.2 GPR54 intracellular signalling through the $G\alpha_q$ pathway

Upon Kp binding to GPR54 it is believed that intracellular signalling occurs through a $G\alpha_q$ pathway. After stimulation, the G-protein $G\alpha$ disassociates from the G-proteins β and γ. $G\alpha$ gains another phosphorylation group through guanosine triphosphate (GTP) conversion to guanosine diphosphate (GDP). The triphospate $G\alpha$ protein activates phospholipase C (PLC) which cleaves phosphatidylinositol 4,5-bisphosphate (PIP2) into inositol 1,4,5-triphosphate (IP3) and diacyl glycerol (DAG). DAG and calcium released from internal stores through IP3 binding of calcium channels on the endoplasmic reticulum go on to

activate protein kinase C (PKC). Among other targets, PKC has the ability to phosphorylate Raf-1 thus stimulating the mitogen-activated protein kinase (MAPK) pathway leading to phosphorylation of MAPK kinase (MEK) followed by phosphorylation of extracellular signal-regulated kinases (pERK).

In a follow-up study by this same group, a proliferation assay was conducted using the same *GPR54* transfected CHO cell line and growth was inhibited with the addition of Kp to the cell culture media [22]. Moreover, Kotani et al. [7] assessed proliferation of *GPR54* overexpressing CHO cells stimulated with Kp and also determined a decrease in proliferation, though the MAP kinase pathway was activated. They did not determine the invasive or metastatic ability of these cells. Stress fiber activation was witnessed by both groups and considered as a possible anti-metastatic mechanism of action.

Since these original discovery papers, there have been conflicting reports of whether stimulation of GPR54 inhibits proliferation as part of the anti-mestastatic behaviour of Kp. Initial studies made the distinction of Kp overexpression specifically having no effect on proliferation while reducing metastatic burden [2, 20]. However, after the discovery of GPR54 as the canonical receptor for Kp, overexpression of *GPR54* was used as an alternative to *KiSS1* overexpression to study anti-metastatic behaviour. Although proliferation was assessed in both cell line models, only *GPR54* overexpressing cells inhibited proliferation. In addition to the studies that have already been examined, further reports are discussed below.

Overexpression of *KiSS1* in the SKOV3 ovarian cell line expressing endogenous GPR54 led to decreased *in vitro* migration and colony formation as well as decreased *in vivo* metastasis formation, but did not affect cell proliferation [23]. In a fibrosarcoma cell line, HT-1080, overexpression of *KiSS1* also led to decreased migration without having an effect on proliferation [24]. Another group

created a stably overexpressing *GPR54* MDA-MB-435 cell line after discovering that endogenous GPR54 was missing from these breast cancer cells. However, unlike the *KiSS1* overexpressing studies, they report a decrease in cell proliferation and colony formation upon Kp stimulation of these transfected cells [25]. Furthermore, *GPR54* was overexpressed in a human papillary thyroid carcinoma cell line and upon Kp stimulation there was inhibition of migration and proliferation [26].

Alternative to creating overexpressing cell lines is to exploit those that have high endogenous levels of either GPR54 or Kp and compare them to cell lines with lower levels. Two GPR54 expressing renal cell carcinoma cell lines of varying Kp expression were compared and determined to have opposite migratory phenotypes upon Kp stimulation. The KU19-20 cells without any detectable Kp mRNA, had decreased migratory behaviour upon Kp stimulation while the highly Kp expressing Caki-1 cells did not. Neither cell line showed a decreased in proliferation upon external Kp stimulation [13, 27]. Similarly, no effect on cell proliferation was seen when Kp was added to the cell culture media of the pancreatic cell lines PANC-1 and AsPc-1 although there was an increase in pERK. However, the highly GPR54 expressing PANC-1 demonstrated a decrease in migration while no effect was seen with the AsPc-1 that expresses GPR54 at lower levels and high levels of Kp as compared to PANC-1. Nor was there a significant difference in the invasive ability of these two lines [28].

Both ligand and receptor are highly expressed in the placenta, which can be used as a model for controlled invasion [8, 14]. To assess epithelial GPR54

and Kp in normal physiology, first term trophoblast explant tissue was used to study the effects of Kp on cells with endogenous GPR54. Upon Kp-10 stimulation of this tissue, extravillous outgrowth ceased, and this was determined to be an effect of migration arrest and not due to decreased proliferation [12].

Currently it is unknown why *GPR54* overexpressing cell lines uniquely induce an anti-proliferative phenotype as compared to other cell line models that have been used to study Kp-GPR54 signalling. Regardless, all models have demonstrated that GPR54 activation through Kp stimulates anti-invasive and anti-metastatic behaviour.

1.1.2 Kisspeptin in clinical samples

As Kp and GPR54 have anti-metastatic ability, loss of either ligand or receptor in human tumour samples would be expected to correlate with a more invasive phenotype. Similarly, the gain of either ligand or receptor would be expected to correlate with a less invasive phenotype. The difficulty that has been encountered in supporting these hypotheses has been the manufacture of reliable antibodies, as perhaps is the difficulty with any novel peptide. As an example, it has been recently reported that a human Kp antibody, previously believed to be specific, cross-reacts with the human neuropeptide FF [29]. This RF-amide peptide is primarily expressed in the central nervous system, however it can also be measured in human plasma [30, 31]. To overcome this antibody issue, mRNA *in situ* hybridization and quantitative real-time PCR have been employed in a number of studies. Expression of the ligand and receptor in

tumours has been compared to a variety of patient characteristics to establish whether *KiSS1* and/or *GPR54* expression correlate with invasion. Specifically, gene expression has been correlated with tissue invasive phenotype (normal and metastatic), clinicopathological markers (grade and stage), and patient survival (disease recurrence and overall survival).

With the discovery of GPR54 as the receptor for Kp, the expression of both ligand and receptor were initially assessed in a small panel of tumours of various tissue type: four breast, two uterus, five ovary, one small intestine, and two colon. These tumours generally had increased levels of *GPR54* as compared to their normal adjacent tissue while *KiSS1* expression seemed to be lacking in both tumours and in normal tissue [8]. *GPR54* is overexpressed in papillary thyroid cancers as compared to follicular carcinomas or normal tissues, but due to small sample size no significant correlations could be determined [32]. In renal cell carcinoma it was demonstrated by real-time PCR and IHC that higher levels of *GPR54* were expressed in cancer compared to normal tissue while *KiSS1* was similarly expressed in both normal renal tissue and disease [27]. Conversely, in the normal pancreas the average *KiSS1* expression is higher than the average expression in tumour samples while the opposite is true for GPR54 [28].

Several research groups used clinical samples with linked outcome data allowing for comparison of *KiSS1* and *GPR54* expression with prognosis. In gastric cancer, downregulation of *KiSS1* is significantly associated with venous invasion and disease recurrence. Furthermore, loss of expression correlates with poor prognosis in overall and disease specific survival independent of stage [33].

Similarly, in esophageal squamous cell carcinoma loss of either one or both *GPR54* and *KiSS1* mRNA correlates significantly with poor prognosis in disease specific survival and correlates independently with lymph node metastasis [34]. For bladder cancer, loss of *KiSS1* expression significantly correlates with poor prognosis in overall survival [35].

There are two reports of *KiSS1/GPR54* expression in clinical cases that equate expression as a poor prognostic marker. In hepatocellular cancer, overexpression of *KiSS1* and *GPR54* as determined by quantitative PCR correlated significantly with advanced stage that is a marker for poor prognosis [36]. These results have since been confirmed using IHC where Kp was an independent prognostic marker for poor prognosis in both disease specific and overall survival [37]. As this study used the Kp polyclonal rabbit antibody that has since been shown to be non-specific, the validity of these results are questionable. Additionally, in a panel of 124 breast cancers, KiSS1 expression correlated with poor prognosis in disease specific survival [38]. However, the real-time PCR was normalized to cytokeratin-19 (CK-19) expression within the same tumour sample. CK-19 has variable expression throughout breast tumours and is itself a marker for poor prognosis [39].

For those studies without outcome data, expression of *KiSS1* and/or *GPR54* in invasive disease was compared to more benign phenotypes. In melanoma, early skin cancer lesions and benign cases all expressed *KiSS1* as compared to the larger lesions and metastatic disease that exhibited *KiSS1* expression in only half of the cases [40]. Furthermore, cases with loss of *KiSS1*

expression significantly correlated with loss of heterozygosity (LOH) on chromosome 6 (q16.3-23), the region believed responsible for Kp regulation [40]. In brain metastases arising from breast cancer, *KiSS1* expression was lost compared to the primary lesion [41]. These two studies have similar results as those with linked clinical outcome; they demonstrate that loss of *KiSS1* correlates with a more invasive disease.

When assessing *KiSS1* and *GPR54* expression in clinical samples, the distinction between anti-tumourigenic and anti-metastatic is important to consider. Many tumours, across a variety of tissues, express increased *KiSS1* and *GPR54* expression as compared to their normal counterparts, which may intuitively suggest that Kp and GPR54 are not representative of favourable prognosis. However, when *KiSS1* and *GPR54* expression are linked with clinical outcome data, expression does correlate with favourable prognosis in almost all clinical samples that have been studied to date. This observation is supported by those studies that compare loss of *KiSS1* expression in metastatic lesions with primary tumours. Prognosis is an important indicator of invasive disease, as most cancers are not necessarily lethal as primary tumours; it is often the metastatic ability and invasiveness of cancer that leads to death of the patient [42, 43]. As *KiSS1* and *GPR54* are expressed more highly in tumours compared to normal tissues, yet lost in more invasive disease, this supports the hypothesis that Kp-GPR54 are anti-metastatic and not anti-tumourigenic. Table 1.1 summarizes all the statistically significant results exploring *KiSS1* and *GPR54* expression in clinical cases as discussed above.

Table 1.1 Summary of statistically significant *KiSS1* and *GPR54* expression in clinical cases

Malignancy	Gene(s) Analyzed	Cases	Assay Used	Comparison	Significant Correlation	Survival Analysis	Multivariable Analysis	Reference
Gastric Adenocarcinoma	*KiSS1*	40	RNase protection	Compared to adjacent normal mucosa	Loss correlates with venous invasion	Loss correlates with DSS and OS	Independent marker	Dhar, 2004
Ductal and Lobular Breast Cancer	*KiSS1* and *GPR54*	124	QPCR	QPCR normalized to CK-19	*KiSS1* exp correlates with node positivity	*KiSS1* exp correlates with DSS	Not done	Martin, 2005
Hepatocellular Carcinoma	*KiSS1* and *GPR54*	60	QPCR	Compared to 8 normal and 60 cirrohtic livers	Exp correlates with higher stage	Exp correlates with DSS	Not done	Ikeguchi, 2003
Brain Metastasis, Primary Breast	*KiSS1*	5	QPCR	Compared to 10 primary tumours	Loss of *KiSS1* in metastases	Not done	Not done	Stark, 2005
Esophageal Squamous Cell Carcinoma	*KiSS1* and *GPR54*	71	QPCR	Compared to adjacent normal tissue	Both inversely correlate with node positivity	Loss correlates with DSS	Independent marker	Ikeguchi, 2004
Bladder Transitional Cell Carcinoma	*KiSS1*	15	cDNA mircroarray	Compared to 9 cell lines	Exp correlates with non-invasive tumours	Exp correlates with OS	Not done	Sanchez-Carbayo, 2003
		25 paired, 173	RNA in situ	25 compared to normal urothelium	Loss correlates with venous invasion	Exp correlates with OS	Not done	Sanchez-Carbayo, 2003
Pancreatic Cancer	*KiSS1* and *GPR54*	30	QPCR	Compared to 5 normal	Loss of *KiSS1*, Exp *GPR54* in cancer	Not done	Not done	Masui, 2004
Papillary and Follicular Thyroid Cancer	*KiSS1* and *GPR54*	23	QPCR	Compared to 11 normal 2 benign	Papillary exp increased over follicular	Not done	Not done	Ringel, 2002
Renal Cell Carcinoma	*KiSS1* and *GPR54*	25	QPCR	Compared to normal renal cortex	*GPR54* higher in RCC compared to renal cortex	Not done	Not done	Shoji, 2008

Exp=expression, DSS=disease specific survival, OS=overall survival

1.2 Neuroendocrine Kisspeptin

It has been well established that sexual reproduction in mammals relies on the activation of the hypothalamic-pituitary-gonadal axis. This axis is mediated by gonadotropin releasing hormone (GnRH) that is released from neurons in the hypothalamus to stimulate gonadotropin release from the anterior pituitary. The gonadotropes release the gonadotropins, lutenizing hormone (LH) and follicular stimulating hormone (FSH), into circulation to act upon the gonads in an endocrine fashion to induce sex hormone release and stimulate gametogenesis [44]. The estrogen and androgen sex hormones then have a negative feedback effect on the hypothalamus that reduces the levels of gonadotropins within the system. For many years the mechanism of this feedback effect was a mystery as the GnRH expressing neurons do not express any of the steroid hormone receptors [45, 46]. Due to this curious circumstance, it was evident that an important regulator in the mammalian reproductive axis was missing.

1.2.1 Kisspeptin regulation of GnRH through GPR54

Kisspeptin was first linked to the hypothalamic-pituitary-gonadal axis by the creation of a *Gpr54* knockout mouse and the discovery of an inactivating *GPR54* mutation in humans. Both mutated species demonstrated hypogonadotropic hypogonadism; the absence of sexual maturation due to decreased gonadotropins in circulation [47]. Since this groundbreaking discovery, GnRH

regulation through Kp release has been studied in many species including the lower vertebrates [48-50].

Activation of the hypothalamic-pituitary-gonadal axis has been determined by GnRH release with accompanying LH and FSH increase upon Kp stimulation in; mice [51, 52], rats [53-59], sheep [52, 60], monkeys [61, 62] and humans [17, 18]. This kisspeptin activity has been demonstrated to be effective both peripherally and centrally and is GPR54 specific [52, 63, 64]. GPR54 has been colocalized to GnRH neurons in the hypothalamus [52, 54], and subsequent activation of GPR54 by kisspeptin on these neurons results in GnRH neuronal activity [55, 59, 65, 66] that is concentration dependent [67] and directly releases GnRH [52, 68, 69] that can be inhibited by a GnRH antagonist [51, 54, 55, 57, 61, 62].

Though *GPR54* is highly expressed in the pituitary, Kp does not seem to promote the release of the gonadotropins from the pituitary directly. In rats, pituitary explants and primary cell cultures stimulated with Kp did not have an effect on LH or FSH release [55, 59]. One group did show LH release from pituitary tissues at a much lower level than GnRH and without a similar effect on FSH [56, 57]. Conversely, LH was released from primary rat pituitary cells at higher concentrations upon Kp stimulation than when GnRH was used, but this was sex specific to males. Furthermore, calcium flux was also witnessed in a small subset of the pituitary cells (~10%) in this study, the authors believing these cells to be the somatotrophs and gonadotrophs [70].

In the ewe, though there was release of LH from the primary culture of pituitary cells cultured from the follicular phase after Kp stimulation *in vitro*, this behaviour was absent in *in vivo* studies [71]. The trend seems to be that there is only an effect when the pituitary is reduced to a single cell layer, but not while it is in its native three-dimensional state.

1.2.2 Hormonal feedback regulation mediated by Kp and GPR54 in rodents

In murine models, *Kiss1* is expressed in the anteroventral periventricular nucleus (AVPV) and the arcuate nucleus (ARC) in the hypothalamus. This has been determined by immunohistochemical (IHC) analysis and RNA *in situ* hybridization [51, 72-74]. These neuron processes lie adjacent to GnRH neuron bodies suggesting their direct effect on GnRH release while not being co-localized directly with the GnRH neuron [75], as is the case for the GPR54 receptor [52, 55]. Furthermore, estrogen receptor alpha (ERα) has been localized directly in the *Kiss1* expressing neurons [73, 74] without being co-localized to the GnRH neurons [76]. Prior to the discovery of Kp and GPR54, the lack of ERα on GnRH neurons while still being affected by estrogen positive and negative feedback had been a mystery. Due to the position of *Kiss1* expressing neurons and ERα, it is thought that the Kp neurons mediate the feedback effects on the hypothalamus.

Within the male mouse, *Kiss1* expression is upregulated in the ARC after castration [74]. This effect can be reversed by implantion of testosterone that seems to be relayed through the *Kiss1* expressing neurons through both ERα

and the androgen receptor (AR). Dihydrotestosterone (DHT) only reduced *Kiss1* expression by half in castrated mice while both testosterone and estrogen replacement completely reduced the elevated *Kiss1* expression [74]. Unlike DHT, testosterone can be aromatized to estrogen suggesting that both the AR and ERα were in use. This change in *Kiss1* expression due to castration was still observed in the ERαKO and non-functional AR mice suggesting that testosterone regulates *Kiss1* expression either through the AR or through aromatization of testosterone and the ERα [74].

Similarly, ovarectomized female mice have an increase in *Kiss1* expression within the ARC that is reduced by estrogen implants [77]. However, the AVPV in female mice seems to regulate the positive feedback effect necessary for the LH surge, as there is a decrease in *Kiss1* expression within the AVPV in ovariectomized mice that is increased by estrogen replacement [73]. The ERα expressing *Kiss1* neurons in the AVPV also express the progesterone receptor [72] that is necessary for the LH surge required for female ovulation and GnRH activity at the time of the LH surge [72].

In male and female rats, *Gpr54* and *Kiss1* RNA expression in the hypothalamus was maximal at puberty compared to postnatal, prepubertal and adult rats [78]. In castrated males, *Kiss1* expression was elevated and this effect was abrogated with testosterone replacement while in females a similar effect was noticed with estrogen replacement in ovariectomized rats [78, 79]. Continous Kp stimulation in rats showed desensitization of the axis by a decrease in LH

overtime although *GnRH* RNA levels did not decrease overtime, suggesting that Kp and GPR54 have a role in GnRH release, not expression [57]

Specifically, *Kiss1* is highly expressed in the ARC as determined by QPCR and IHC and these neurons also express ERα [79]. Female ovariectomized rats have decreased Kp expression in the AVPV as compared to intact females. Further, estrogen replacement in ovariectomized rats increased *Kiss1* expression in the AVPV [80, 81]. Comparatively, intact male rats have very few *Kiss1* neurons and subsequently very little *Kiss1* expression in the AVPV [80, 81]. However, Kp neurons were highly expressed in the ARC of both gonadectomized male and female rats, and these levels were reduced with the presence of hormonal implants [80, 81].

In female rats, *Kiss1* and *GPR54* had maximum expression during the diestrus phase of the estrus cycle [78, 79]. This change in *Kiss1* expression throughout the estrus cycle was variable depending on which region of the hypothalamus was being studied. In the AVPV, *Kiss1* had the highest expression during proestrus and the lowest expression at diestrus, yet in the ARC *Kiss1* had the highest expression at diestrus and lowest in proestrus [80].

Intact female rats failed to undergo ovulation or have a LH increase when pretreated with ERα antagonist even after intracerebral injection with Kp-10. This was compared to LH release and ovulation after GnRH treatment [82]. No such effect was reported when mice were pretreated with an ERβ antagonist prior to injection, and only a modest decline in LH was seen after progesterone receptor

(PR) antogonist pretreatment. Again, this effect was not witnessed in the GnRH injected rats [82].

Similarly, PR and ERα were required in a normal cycling female treated with empty vehicle for FSH release for the preovulatory phase as was seen in the LH release studies [83]. While both ERα and PR antagonists blocked FSH release stimulated by Kp-10, as seen for LH, there was also a decrease in FSH release with ERβ. For GnRH injections which were unaffected by ERα, ERβ, PR antagonist pretreatment in the release of LH, ERα and PR antogonists decreased FSH release after GnRH stimulation [83].

To summarize the above scientific findings; the AVPV is recognized as the hypothalamic region responsible for positive feedback and the subsequent LH surge required for ovulation, while the ARC is responsible for the negative feedback within the Kp-GPR54 regulated hypothalamic-pituitary-gonadal axis in rodents (Figure 1.3).

1.2.3 Hormonal regulation mediated by Kp and GPR54 in primates

In monkeys, when human Kp-10 was continuously administered over a 4 day period there was an initial spike in LH secretion, followed by LH returning to normal levels after the first day of continuous infusion. By the fourth day, although N-methyl-D-aspartate acid (NMDA) and GnRH could elicit a LH surge, there was no response from a bolus Kp-10 injection. However, 21 hours after cessation of continuous Kp stimulation, LH was once again responsive to a single bolus of Kp-10 [84]. Further, Kp is released in a pulsatile fashion similar to

that of GnRH (LHRH-1) [69]. Kp is also increased in the stalk-median eminance of midpubertal monkeys as compared to prepubertal and early pubertal monkeys [69].

KiSS1 has been localized to the ARC of the medial basal hypothalamus (MBH) of the castrated male monkey [85, 86]. Assessing hormonal feedback by testosterone implant into castrated males found that *KiSS1* expression was decreased in the MBH with testosterone treatment while *KiSS1* RNA was low in the preoptic area (POA) regardless of treatment. *GPR54* RNA levels were unaffected for this study [86]. Comparatively, *KiSS1* expression was increased in the infundibular nucleus of the MBH of ovariectomized female monkeys [87, 88] as compared to the estrogen replacement group of animals [88]. However, there was no change in expression found in the preoptic area for either species [87, 88]. The primate infundibular nucleus has been equated to the arcuate nucleus (ARC) in other species, the site for Kp negative feedback [89, 90] and the preoptic area equated to the anteroventral periventricular (AVPV) found in rodents [88].

Menopause in human females can be used as a castration comparison model as has been done in experimental animals [91]. Using neuronal tissue obtained from deceased women, there was an increase in *KiSS1* mRNA expression in the infundibular nucleus of post-menopausal woman as compared to pre-menopausal and this was reflected in postmenopausal female monkeys [88]. This suggests that there is a similar negative feedback loop affecting *KiSS1* expression in humans; however further studies are required.

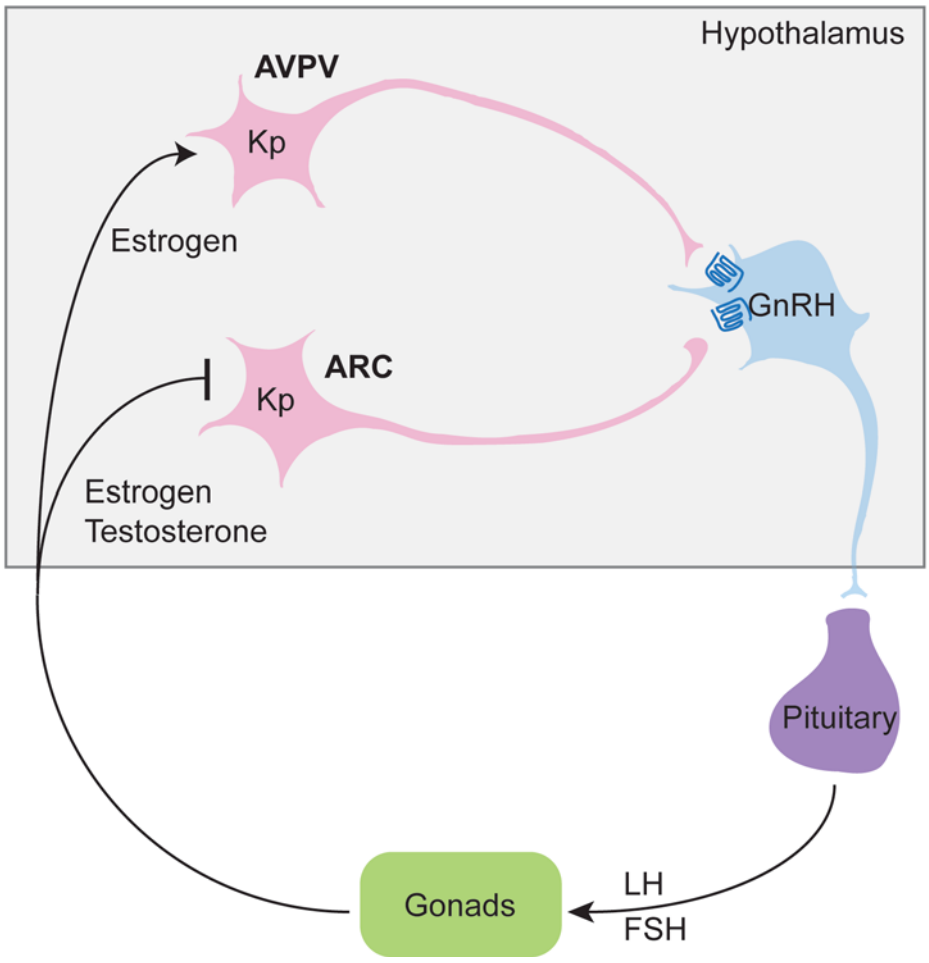

Figure 1.3 Kp and GPR54 hormonal feedback regulation in the rodent
Within the hypothalamus the arcuate nucleus (ARC) mediates the negative feedback through Kp expressing neurons in both male and female rodents. The anteroventral periventricular nucleus (AVPV) is specific to female positive feedback regulation required for ovulation. Kp is released to stimulate GPR54 located on the GnRH neuron also within the hypothalamus that then signals to the pituitary to release the gonadotropins, LH and FSH that then go on to stimulate sex hormone release from the gonads (Modified from reviews [92-94]).

1.2.4 GPR54/Kisspeptin mutations in humans

The discovery of a homozygous single nucleotide mutation in exon 3 (L148S) of *GPR54* in 6 individuals with idiopathic hypogonadotropic hypogonadism (IHH) from a consanguineous family, led to the discovery of kisspeptin's significant role in stimulating the endocrine cascade [47]. In the same publication, an IHH patient was discovered to be a compound heterozygote, with one allele coding for a truncating mutation (R331X) and the other allele with a nonstop mutation (X399R), both in exon 5. Functional studies revealed that there was a decrease in GPR54 activity in the L148S point mutation by 65% and a decreased by 67% for the R331X premature stop codon. Since the nonstop mutation X399R affected GPR54 conformation, it could not be used for functional studies, but the simple addition of arginine to the end of GPR54 was enough to reduce activity by 61%. Simultaneously, a homozygous deletion also in exon 5 was found to cause IHH in a consanguineous family with five affected children [95]. This deletion leads to a reading frame shift resulting in an altered protein sequence beginning from the third intracellular loop of GPR54. It is as yet unknown if this deletion leads to complete loss of expression of this protein or simply dysregulation of GPR54 function.

Since these initial discoveries, other mutations have since been reported. Two of these mutations were found in one isolated hypogonadotropic hypogonadism patient; a transversion in exon 4 from the T nucleic acid to C resulting in a C223R change in amino acid sequence and a transversion in exon 5 G>T resulting in an R297L sequence change thus creating a compound

heterozygote. The C223R mutation was found to have a more significant decrease in GPR54 activity (80%) than the R297L (15%). Both the asymptomatic mother and brother of the proband were heterozygous for the R297L mutation [96].

Another frameshift mutation, arising from a single nucleotide insertion in exon 5, created the IHH phenotype in a homozygous patient [97]. This insertion results in an elongated 441 amino acid protein sequence for GPR54 as compared to the normal 398 amino acids. There were no functional studies to assess potential decrease of GPR54 activity in this case, although due to a probable conformation change that would arise from the elongated protein it is most likely that these studies are not possible. The most recent report of a GPR54 mutation resulting in IHH was published last year. A single homozygous point mutation in exon 2 (L102P) was found in 5 patients from two separate consanguineous families resulting in decreased Kp binding affinity and complete loss of GPR54 signal [98].

GPR54 signaling through Kp binding activates calcium flux specific for the G-protein coupled receptors of the $G\alpha_q$ subfamily [7, 8, 14]. GPR54 activity can thus be measured by calcium release assays, inositol phosphatase (IP3) activity and has been elucidated to signal through the MAP kinase pathway allowing upregulation of pERK to also be used as a marker for GPR54 activity [7, 24, 28, 32]. By assessing at which point GPR54 activity is lost, one could hypothesize or elucidate how the mutation is affecting downstream events. For the L102P point mutation affecting the first extracellular loop of GPR54, decreased Kp binding

was determined to be the consequence of the mutation. For the deletion and insertion resulting in a frameshift and elongated GPR54, it would be reasonable to assume that an elongated protein would result in a conformation change that would target the receptor for the ubiquitin-proteasome-degradation pathway and result in the loss of the receptor. Similarly, the R331X point mutation giving rise to a stop codon may result in a truncated protein being targeted for degradation, or perhaps the intracellular portion required for signaling is lost. The second extracellular mutation R297L may also result in loss of Kp affinity for GPR54, however it seems this is a much milder affect than the L102P mutation since there is only 15% decrease in GPR54 activity. The second mutation in this compound heterozygote, C223R, [99] results in a change of charge from the polar cysteine to the positive arginine. This strong positive charge may be too closely positioned to the hydrophobic plasma membrane resulting in a displaced intracellular loop.

The first in-depth functional analysis of one of the GPR54 loss-of-function mutations was recently published [100]. By overexpressing the L148S mutation with an epitope tag and through the completion of a series of functional studies, it was determined that it is the second intracellular loop of GPR54 that is specific for Gα protein coupling to the receptor. The loss of GPR54 signalling was due to the inability of GPR54 to undergo a conformational change allowing the Gα protein to dissociate from the G$\beta\gamma$ subunit, thus arresting the signalling cascade and not due to the inability of Gα protein to interact with GPR54. Further, they

determined that the overexpression of the $G\alpha_q$ subunit partially rescued the phenotype.

All the aforementioned mutations are loss of function mutations. Recently, the first activating GPR54 mutation was published [99]. An 8 year old female presenting with precocious puberty was found to have a single heterozygous point mutation from C>G resulting into an amino acid change from arginine to proline. This mutation led to prolonged GPR54 signalling with a longer response by the MAP kinase pathway. It is interesting to note that the activating mutation is intracellular rather than extracellular. Extracellular over-activation of GPR54 such as increased Kp binding has been shown to desensitize GPR54 [68, 84].

There has yet to be discovered an inactivating or activating *KiSS1* mutation. However, the creation of *Kiss1* knockout mice recapitulated the phenotype expressed by the *Gpr54* knockouts. All knockout mice for either *Gpr54* or *Kiss1* have resulted in the same loss-of-sexual-maturation phenotype [47, 63, 64, 101, 102]. Figure 1.4 summarizes all the human genomic mutations resulting in either loss of GPR54 function or gain of function that have been discussed.

1.2.5 *Kiss1* and *Gpr54* knockout mouse models

There are currently four *Gpr54* knockout mouse models [47, 63, 64, 102], and two *Kiss1* knockout mice that have been created [64, 101]. All six of the knockout mice have the same hypogonadotrophic hypogonadism phenotype as seen in *GPR54* mutated humans. The males have small testes, microphallus and smaller

body weights, while the females have smaller ovaries, threadlike uteri, and delayed vaginal openings indicative of a lack of sexual maturation. Furthermore, these mice are sterile due to disruption of oogenesis and spermatogenesis. The *Gpr54* knockout mice also fail to demonstrate normal sexual behaviour, but this can be reversed with sufficient sex steroid hormone replacement [103].

The knockout mice exhibit low levels of the gonadotropins, LH and FSH, as well as low levels of sex steroids; testosterone in males and 17-β-estradiol in females. *Gnrh* expression within the hypothalamus of the knockout mice is similar to that of wildtype mice indicating that loss of *Gnrh* expression is not the mechanism behind the low gonadotropin levels, but more likely due to the inability of GnRH to be released [47, 52, 101]. As *Gpr54* is intact in the *Kiss1* knockout mice, introducing Kp back into the system of these mice releases the gonadotropins [64, 101]. This is not the case for the *Gpr54* knockout mice demonstrating that the hypothalamic-pituitary axis is specifically regulated through GPR54.

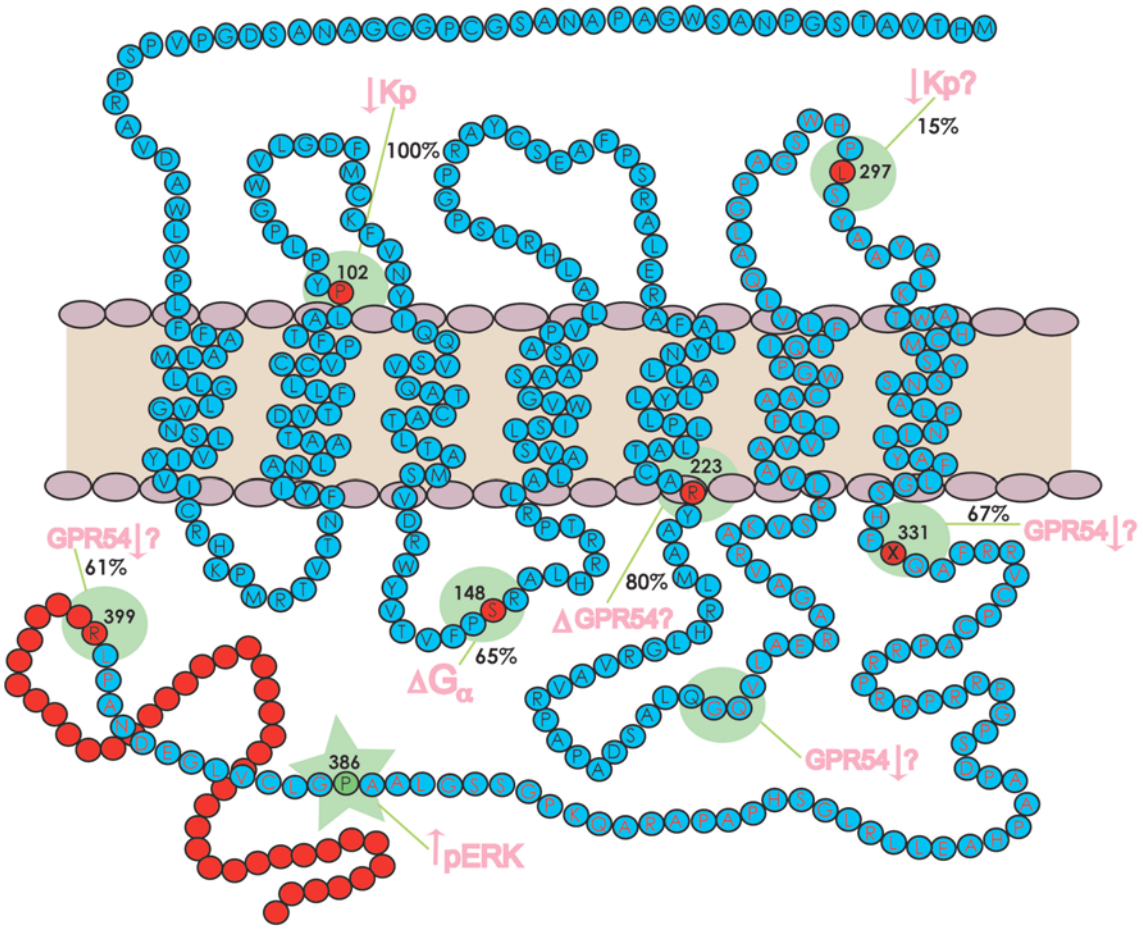

Figure 1.4 Summary of human GPR54 mutations and their effects

Green circles represent inactivating mutations while the green star represents the activating mutation. Red amino acids represent the specific deactivating mutation within the protein sequence while the green amino acid represents the activating mutation. Pink text and percentage represents the effect of the mutation and by how much the activity of GPR54 has been affected when known. Δ represents conformational change, downward arrows represent decrease in either Kp binding or GPR54 production, upward arrow represent prolonged pERK activity (Modified from review article [104]).

1.2.6 Circulating kisspeptins

Kp was discovered as the ligand for GPR54 after being isolated from placental fractions and both ligand and receptor are highly expressed in the placenta [2, 7, 8, 14]. Originally, Kp and GPR54 were found to be more highly expressed in early pregnancy as compared to term placentas [12, 105], but this has since been refuted [106, 107]. Kp and GPR54 have decreased expression in more invasive choriocarcinoma cell lines as compared to healthy placenta or molar pregnancies [105]. Since Kp has the protein sequence of a secreted peptide, it follows that one would try to measure Kp in a blood sample. Although Kp is barely measurable in men and non-pregnant women, it is at 10,000 fold higher in plasma of pregnant women, reaching its highest levels by the third trimester of pregnancy [106, 107]. It is also elevated in the umbilical cord blood of newborns though not as greatly as the maternal plasma [108]. Although, Horikoshi et al. [107] showed by RNA in situ hybridization and IHC that Kp is expressed in term placenta, circulating Kp quickly falls back to normal levels after birth.

The ability to measure Kp in blood samples led to the exploration of Kp as a serum biomarker for disease. Small for gestational age babies (SGA) had lower levels of metastin in their mother's serum as compared to an equal number of control normal pregnancies [109]. β-HCG levels suggested that the SGA was not due to a decrease in placental size and therefore a possible cause of SGA could be a decreased in invasive ability of the placenta. This would support the role of Kp and GPR54 as regulators of trophoblast invasion during pregnancy.

With respect to cancer, Kp was measured in plasma of women with gestational trophoblastic neoplasia, a cancer of placental origin, and were shown

to have increased Kp [106]. This increased Kp dropped back to normal physiological levels once the tumour had been removed surgically and the patient had undergone chemotherapy. The disadvantage in this instance is that hCG is already an establish serum marker for the diagnosis and follow-up of this disease. Since many tumours have been documented to express Kp, it is surprising there have not been more publications demonstrating increased Kp levels in cancer patients.

One would assume that since it is a normal physiological event to have extremely high levels of Kp in serum during pregnancy that there should be no adverse consequence to this phenomenon. In 2005 the first study to inject Kp into humans was published. The study showed that upon intravenous injection of Kp into healthy adult male volunteers, LH, FHS and testosterone levels increased while sex hormone binding globulin (SHBG) and Inibin B levels were unaffected. Most importantly there were no reports of any side effects resulting from the treatment [17]. A similar study was conducting in pre-menopausal healthy females with Kp injected as a single bolus in the abdomen as compared to the iv infusion in men. Plasma kisspeptin levels do not vary throughout the menstrual cycle, however with the injection of Kp there were increased levels of LH and FSH, but the most significant increase for both gonadotropins occurred during the preovulatory phase. Estrogen and progesterone were significantly increased after Kp injection in the luteal phase only [18]. Again there were no adverse affects from the Kp injection.

Since the human injection studies, the same group determined that Kp injection into male rats causes testicular degeneration [15]. Originally thought to be a consequence of prolonged exposure to high Kp levels, it was recently determined that a single peripheral bolus of Kp could cause testicular damage as early as 12 hours post injection. Interestingly, no other tissues were harmed and a similar effect is seen when rats are injected with GnRH [110]. This effect was prevented when mice were pretreated with a GnRH receptor antagonist, suggesting these adverse effects were a result of overstimulation of the hypothalamic-pituitary-axis. This raises the importance of properly assessing suitable Kp concentrations when considering any further human injections, as testicular degeneration could be avoided with still maximal gonadotropin release by adjusting the dose of Kp.

1.3 Hypotheses and Specific Aims

1.3.1 Hypotheses

(i) Expression of GPR54/kisspeptin in epithelial malignancies is predictive of disease outcome and (ii) altering endogenous GPR54 signalling in malignant epithelial cells could alter their metastatic properties.

1.3.2 Specific Aims

(i) The first aim was to determine the clinical significance of Kp and GPR54 expression in cancer. Although there is extensive literature documenting *GPR54*

and *KiSS1* RNA expression in a variety of tumours, there have been few publications based on the immunohistochemical analysis of these two proteins. These results are presented in Chapters 2 and 3. Chapter 2 focuses on the exploration of Kp and GPR54 expression across a panel of cancers while Chapter 3 focuses specifically on Kp and GPR54 expression in ovarian cancer and the related clinical outcome of these patients.

Following the discovery that Kp and GPR54 could be detected in a variety of tumours, we set out to determine if Kp overexpression could then be measured in the plasma of cancer patients. Chapter 4 discusses these results and further explores the initial findings from Chapter 3.

(ii) The second aim was to find breast and/or ovarian cell lines endogenously expressing and signalling through GPR54, to examine the consequences of abrogating epithelial signalling. Previous studies on the anti-metastatic effects of Kp-GPR54 signalling in malignant cells have not addressed endogenous signalling in breast or ovarian cancer. Chapter 5 addresses these findings, with respect to GPR54 expression and signalling in breast and ovarian cells lines, and the issues we encountered while trying to establish this model.

(iii) The third and final aim was to uncover gene loci whose regulation may depend directly on GPR54 signalling in a physiological context. The original objective was to determine whether there are common genes regulated by GPR54 signalling in malignant cells, in comparison with physiological GPR54

signalling in the hypothalamus. To discover the hypothalamic gene loci, we conducted differential transcript analysis of the GPR54 and kisspeptin knockout mice hypothalamus. Chapter 6 describes candidate GPR54 regulated genes that were discovered from an Affymetrix and large-scale real-time PCR analysis of microdissected hypothalamic tissue. This chapter also addresses experimentally which transcripts are hormonally regulated and which are independent of hormonal action.

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2 *GPR54* AND *KISS1* EXPRESSION IN CLINICAL CASES: ASSESSMENT OF PROSTATE, TESTICULAR, BREAST, AND OVARIAN CANCER TISSUE MICROARRAYS[1]

Objective: To analyze GPR54 and kisspeptin by immunohistochemistry in a variety of cancers and determine any prognostic significance using linked clinical outcome data when available.

[1] A version of this chapter will be submitted for publication. Prentice LM, Leung S, Köbel M, McKinney S, Goktepe O, Rajput A, Mehl E, Gilks CB, Huntsman DG, and Aparicio SAJ. *GPR54* and *KiSS1* expression in clinical cases: assessment of prostate, testicular, breast, and ovarian cancer tissue microarrays.

2.1 Introduction

With the discovery of the *KiSS1* gene as a metastasis suppressor, many researchers set out to explore *KiSS1* expression in a multitude of cancers to determine if the kisspeptin (Kp) protein could be used as a prognostic biomarker. Similarly, expression of the Kp receptor, GPR54, was explored alongside Kp in several studies. The difficulty in achieving this aim was establishing specific antibodies for both Kp and GPR54. Therefore many of these studies utilized RNA *in situ* hybridization or real-time PCR (QPCR) as an alternative to immunohistochemical (IHC) analysis.

The normal physiological function of Kp and GPR54 is to regulate the hypothalamic-pituitary-gonadal axis, however both are expressed in tissues outside of the hypothalamus. *KiSS1* is normally expressed at low levels in the testes, liver, pancreas, and small intestine and highly expressed in the placenta [1, 2]. GPR54 is also most highly expressed in the placenta but also has high expression in the pituitary and pancreas. GPR54 is expressed at lower levels in the testes, spleen, thymus, and lymph nodes [1-3].

To assess the prognostic significance of this ligand-receptor pair in clinical cases, *KiSS1* and *GPR54* expression has been examined in a variety of cancers. In almost all cases loss of Kp and/or GPR54 has been correlated with poor prognosis or advanced disease. This finding has been true for gastric, esophageal, bladder, melanoma, pancreatic, thyroid, renal and breast cancer [4-11]. The one anomaly has been hepatocellular cancer where a more advanced

disease was associated with overexpression of *KiSS1* and *GPR54* as determined by QPCR [12].

Using immunohistochemistry and tissue microarray technology (TMA), we assessed Kp and GPR54 expression in cancers that had not been previously evaluated: ovarian, testicular, and prostate. We also assessed a large 438 case breast cancer TMA with clinically linked outcome data to assess prognostic significance. Fluorescent *in situ* hybridization was also employed to determine gene copy number as a possible mechanism for *KiSS1* and *GPR54* expression in the breast and ovarian cancer tissue microarrays.

2.2 Material and Methods

2.2.1 Tissue microarrays

Breast cancer array

The breast cancer array was constructed using formalin-fixed, paraffin embedded tissue blocks received from the Department of Pathology at Vancouver General Hospital during the period 1974-1995. Case selection and tissue microarray construction have been described previously [13-15]. Briefly, representative areas of carcinoma were selected and marked on the hematoxylin and eosin slides and corresponding tissue blocks for TMA construction. The TMAs were assembled using a tissue-arraying instrument (Beecher Instruments, Silver Springs, MD), with two 0.6 mm cores per case. Outcome data were available for all 438 patients, with median follow-up of 15.4 years (range 6.3-26.6 years). Ethical approval was obtained to perform this study from the Clinical Research

Ethics Board of the University of British Columbia. The clinicopathological characteristics of the cohort are listed in Table 2.1.

Prostate cancer array

The prostate array consisted of 84 quadruplicate 0.6 mm cores obtained from formalin-fixed paraffin embedded tissue blocks as previously described [16]. The samples were obtained from radical prostatectomies from non-treated patients of varying Gleason grade as reviewed by a pathologist. The clinicopathological characteristics of the cohort are listed in Table 2.2.

Germ cell testis array

Duplicate 0.6 mm cores from paraffin embedded germ cell tumours (33 seminomas, 23 pure embryonal carcinomas and 10 mixed embryonal carcinomas) obtained from the Brigham and Women's Hospital were used to create the testicular TMA under the Harvard Medical School Institutional Review Board guidelines [17]. The clinicopathological characteristics for this cohort were unavailable.

Ovarian array

This small ovarian tissue microarray consists of 49 ovarian cancer samples that were obtained from patients who underwent debulking surgery from January 2004 until September 2005 at Vancouver General Hospital and the British Columbia Cancer Agency. Patients treated with pre-operative chemotherapy

Table 2.1 Clinicopathological characteristics of the breast TMA

Parameter	N
Histology	
Invasive Ductal	372
Invasive Lobular	40
Node	
Negative	236
Positive	134
Grade	
1	84
2	219
3	100
Biomarkers	
ER <1%	78
ER >=1%	287
Her2 neg	240
Her2 weak	69
Her2 strong	35
Tumour Size	
<=2cm	185
>2cm and <=5cm	186
>5cm	34
Age (years)	
Mean (SD)	60.3 (14.1)

Table 2.2 Clinicopathological characteristics of the prostate TMA

Parameter	N
Histology	
BPH	16
GL3	38
GL4	7
GL5	23
Clinical Stage	
T1 a, b, c	30
T2 a, b	9
T3 a, b	1
Pathological Stage	
T2 a, b	54
T3 a, b	10
Age (years)	
Median	66
Range (Min – Max)	46-89

were omitted [18]. TMA construction was similar to that described in the breast cancer array and ethical approval was obtained from the University of British Columbia Ethics Board. The clinicopathological characteristics of the cohort are listed in Table 2.3.

2.2.2 Manual kisspeptin immunohistochemistry

For the breast cancer TMA, kisspeptin immunohistochemistry (IHC) was performed manually. Sections from formalin-fixed and paraffin-embedded tissues were deparaffinized with xylene and rehydrated with a graded series of alcohols. Wet heat-induced antigen retrieval was performed in a steamer for 20 min with a modified citrate buffer (pH 6.1, Dako, Mississauga, ON). Following antigen retrieval, sections were treated with 3% hydrogen peroxide (H_2O_2) in phosphate buffered saline (PBS) for 30 min to quench endogenous peroxidase activity. All of the aforementioned steps were followed by three washes with PBS for 5 min each. Slides were subsequently blocked for 30 min with serum-free protein block (Dako) and incubated overnight at 4°C with a polyclonal goat anti-KiSS-1 antibody (C-20, Santa Cruz Biotechnology, Santa Cruz, CA) diluted 1:400 in serum-free protein block. Kisspeptin immunoreactivity (IR) was detected with the CSA II biotin-free tyramide signal amplification system and 3,3'-diaminobenzidine chromogen solution (Dako). Specifically, rabbit anti-goat-horseradish peroxidase (HRP) was applied for 15 min followed by fluorescyl-tyramide amplification reagent for 15 min and anti-fluorescein-HRP for 15 min. All of the steps subsequent to the incubation with primary antibody were followed by three

Table 2.3 Clinicopathological characteristics of the ovarian TMA

Parameter	N
Histopathological Subtype	
Serous	40
Endometrioid	6
Clear Cell	4
Serous Borderline	1
Grade	
1	4
2	7
3	39
Stage	
1	7
2	7
3	30
4	7
Age (years)	
Mean (SD)	59.5 (12.2)
Median	57.0

washes with tris-buffered saline containing 1% Tween (TBST) for 5 min each. Slides were counterstained with Harris hematoxylin (Sigma-Aldrich, Oakville, ON) and mounted in a xylene-based mounting medium. Based on previously published data showing cell-type restriction of GPR54 and kisspeptins in different trophoblast layers of human placenta (Bilban 2004), less than 10-week old human placenta was used as a specificity control (courtesy of Vancouver Coastal Health archives), in conjunction with two blocking peptides (21 residues and 54 residues). Omission of the primary antibody was used as a negative control.

2.2.3 Automated GPR54 and kisspeptin immunohistochemistry

All of the TMAs were stained using an automated system for both GPR54 and Kp IHC, except for the breast TMA that is described above. TMA 4 μm sections were processed using a Ventana Discovery XT automated system (Ventana Medical Systems, Tucson, AZ) as per manufacturer's protocol with proprietary reagents. After slides were baked at 60°C for 1 h, they were deparaffinized on the automated system with EZ Prep solution (Ventana). Heat-induced antigen retrieval method was used in Cell Conditioning solution (CC1- Tris based EDTA buffer, pH 8.0, Ventana). The polyclonal rabbit GPR54 antibody was obtained from MBL International Corporation (Woburn, MA) specific for the N-terminal extracellular domain (catalogue number LS-A1929) and used with heat at a 1:25 concentration in Ventana antibody diluent. The polyclonal goat KiSS1 antibody from Santa Cruz (Santa Cuz, CA) was incubated for 2 h without heat at 1:10 dilution. For GPR54, the Ventana Universal Secondary Antibody was used for 32

min at 37°C, while a rabbit anti-goat (Jackson ImmunoResearch, West Grove, PA) at 1:500 dilution was used for KiSS1. The detection system used for GPR54 was the Ventana DABMap kit, while the Ultra detection system (Ventana) was used for KiSS1. All slides were counterstained with Hematoxylin and treated with a proprietary bluing agent (Ventana) and washes were conducted with the Ventana Reaction Buffer. Dehydration steps and coverslip procedure were completed manually as per manufacturer's recommendations. Specificity was determined by Western blot for GPR54 and by using less than 10-week old human placenta as a positive control and omission of primary antibody as a negative control for both antibodies.

2.2.4 IHC scoring

Kp and GPR54 IHC scoring was assessed by a pathologist (Martin Köbel or Ashish Rajput) for all tissues except Kp in the ovarian carcinomas. For all TMAs, GPR54 was scored as 0 for tissues with fewer than 5% of staining, +1 for tissues that had at least 5% of tumour cells with dark staining, and +2 for tissues that had greater than 75% of tumour cells with dark staining. For all the TMAs except the small ovarian cancer array, Kp was scored at 0 for completely negative tissues, +1 for any detectable staining, and +2 for intense signal. For the ovarian cancer array, Kp was scored solely by the author (Leah Prentice) without the benefit of a trained pathologist and was considered 0 for completely negative tissues, +1 for very weak staining, +2 for moderate staining, and +3 for intense staining.

2.2.5 Photomicrographs

The TMAs were digitally scanned with a BLISS (Bacus Laboratories Inc., Slide Scanner) automated system (Bacus Laboratories, Lombard, IL) as previously described [19]. These images are publically available on our webslide server: http://www.gpecimage.ubc.ca/tma/web/viewer.php.

2.2.6 Fluorescent in situ hybridization (FISH)

Six mm thick sections of the TMAs were treated for fluoresecent in situ hybridization (FISH) as described elsewhere [20, 21]. Locus specific FISH analysis for *KiSS1* and *GPR54* were performed using the BACs CTD-3195E18, CTD-2582H10, and CTD3009K5 for *GPR54* and RP11-673P13, CTD-2145H2, and RP11-203F10 for *KiSS1* (BACPAC Resources Centre, Children's Hospital Oakland Research Institute). All BACs were directly labeled with Spectrum Green using a Nick translation kit (Vysis, IL, USA), and validated for specificity using normal metaphases from human blood cells. A centromeric probe for chromosome 1 and a telomeric probe control for 19q both labeled in spectrum orange were used as controls for aneusomy for *KiSS1* and *GPR54* respectively (Vysis, IL, USA). FISH was performed using Vysis reagents according to the manufacturer's protocols (Vysis, IL, USA) and slides were counterstained with 4,6-diamidino 2-phenylindole (DAPI). FISH signals and patterns were identified on a Zeiss Axioplan epifluorescent microscope and were scored either manually (oil immersion 100X) or using Metasystems Metafer software (MetaSystems Group, Inc. Belmont, MA) and enumerated in approximately 40 morphologically

intact and non-overlapping nuclei. Amplification was defined as an amplification ratio of 1.5 or greater, and loss was defined as a ratio of 0.6 or less.

2.2.7 Reverse transcription

The ovarian TMA had matched RNA available from Press et al. [18] for most of the cases. One microgram of RNA was DNAse treated prior to reverse transcription to eliminate any contaminating genomic DNA from the sample. Samples were incubated at RT with DNAse I (Invitrogen, Carlsbad, CA) for 15 min before the addition of 25 mM EDTA (Invitrogen, Carlsbad, CA) and heated to 65°C for 10 min to stop the reaction as per manufacturer's recommendation. Samples were then immediately put on ice to cool. Random hexamers at 250 ng/ul (Invitrogen, Carlsbad, CA), 10 μM dNTPs (Invitrogen, Carlsbad, CA), 400 U MMLV-Reverse transcriptase enzyme (Invitrogen, Carlsbad, CA), 0.1 M DDT (Invitrogen, Carlsbad, CA), with the accompanying reverse transcriptase buffer (250 mM Tris-HCI pH 8.3, 375 mM KCl, 15 mM $MgCl_2$), 8 U RNase OUT enzyme, and water were added to the DNAse treated samples. Samples were incubated at 23°C for 10 min then incubated at 37°C for 1 h, and the reaction was stopped by a 10 min 65°C incubation step.

2.2.8 Quantitative real-time PCR (QPCR)

The Applied Biosystems (ABI) 7900HT Fast Real-Time System was used to amplify and detect *KiSS1* and *GPR54* mRNA transcripts (Applied Biosystems, Foster City, CA). Gene specific probe and primer sets were ordered as 20x target

assays from ABI specifically covering exon boundaries for human *KiSS1* (Hs00158486_m1) and *GPR54* (Hs00261399_m1). The 18s rRNA subunit (Hs99999901_s1) expression levels at 1:250 dilution were used as an endogenous loading control before comparative normalization. Using RNA generously donated by Dr. Lindsay Brown, *KiSS1* expression was normalized to RNA from IOSE epithelial ovarian cells using relative quantification. *GPR54* expression was normalized to RNA from MDA-MB-231 cells, as IOSE cells did not express GPR54.

2.2.9 Statistics

For the breast TMA Kp and GPR54 correlations with clinicopathological markers and survival, were assessed using statistical software SPSS 16.0. For boxplots demonstrating the *GPR54* and *KiSS1* gene copy number and IHC relationship, R 2.8 software was used. For the small ovarian TMA, Kp and GPR54 continuous variables (QPCR results) were compared by linear regression and compared to discrete variables (FISH ratios, IHC scores) using Fischer's ANOVA (analysis of variation). Discrete variables compared to discrete variables were analyzed by Pearson's chi-square.

2.3 Results

2.3.1 GPR54 is more highly expressed in the more benign subtype of prostate and testicular cancers

We assessed a prostate tissue microarray for Kp and GPR54 expression by immunohistochemistry. Contained within the prostate array were cases of benign prostatic hyperplasia (BPH) as well as cases that ranged in Gleason score from GL3-GL5. Of these cases, GPR54 was most frequently and most highly expressed in the BPH (85% of cases had an IHC score of +2). There was no discernable difference between the remaining Gleason grades, nor was there any detectable differences between the kisspeptin expressing cases (Figure 2.1 A). The lack of any obvious expression pattern within subtype for Kp could also be due to the lack of Kp positivity in this tissue. Of 72 scorable cases, only 6 had any detectable Kp (8%).

The testicular array consisted of germ cell tumours, either seminomas, or nonseminomas (embryonal mixed or embryonal pure). Of these cancers, the seminomas have a better prognosis and 32/33 (97%) cases had high GPR54 expression (IHC score of +2). KiSS1 was also most highly and most frequently expressed in this subtype with an average IHC score of +1 (Fig 2.1 B). Overall, Kp and GPR54 were more frequently and highly expressed in the testicular cancers rather than the prostate cancers.

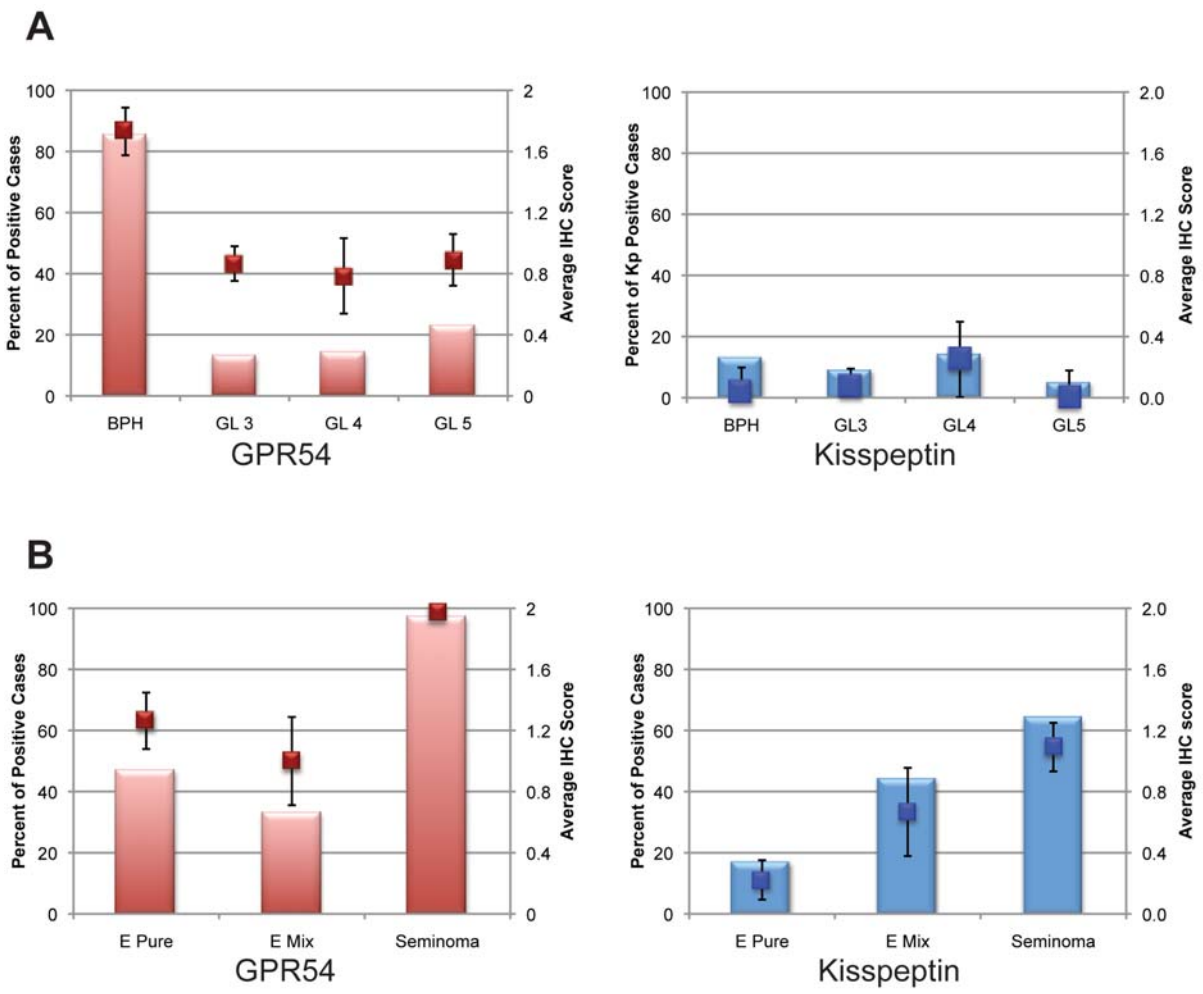


Figure 2.1 GPR54 and Kp expression in male cancers

Frequency of expression is measured on the left y-axis as percent of positive cases represented by bars, while strength of expression is the average IHC score within a particular subtype measured by the right y-axis and is represented by squares. A) Prostate cancer TMA. B) Germ cell TMA (GPR54 in red on left side; Kp in blue on right side). Error bars for the average IHC scores represents the standard error across all samples within a particular subtype. E=embryonal.

2.3.2 Loss of GPR54 in breast cancer is a poor prognostic marker in node positive patients.

To assess whether Kp and GPR54 expression have prognostic significance, a breast cancer TMA with linked clinical outcome data was used. Both Kp and GPR54 were grouped individually into negative and positive cases based on their IHC expression. As GPR54 is normally expressed in breast tissue (Fig 2.2 A), the negative expression group consisted of cases with an IHC score of 0 and was considered as GPR54 loss. The GPR54 positive group consisted of the remaining +2 and +1 IHC cases. Kp is not ordinarily expressed in breast tissue (Figure 2.2 A), and those with an IHC score of +2 were considered as positive. The remaining 0 and +1 cases were grouped and classified as Kp negative.

Loss of GPR54 was significantly associated with poor prognosis in node positive patients in overall and disease specific survival (Figure 2.2 B), but this did not maintain significance within multivariable analysis against age at diagnosis and Her2 positivity (Table 2.4). Though Kp expression alone did not correlate with survival, breast cancer cases expressing both Kp and GPR54 have a significantly more favourable prognosis when compared to those that had both loss of Kp and GPR54 in disease specific survival (Figure 2.2 C).

Kp and GPR54 expression did not significantly correlate with any other clinicopathological markers such as estrogen receptor status, or Her2 expression after a correction for multiple comparisons (Tables 2.5 and 2.6 respectively). There was a slight positive correlation between the highest Kp and GPR54 expressors (+2) (Kendall's tau-b= 0.199, p=0.0013, Table 2.5).

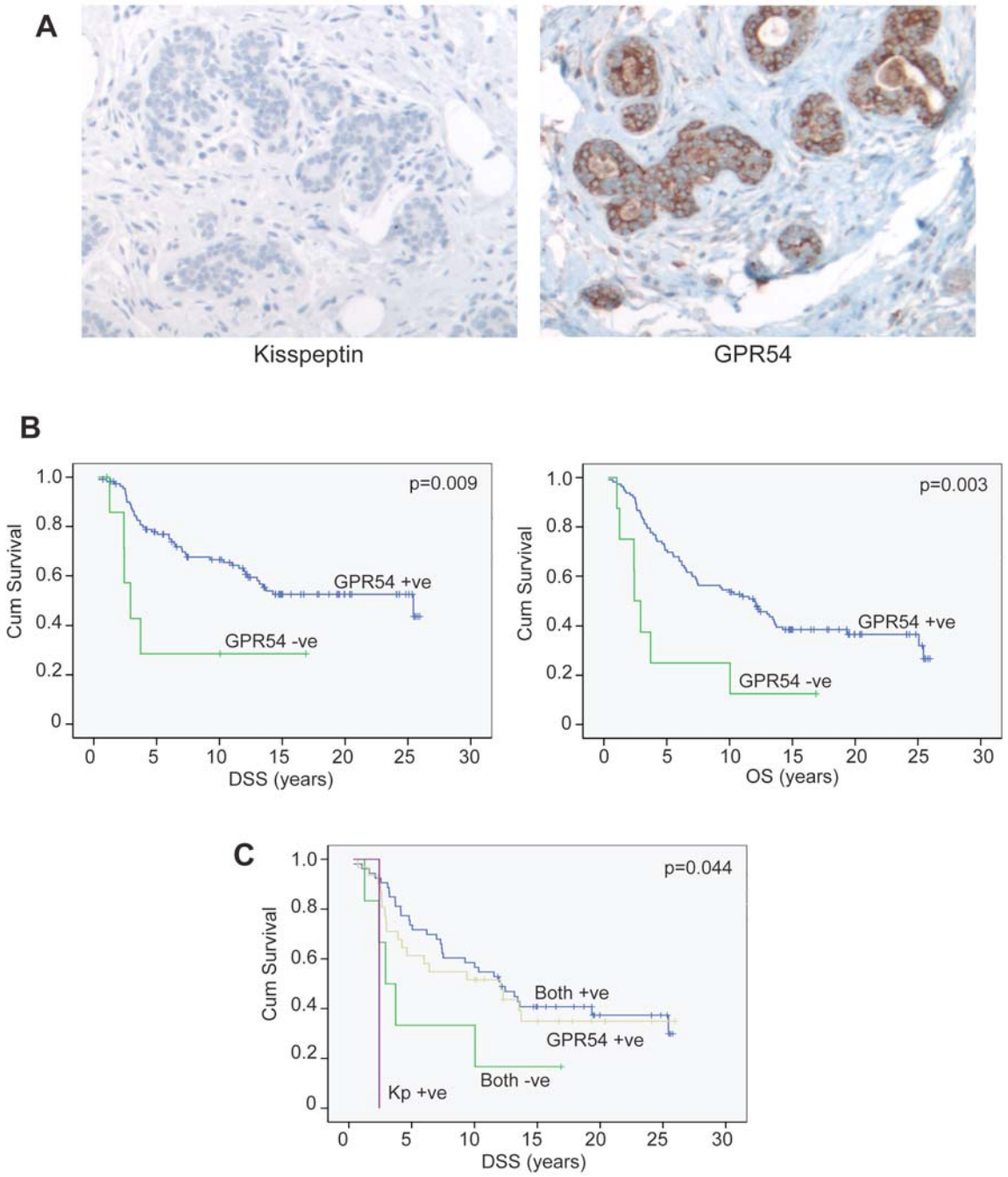


Figure 2.2 Kp and GPR54 expression in breast cancer

A) Representative micrographs of Kp (left) and GPR54 (right) expression in normal human breast tissue. B) Kaplan-Meier survival plots with cumulative survival on the y-axis and time in years on the x-axis for disease specific (DSS) and overall survival (OS). C) Kaplan-Meir survival plot including both Kp and GPR54.

Table 2.4 Multivariable analysis in node positive breast cancer patients

Source	Hazard Ratio	95% CI L	95% CI U	p-value
GPR54	0.828	0.291	2.352	0.723
Grade	0.941	0.545	1.625	0.827
ER	0.973	0.370	2.557	0.955
Age at Dx	1.030	1.008	1.053	0.007
Tumour Size	1.089	0.896	1.324	0.390
Her2	9.627	2.940	31.524	0.000

Table 2.5 Kp IHC correlation with clinicopathological markers

Biomarker	Kendall's tau-b	Raw p-value
GPR54 [0,1] vs. [2]	0.199	0.001*
KI67 cut off at 13.5%	-0.136	0.028
Grade [1,2] vs.[3]	-0.117	0.051
Her2 [0,1] vs. [2]	-0.120	0.073
PR cut off at 1%	0.081	0.240
BCL2 [0] vs. [1,2]	0.076	0.241
ER cut off at 1%	0.060	0.408
Age cut off at 50 yrs	0.051	0.456
CK5/6 [0] vs. [1,2]	-0.041	0.578
KISS1 FISH cut off at 1.5	-0.053	0.592
EGFR [0] vs. [1,2]	-0.031	0.685
Nodal status	-0.022	0.777
Tumor size cut off at 2 cm	0.012	0.894

*Maintains significance with consideration for multiple comparisons.

Table 2.6 GPR54 IHC correlations with clinicopathological markers

Biomarker	Kendall's tau-b	Raw p-value
Tumor size cut off at 2 cm	-0.116	0.036
PR cut off at 1%	-0.093	0.165
KI67 cut off at 13.5%	0.076	0.207
EGFR [0] vs. [1,2]	0.070	0.331
Grade [1,2] vs.[3]	0.046	0.492
Nodal status	0.031	0.492
BCL2 [0] vs. [1,2]	-0.043	0.497
Her2 [0,1] vs. [2]	-0.025	0.718
ER cut off at 1%	0.009	0.809
Age cut off at 50 yrs	-0.028	0.820
CK5/6 [0] vs. [1,2]	0.003	1.000
GPR54 FISH cut off at 1.4	0.062	1.000

2.3.3 GPR54 and Kp expression is not mediated through gene copy number in breast cancer

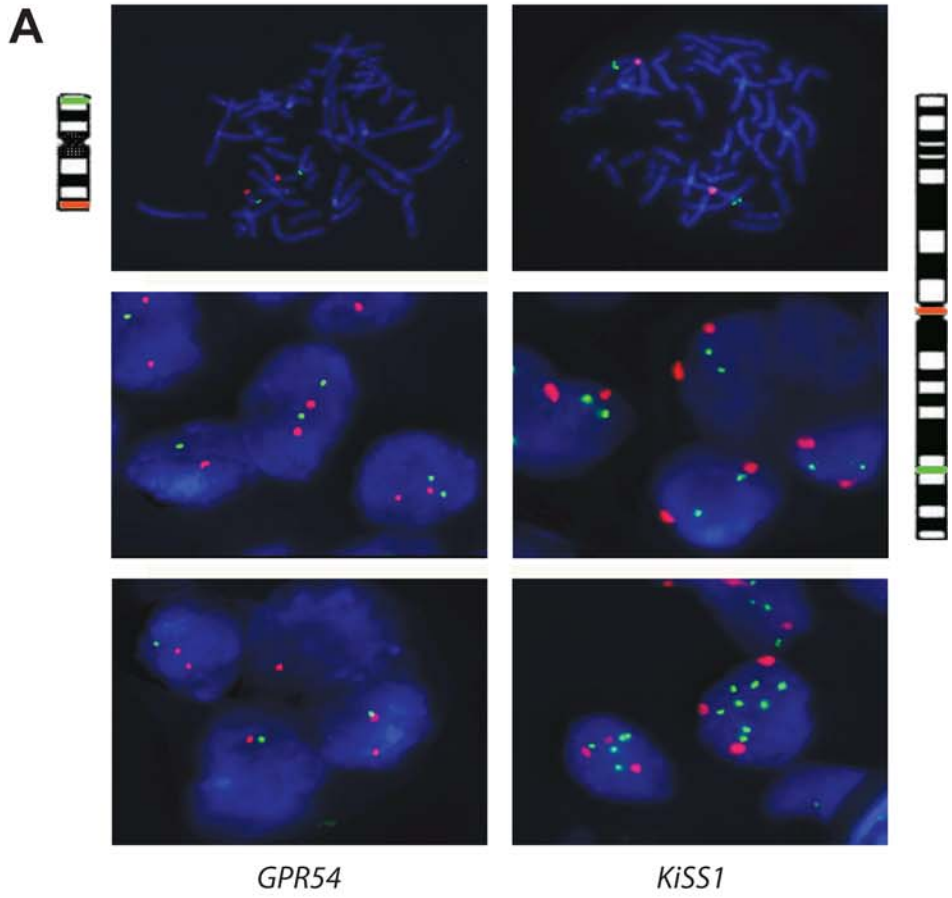
To assess gene copy number as a possible mechanism for either increase or loss of Kp and/or GPR54 expression, a fluorescent *in situ* hybridization (FISH) assay was established and applied to the breast cancer TMA (Figure 2.3 A). Gene copy number is compared to a reference probe, in this instance either centromere 1 for *KiSS1* or telomere 19q for *GPR54*, to take into account any abnormal gene copy number that may be due to chromosomal aneuploidy. For *GPR54* analysis there were 191 breast cancer cases with scorable results. Of these, 27 (14%) had loss of *GPR54* using a ratio of 0.6 or less, and 4 had gain of *GPR54* using a ratio of 1.5 or greater. For the *KiSS1* locus, 29 of 233 scorable cases were amplified (12.4%) and one had loss of gene copy number, using the same ratio parameters described for *GPR54*. *KiSS1* amplification ratio did not correlate with Kp expression (Spearman's ρ=-.05, p=0.529), while surprisingly *GPR54* gene copy number had a slight negative correlation with the IHC results (ρ=-.195, p=0.019; Figure 2.3 B). For *GPR54*, gene copy number was used rather than a ratio of loss to take into account any low ratios arising from abnormal chromosomal gain.

2.3.4 GPR54 and Kp expression is not mediated through gene copy number in ovarian cancer

Using a small ovarian cancer array, *GPR54* and *KiSS1* gene copy number was determined by FISH, mRNA expression was determined by real-time PCR, and protein expression was determined by IHC (Tables 2.7 and 2.8 respectively).

Figure 2.3 *GPR54* and *KiSS1* gene copy number in breast cancer

A) Fluorescent in situ hybridization (FISH) was used to determine gene copy number for *GPR54* (left) and *KiSS1* (right). Top panel shows gene expression in normal metaphase demonstrating probe specificity; middle panel shows normal gene copy number in tumours. Bottom panel shows loss for *GPR54* (green) as compared to 19q telomeric control (orange), while KiSS1 (green) is amplified compared to centromere 1 control (orange). Locations of the probes are shown on the corresponding idiograms of the chromosome being studied. B) Boxplots comparing gene copy number for *GPR54* with IHC score (left) or gene ratio for *KiSS1* with IHC score (right).


A
GPR54
KiSS1

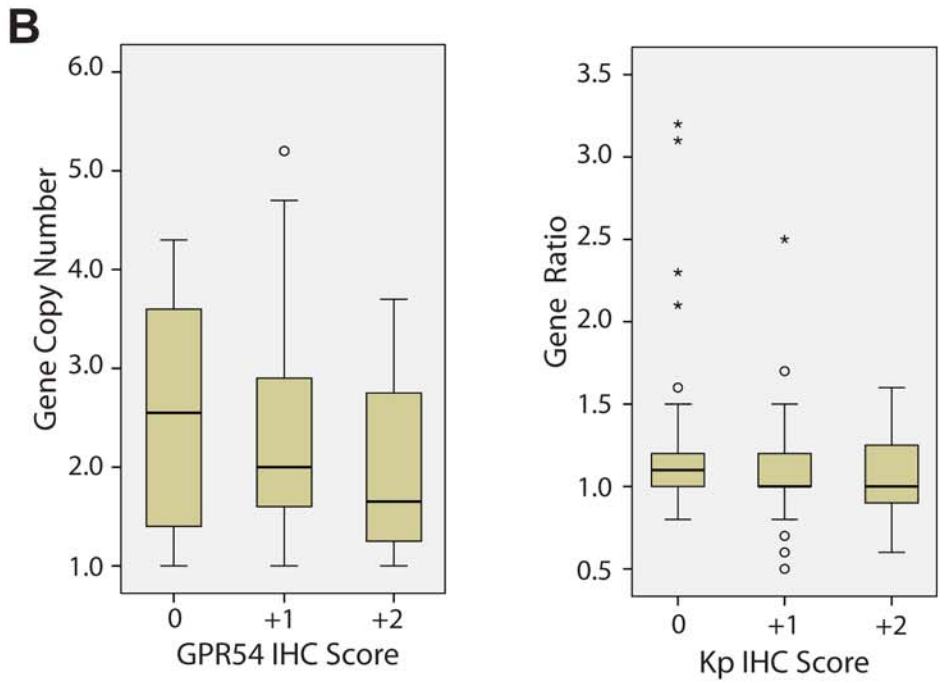

B
Gene Copy Number
6.0
5.0
4.0
3.0
2.0
1.0
0
+1
+2
GPR54 IHC Score
Gene Ratio
3.5
3.0
2.5
2.0
1.5
1.0
0.5
0
+1
+2
Kp IHC Score

Table 2.7 *GPR54* gene ratio and expression in ovarian cancer

Tumor	QPCR	FISH Ratio	IHC	Histopathology
198	56.16	x	x	Clear cell
213	0.66	x	x	Clear cell
219	8.65	x	x	Clear cell
392	1.04	x	2	Clear cell
242	1.12	1.0	2	Endometrioid
281	9.43	x	2	Endometrioid
334	3.28	1.0	2	Endometrioid
156	0.91	1.1	0	Endometrioid
343	x	x	x	Endometrioid
172	0.45	1.1	2	Mixed carcinoma
186	0.46	1.1	2	Mixed carcinoma
240	2.73	0.8	2	Mixed carcinoma
327	0.98	x	1	Mixed carcinoma
280	0.21	1.5	0	Poorly differentiated
336	0.04	1.0	2	Poorly differentiated
161	0.96	x	2	Poorly differentiated
201	3.55	x	1	Poorly differentiated
208	0.37	x	x	Poorly differentiated
273	7.09	x	0	Poorly differentiated
305	0.69	x	2	Poorly differentiated
363	0.06	0.4	2	Primary peritoneal
221	7.27	1.1	2	Serous
324	1.17	x	2	Serous
163	2.46	0.9	1	Serous
178	1.56	1.0	1	Serous
195	4.47	2.0	1	Serous
212	1.38	1.2	2	Serous
217	0.79	1.1	0	Serous
223	2.22	1.1	2	Serous
229	0.41	0.6	0	Serous
236	0.10	x	x	Serous
239	1.82	1.1	2	Serous
254	0.04	x	1	Serous
283	1.99	x	2	Serous
293	0.39	0.5	2	Serous
297	0.91	x	x	Serous
309	0.67	x	x	Serous
319	1.36	x	1	Serous
330	1.46	0.6	2	Serous
332	0.62	1.0	2	Serous
344	1.37	1.1	2	Serous
345	0.92	x	2	Serous
366	0.31	x	2	Serous
372	6.00	1.1	0	Serous
379	0.96	x	2	Serous
384	6.50	x	x	Serous
388	13.68	1.0	1	Serous
394	x	0.6	2	Serous
329	0.03	0.9	2	Serous

Table 2.8 *KiSS1* gene ratio and expression in ovarian cancer

Tumor	QPCR	FISH Ratio	IHC	Histopathology
198	0.05	0.7	3	Clear cell
213	0.59	x	3	Clear cell
219	1.66	x	3	Clear cell
392	1.16	1.6	3	Clear cell
242	1.77	x	1	Endometrioid
281	9.27	1.1	2	Endometrioid
334	0.33	1.0	1	Endometrioid
156	7.44	x	1	Endometrioid
343	0.21	x	0	Endometrioid
172	7.20	1.7	3	Mixed carcinoma
186	x	1.0	2	Mixed carcinoma
240	1.20	1.1	2	Mixed carcinoma
327	1.36	1.1	0	Mixed carcinoma
280	2.25	0.9	0	Poorly differentiated
336	2.62	0.8	1	Poorly differentiated
161	5.12	x	1	Poorly differentiated
201	2.68	x	0	Poorly differentiated
208	0.04	x	1	Poorly differentiated
273	3.27	x	0	Poorly differentiated
305	1.69	1.1	2	Poorly differentiated
363	0.28	0.7	0	Primary peritoneal
221	0.92	1.2	0	Serous
324	0.75	0.9	0	Serous
163	1.42	1.4	0	Serous
178	0.94	0.5	0	Serous
195	x	0.9	0	Serous
212	0.30	1.5	0	Serous
217	3.02	1.0	0	Serous
223	0.77	0.9	1	Serous
229	3.01	0.8	0	Serous
236	0.34	x	0	Serous
239	57.18	1.4	0	Serous
254	0.33	0.7	0	Serous
283	2.36	1.1	0	Serous
293	0.98	1.1	1	Serous
297	0.02	x	0	Serous
309	0.17	x	2	Serous
319	0.05	1.1	1	Serous
329	0.27	1.1	1	Serous
330	3.75	1.0	1	Serous
332	1.95	1.2	1	Serous
344	x	1.0	0	Serous
345	0.34	1.4	0	Serous
366	0.64	1.3	0	Serous
372	1.91	0.9	0	Serous
379	1.27	1.2	0	Serous
384	0.53	x	0	Serous
388	1.05	1.3	0	Serous
394	x	0.9	0	Serous

For *GPR54*, 5/26 cases had a FISH ratio of 0.6 or less, 14/47 had a QPCR fold change of 2 or greater, and 6/40 cases were negative for GPR54 protein (IHC score of 0). There was a slight yet significant positive correlation between FISH gene ratio and real-time PCR expression (r^2=0.167, p=0.03822). However there was no significant correlation between either gene ratio and IHC or QPCR and IHC. GPR54 IHC was only resolved in 1 of 4 clear cell cases and did not appear to favour any particular subtype.

For *KiSS1*, 3/36 cases showed gene amplification by FISH using a cut-off ratio of 1.5 or greater, 13/45 cases showed an expression fold change of 2 or greater for QPCR, and 22/49 cases showed Kp expression by IHC (IHC scores +1, +2, and +3 grouped). None of these parameters significantly correlated with one another, most specifically gene ratio with IHC. Of note, all four cases of the ovarian cancer clear cell subtype had the highest Kp IHC scores.

2.4 Conclusions

We have demonstrated for the first time that GPR54 is more highly expressed and at a greater frequency in benign prostate hyperplasia as compared to malignant disease. Benign prostatic hyperplasia results in the enlargement of the prostate that leads to urinary tract problems but is not believed to be a premalignant lesion [22]. Kp and GPR54 protein expression in normal prostate tissue is currently unknown, and therefore it cannot be determined if GPR54 expression is gained in benign prostatic hyperplasia or lost in prostate cancer. However, high GPR54 expression in benign disease while having lower

expression in malignant disease is consistent with the hypothesis that gain of expression is correlated with a more favourable phenotype. Comparatively Kp expression is a rare event in prostate disease.

Germ-cell cancers are the most common type of cancer in young men and are separated into seminomas and nonseminomas. Seminomas are generally confined to the testis and not considered as high risk. Conversely, half of the patients that are diagnosed with nonseminomas have metastatic spread [23]. Almost all the seminomas examined in this TMA had the highest GPR54 expression, only one case had moderate GPR54 expression. Kp also had the highest expression in seminomas though the results were not as striking as for GPR54.

Part of assessing testicular germ cell cancers for risk and confirming diagnosis is the association with serum markers. Nonseminomas may express α-fetoprotein (yolk sac tumours) or human chorionic gonadotropin (hCG; choriocarcinomas) and lactate dehydrogenase (LDH) though the latter is not specific to germ cell tumours. Seminomas have been shown to express hCG in plasma in 20% of patients. In general the higher the concentration of the serum marker the more advanced the cancer and the higher the stage [23]. hCG is also highly expressed in gestational trophoblast neoplasia (GTN), a malignant tumour of placental origin. This is not surprising as hCG is secreted from normal healthy placenta, as is Kp [24, 25]. It was recently discovered that Kp is also elevated in GTN and decreases during treatment until it reaches the normal concentrations once the tumour is completely removed [24]. Due to the similarity in Kp and hCG

expression in GTN, this raises the possibility that Kp may also be secreted from testicular germ cell tumours that secrete hCG. As Kp is more highly expressed in the seminomas, and least in the embryonal pure nonseminomas, it may be used as a plasma marker to distinguish between subtype of germ cell tumours and aid in determining treatment. This is an area of research yet to be assessed.

In breast cancer, loss of GPR54 correlates with poor prognosis in node positive patients. Node positivity is associated with increased risk for metastasis and metastasis is the main cause of death in breast cancer patients [26]. This correlation was significant in disease specific and overall survival but was not an independent marker when compared to other well established risk factors such as Her2 positivity. Due to this outcome and that Kp was insignificant in all analysis make this ligand-receptor pair a poor consideration as biomarkers in breast cancer. However, these findings support our hypothesis of GPR54 expression being associated with favourable outcome.

To elucidate a possible mechanism for Kp and GPR54 protein expression, we assessed gene copy number and RNA expression of this ligand-receptor pair. We were unable to find any significant correlation between DNA, RNA, and protein, however this is not the first report of unmatched expression. Specifically, *Kiss1* expression as determined by RNA *in situ* hybridization was localized in the arucate nucleus (ARC), the anteroventral periventricular nucleus (AVPV) and the periventricular nucleus (PeN) of the female mouse hypothalamus [27, 28], but only located in the AVPV and ARC by IHC [28]. Furthermore, *Kiss1* mRNA was

much lower and sometimes completely absent in the brain of rats as compared to Kp protein as determined by IHC [29].

Kp is a secreted protein and tissues with high RNA expression may be providing Kp for neighbouring tissues or for endocrine signalling. Depending on the speed at which Kp is manufactured and released from the cell, Kp protein may not be measureable in the Kp donating tissues and this may account for differences between RNA and protein expression. Alternatively, Kp has an extremely short half-life of 27.6 ± 1.1 min [30] in plasma, which may reflect a short half-life within tissues. Kp may be more rapidly degraded in formalin-fixed, paraffin embedded tissues used in TMAs as compared to frozen tissues that are used to provide RNA for the analysis.

For GPR54, a rapid change in gene expression may not be reflected immediately in protein concentration for a seven-transmembrane protein. The half-life of GPR54 has yet to be determined, but transmembrane proteins may be internalized and recycled back to the surface without actual degradation of protein thus increasing the half-life significantly [31]. Therefore it follows that there may be low levels of *GPR54* mRNA within tissues that have high levels of GPR54 protein. Further studies are required to definitively account for differences in RNA and protein expression.

We have also determined that gene copy number does not account for gain or loss of either Kp or GPR54 expression in breast and ovarian cancer. Gene copy number can account for increased protein expression as has been seen in HER2/ErbB2. HER2 is overexpressed in 15-25% of ductal breast cancers

and correlates with poor prognosis [32]. Clinically ErbB2 expression is detected by IHC and followed up by FISH when the initial results are unclear. If FISH results determine that Her2 is amplified, then treatment for the patient will follow the regiment of Herceptin treatment to block the ErbB2 receptor. However, gene copy number is not always responsible for protein expression as in the case of ERα [33].

This is the first time GPR54 and Kp expression has been determined by IHC in testicular, prostate, and ovarian cancer. Although previously assessed in breast cancer, this is the first report of expression determined in a large cohort with linked clinical outcome data. In all of these diseases, expression correlated with the characteristics linked to a more favourable prognosis. This research supports the current literature that expression of this ligand-receptor pair is a favourable prognostic marker. However, further studies are required before either Kp or GPR54 could be considered as reliable cancer biomarkers.

2.5 References

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3 KISSPEPTIN AND GPR54 IMMUNOREACTIVITY IN A COHORT OF 518 PATIENTS DEFINES FAVOURABLE PROGNOSIS AND CLEAR CELL SUBTYPE IN OVARIAN CARCINOMA[2]

Objective: To analyze GPR54 and kisspeptin by immunohistochemistry in ovarian cancer and determine any potential prognostic significance using linked clinical outcome data.

[2] A version of this chapter has been published. Prentice LM, Klausen C, Kalloger S, Kobel M, McKinney S, Santos JL, Kenney C, Mehl E, Gilks CB, Leung P, Swenerton K, Huntsman DG, and Aparicio SAJ. Kisspeptin and GPR54 immunoreactivity in a cohort of 518 patients defines favourable prognosis and clear cell subtype in ovarian carcinoma. BMC Med. (2007);5:33.

3.1 Introduction

The early diagnosis and management of ovarian cancer is a major area of unmet medical need. Central to the lack of progress in clinical management, has been the virtual absence of prognostic or predictive molecular markers for ovarian cancer. Key to addressing these questions is the availability of sufficiently large, clinically annotated tissue microarrays (TMA) that offer the prospect of defining the prognostic or predictive value of any given molecular marker. Therefore we have constructed a large ovarian cancer TMA (518 patients) with associated clinical demographic and outcome information and have used this to systematically address the value of possible biomarkers of disease prognosis. In the present study, we have tested the prognostic value of kisspeptin and GPR54 immunoreactivity in ovarian cancer.

Kisspeptins (Kp-54, Kp-14, Kp-13, Kp-10) are the canonical, physiologically occurring and high affinity RF-amide peptide ligands that activate transmembrane signalling via a classical (7TM1) family G-protein coupled receptor, GPR54. Kisspeptins were first discovered through microcell mediated chromosome transfer experiments that defined the *KiSS1* locus as a suppressor of melanoma tumour metastasis [1, 2]. Subsequently, kisspeptins were associated as endogenous ligands for the GPR54 receptor. Furthermore, a physiological role in the regulation of placental trophoblast invasion has been suggested [3] and in migratory cell lines, activation of GPR54 signalling abrogates migratory behaviour [1, 4-6]. In 2003 we uncovered in human and mouse genetic studies, the major physiological functions of kisspeptin-GPR54

signalling, as being gatekeepers for GnRH release in the hypothalamus [7, 8]. In the absence of functional kisspeptin [9] and GPR54 [8, 10-12] neither humans nor mice undergo puberty and are unable to generate pituitary release of gonadotropins that drive sex-steroid release. Several subsequent physiological studies have confirmed that kisspeptins act as neuroendocrine peptides that switch on or off the GnRH axis in humans and mammals [13-24] and are thus required as physiological regulators of sex-steroid release. The mechanistic relationship between GPR54 regulation of the hypothalamic-pituitary-gonadal axis, and possible effects on epithelial cell migration remains unclear, however several anecdotal studies on human tumours have suggested possible associations of loss/absence of expression, with poor prognosis [25-32].

Recently Zhang et al. [33] and Hata et al. [34] surveyed RNA expression of the *KiSS1* and *GPR54* loci in small cohorts (<100 cases) of ovarian cancer and observe a trend towards favorable prognosis where *KiSS1*/*GPR54* RNA expression is elevated. None of these studies have been sufficiently powered to address cell-type and prognostic associations in major epithelial malignancies. We show in the present study of 518 ovarian cancer cases that kisspeptin and GPR54 immunoreactivity are very significantly associated with a clear cell carcinoma subtype and that both kisspeptins and GPR54 are independent markers for favourable prognosis as determined by multivariable analysis.

3.2 Material and Methods

3.2.1 Ovarian tumour samples and tissue microarray (TMA) construction

Approval for the study was obtained from the ethics committee of the University of British Columbia. Ninety-three percent of women diagnosed with ovarian cancer in British Columbia are treated at the British Columbia Cancer agency (BCCA) and provincial treatment guidelines are followed. Outcomes are tracked via The Cheryl Brown Ovarian Cancer Outcomes Unit as an ovarian cancer database of the BCCA. A total of 3501 patients with invasive epithelial ovarian carcinoma were referred to the BCCA between 1984 and the year 2000. The focus of this study was 834 patients who had ovarian carcinoma with no macroscopic residual disease after surgery. For 202 cases the slides of the primary ovarian tumour were not available for review and these cases are excluded. A gynaecological pathologist (CBG) then performed a blinded full slide review of the remaining 632 cases. Tumour cell type and grade (Silverberg) were assessed; all clear cell carcinomas were considered to be grade 3, as per WHO recommendations. After review, 518 cases of invasive ovarian carcinoma were available in tissue blocks for tissue microarray construction. A representative area of each tumour was selected and a duplicate core tissue microarray (TMA) was constructed (Beecher Instruments, Silver Springs, MD); the cohort is described in Table 3.1. Serial 4 μm sections were cut for immunohistochemical (IHC) analysis.

Table 3.1 Clinicopathological characteristics of the cohort

Parameter	N
Histopathological Subtype	
Adenocarcinoma	4
Clear Cell	132
Endometrioid	125
Mucinous	31
Serous	212
Squamous Cell	1
Transitional	6
Undifferentiated	7
Grade	
1	106
2	114
3	298
Stage	
1	214
2	219
3	85

Age (years)	
Mean (SD)	58 (12.8)
Median	57
Range (Min – Max)	25 - 89

3.2.2 Immunohistochemistry

Kisspeptin

Sections from formalin-fixed and paraffin-embedded tissues were deparaffinized with xylene and rehydrated with a graded series of alcohols. Wet heat-induced antigen retrieval was performed in a steamer for 20 min with a modified citrate buffer (pH 6.1, Dako, Mississauga, ON). Following antigen retrieval, sections were treated with 3% H_2O_2 in PBS for 30 min to quench endogenous peroxidase activity. All of the aforementioned steps were followed by three washes with PBS for 5 min each. Slides were subsequently blocked for 30 min with serum-free protein block (Dako) and incubated overnight at 4°C with a polyclonal goat anti-KiSS-1 antibody (C-20, Santa Cruz Biotechnology, Santa Cruz, CA) diluted 1:400 in serum-free protein block. Kisspeptin immunoreactivity (IR) was detected with the CSA II biotin-free tyramide signal amplification system and 3,3'-diaminobenzidine chromogen solution (Dako). Specifically, rabbit anti-goat-HRP was applied for 15 min followed by fluorescyl-tyramide amplification reagent for 15 min and anti-fluorescein-HRP for 15 min. All of the steps subsequent to the incubation with primary antibody were followed by three washes with TBST for 5 min each. Slides were counterstained with Harris hematoxylin (Sigma-Aldrich, Oakville, ON) and mounted in a xylene-based mounting medium. Based on previously published data showing cell-type restriction of GPR54 and kisspeptins in different trophoblast layers of human placenta [3], less than 10-week old human placenta was used as a specificity control (courtesy of Vancouver Coastal Health archives), in conjunction with two blocking peptides (21 residues and 54

residues – Figure 3.2). Omission of the primary antibody was used as a negative control.

GPR54

TMA 4 µm sections were processed using a Ventana Discovery XT automated system (Ventana Medical Systems, Tucson, AZ) as per manufacturer's protocol with proprietary reagents. After slides were baked at 60°C for 1 h, they were deparaffinized on the automated system with EZ Prep solution (Ventana). Heat-induced antigen retrieval method was used in Cell Conditioning solution (CC1-Tris based EDTA buffer, pH 8.0, Ventana). The polyclonal rabbit GPR54 antibody was obtained from MBL International Corporation (Woburn, MA) specific for the N-terminal extracellular domain (catalogue number LS-A1929) and used with heat at a 1:25 concentration in Ventana antibody diluent. The Ventana Universal Secondary Antibody was used for 32 min at 37°C. The detection system used was the Ventana DABMap kit, and slides were then counterstained with Hematoxylin and treated with a proprietary bluing agent (Ventana). All washes were conducted with the Ventana Reaction Buffer. Dehydration steps and coverslip procedure were completed manually as per manufacturer's recommendations. Specificity was determined by Western blot (Figure 3.1) and by using less than 10-week old human placenta as a positive control (Figure 3.2) and omission of primary antibody as a negative control.

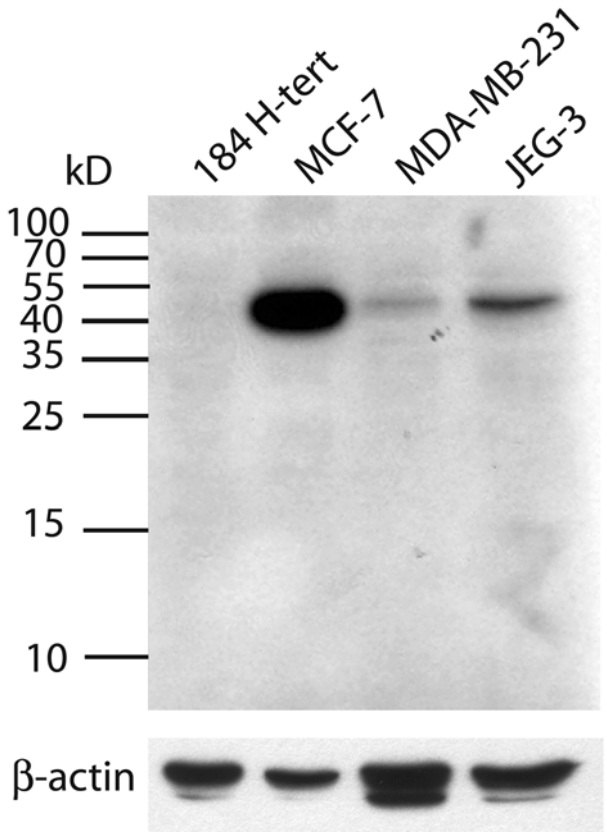

Figure 3.1 Western blot demonstrating GPR54 specificity

30 μg of protein collected using the standard RIPA buffer method was run on 12% SDS-PAGE and transferred to a nitrocellulose membrane for detection with GPR54 MBL antibody for four cell lines. In three of four lanes a single band specific to GPR54 at 42kD is apparent. Loading control β-actin is labeled.

3.2.3 Photomicrographs

The TMA was digitally scanned with a BLISS (Bacus Laboratories Inc., Slide Scanner) automated system (Bacus Laboratories, Lombard, IL) as previously described [35]. These images are publicly available on our webslide server: http://www.gpecimage.ubc.ca/tma/web/viewer.php.

3.2.4 Statistical analysis

Survival time dependant recursive partitioning was used to binarise the raw kisspeptin and GPR54 data. Univariable survival analysis was performed by the generation of Kaplan-Meier curves [36] and differences between the groups were assessed using Log-rank Statistic [37]. Multivariable survival analysis was performed using the Cox Proportional Hazards Model [37, 38]; the adenocarcinoma, squamous cell, transitional, and undifferentiated ovarian subtypes were excluded from multivariable analysis due to insufficient sample size. Contingency tables and the Pearson Chi-square statistic were used to test the change in the distribution of kisspeptin and GPR54 expression across primary cell types [39]. All analyses were performed using JMP version 6.0.3 (SAS Institute, Cary NC, U.S.A.).

3.3 Results

3.3.1 Kisspeptin positivity is an independent marker for favourable prognosis

Kisspeptin-IR was tested on human placenta less than 10 weeks old as a positive control (Figure 3.2). Specifically, there was cell type specificity demonstrated by intense staining in syncytiotrophoblast cells as previously determined [3, 40], but not in other cell layers of the trophoblast. Pre-absorption with two different blocking peptides (metastin (Kp-54, 68-121aa) and kisspeptin 100-120aa), fully blocked kisspeptin-IR, whereas Kp-10 (112-121aa) showed little or no block (Figure 3.2).

For the 518 case ovarian tissue microarray, kisspeptin-IR was scored as 0 for negative cases, +1 for mild staining, and +2 for intense staining (Figure 3.3). Of the 518 cases, 44 stained at +2, 98 had +1 staining intensity, 354 cases were negative for kisspeptin-IR, and 22 cases were uninterpretable. The negative (0) and mildly reactive (+1) cases were grouped for statistical analysis and assigned the designation 0 and considered kisspeptin negative, while the +2 cases were considered kisspeptin positive and designated as 1. Univariable disease specific survival analysis showed that kisspeptin-IR significantly associated with favourable prognosis ($p=0.0023$), as did overall survival ($p=0.0006$, Figure 3.4). Further, multivariable survival analysis including; stage, grade, histological subtype, age and GPR54-IR, indicated kisspeptin-IR as an independent marker for favourable prognosis in disease specific ($p=0.0046$, Table 3.2) and overall survival ($p=0.0170$, Table 3.3).

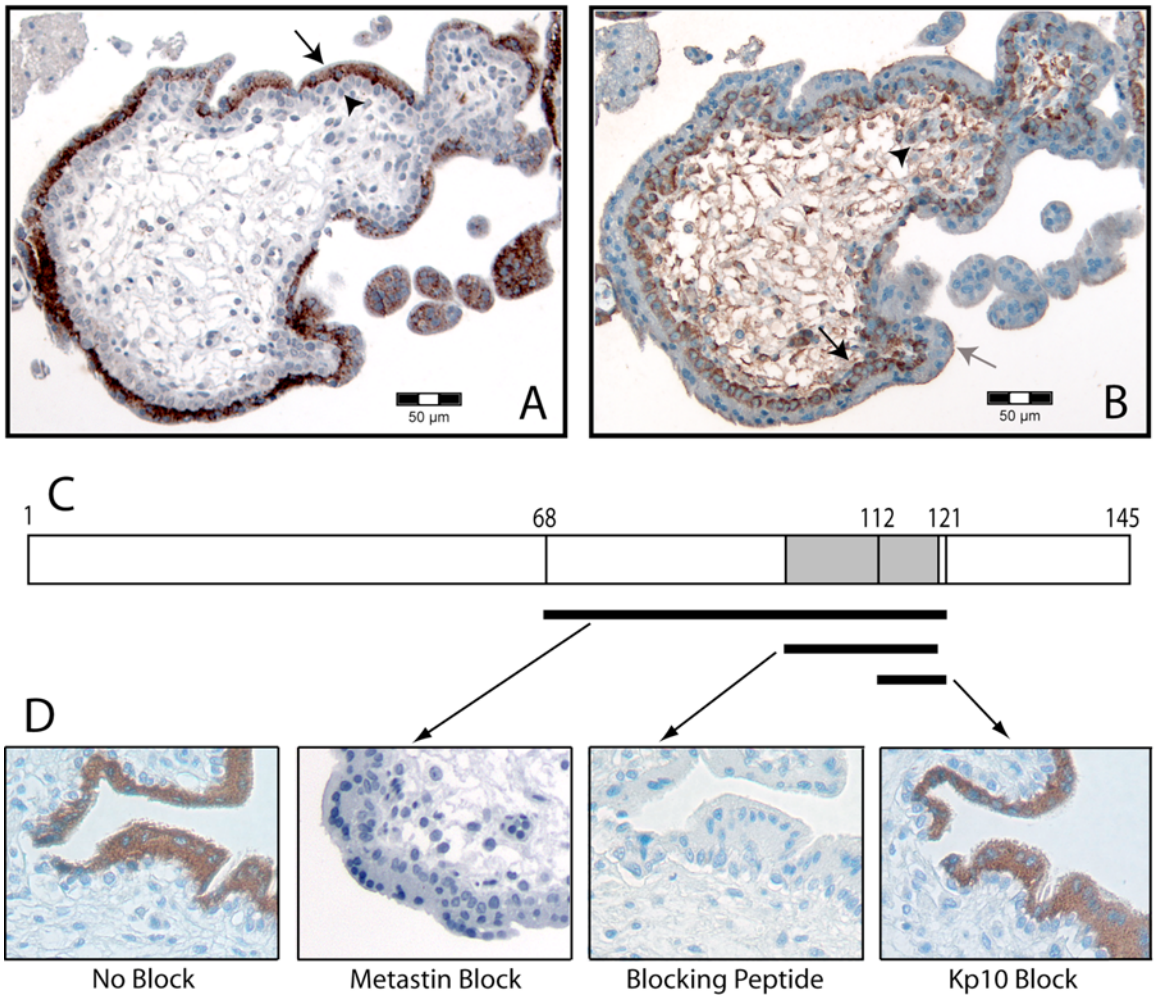

Figure 3.2 Immunohistochemistry controls

Less than 10 week old human placenta used as a positive control. A) Kisspeptin-IR shows intense cell-type specific staining in the syncytiotrophoblasts (black arrow), while the cytotrophoblast layers remain unaffected (black arrowhead). B) GPR54-IR shows intense staining in the villous cytotrophoblasts (black arrow), the extravillous cytotrophoblasts (black arrowhead), and moderate staining on the syncytiotrophoblast membrane (grey arrow). C) Schematic of the 1-145 amino acid (aa) KiSS-1 pro-peptide. Metastin (Kp-54) is encoded within the 68-121 aa sequence, while Kp-10 is encoded within this same region from 112-121 aa. The specific blocking peptide is encoded within the 100-120 aa sequence. D)

Varying kisspeptin-IR was found among the different blocking peptides used. Blocking the primary antibody with full-length metastin (Kp-54) and blocking peptide resulted in complete loss of immunoreactivity, while Kp-10 was unable to block any detectable staining.

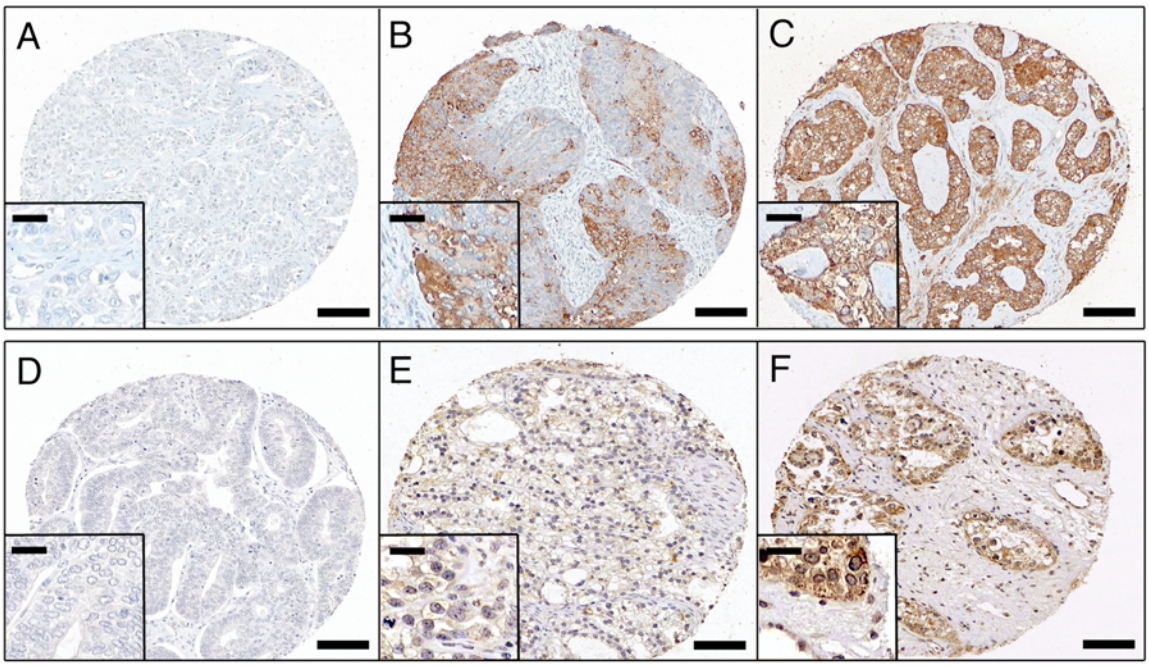

Figure 3.3 Immunoreactivity patterns

Three types of kisspeptin-IR (A-C) and GPR54-IR (D-F) observed in the ovarian TMA. A, D) Representative samples of negative cases show complete lack of staining and are classified as 0. B) Moderate GPR54-IR shows a patchy staining pattern with light and dark brown regions of reactivity shown throughout the tumour core, and E) Mild kisspeptin-IR shows uniform light brown staining throughout the sample: both B and E are classified as +1 immunoreactivity. C, F) Examples of +2 intense immunoreactivity exhibit dark brown staining in all tumour cells. Scale bar represents 100 μm. Insets in each panel show a more detailed view of the staining pattern that is demonstrated in the larger image; inset scale bar represent 25 μm.

3.3.2 GPR54 positivity is an independent marker for favourable prognosis in overall survival

The GPR54 antibody and protocol were tested on less than 10-week old human placenta and specifically stained both villous and extravillous cytotrophoblasts and the syncytiotrophoblasts as described by previous groups [3, 40] (Figure 3.2). Although GPR54 is a 7-transmembrane protein, there was some reactivity in the cytoplasm of some tumour cells (this is not entirely surprising since GPR54 is a transmembrane protein and may be recycled through the cytoplasm) but only membranous staining was taken into consideration while assessing immunoreactivity. Three immunoreactivity patterns were observed within the TMA for GPR54. Specifically, negative or very weak reactivity in less than 5% of cells was designated as 0 (103 cases), while patchy or moderate staining in 5-75% of cells was designated +1 (282 cases), and strong staining in greater than 75% of cells were considered +2 (104 cases, Figure 3.3). The remaining 9 cases were uninterpretable. As with kisspeptin, the 0 and +1 GPR54 cases were group together and considered as loss of receptor and designated 0, while the strong staining +2 cases were considered positive and designated as 1. Univariable survival analysis determined GPR54 as a significant marker for favourable prognosis in disease specific ($p=0.0092$) and overall survival ($p=0.0002$, Figure 3.4). Similar to kisspeptin-IR, GPR54 maintained significance in multivariable overall survival ($p=0.0303$, Table 3.3). However, GPR54 was not found to be a significant independent marker in disease specific survival ($p=0.1118$, Table 3.2).

Figure 3.4 Disease specific and overall survival curves for Kp and GPR54
A) GPR54 expression in disease specific survival B) GPR54 expression in overall survival C) Kp expression in disease specific survival D) Kp expression in overall survival. E) Assessing both GPR54 and Kp expression in disease specific survival F) Assessing both GPR54 and Kp expression in overall survival. Cumulative survival is on the y-axis; time in years is on the x-axis. G+ represents GPR54 positive, G- represents GPR54 negative, K+ represents Kp positive cases, and K- represents Kp negative cases.

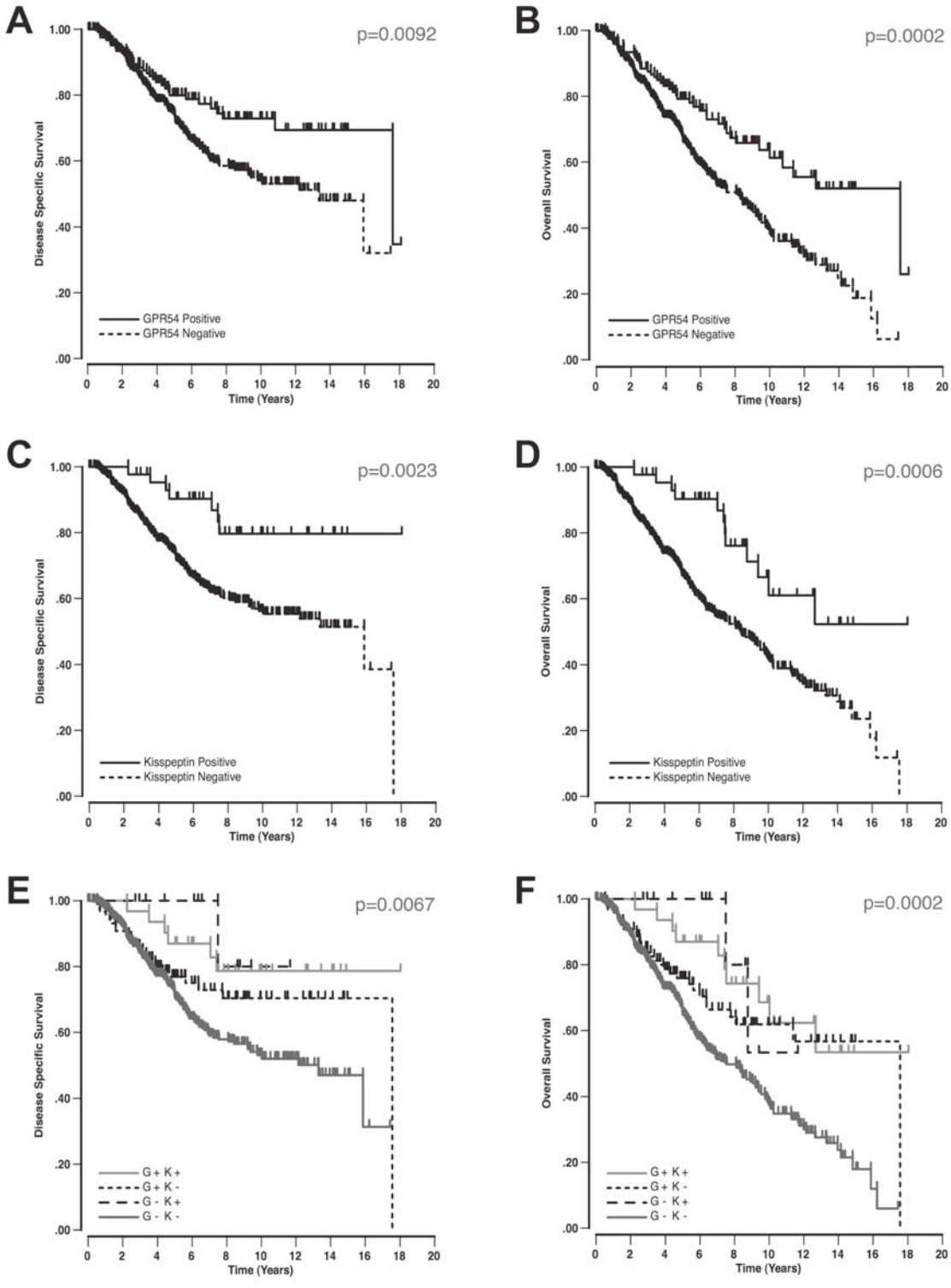
A
p=0.0092
Disease Specific Survival
GPR54 Positive
GPR54 Negative
Time (Years)
B
p=0.0002
Overall Survival
GPR54 Positive
GPR54 Negative
Time (Years)
C
p=0.0023
Disease Specific Survival
Kisspeptin Positive
Kisspeptin Negative
Time (Years)
D
p=0.0006
Overall Survival
Kisspeptin Positive
Kisspeptin Negative
Time (Years)
E
p=0.0067
Disease Specific Survival
G + K +
G + K -
G - K +
G - K -
Time (Years)
F
p=0.0002
Overall Survival
G + K +
G + K -
G - K +
G - K -
Time (Years)

Table 3.2 Multivariable disease specific proportional hazards

Parameter	Risk Ratio (95% CI)	p-value
Stage		<0.0001**
1	0.6404 (0.4901 to 0.8278)	
2	0.7149 (0.5647 to 0.8994)	
3	1.0000	
Histological Grade		0.0720
1	0.6234 (0.3829 to 0.9808)	
2	1.3899 (1.0193 to 1.8888)	
3	1.0000	
Subtype		0.2508
Clear cell	1.4519 (0.8853 to 2.3847)	
Endometrioid	0.6804 (0.4105 to 1.1032)	
Mucinous	1.0721 (0.5119 to 1.9699)	
Serous	1.0000	
Age		0.0747
	N/A*	
GPR54		0.1118
Positive	0.6475 (0.3738 to 1.1052)	
Negative	1.0000	
Kisspeptin		0.0046**
Positive	0.3508 (0.1426 to 0.7408)	
Negative	1.0000	

*RR for age is not available because it is a continuous variable
** Represents statistical significance

Table 3.3 Multivariable overall proportional hazards

Parameter	Risk Ratio (95% CI)	p-value
Stage		<0.0001**
1	0.7258 (0.5842 to 0.8961)	
2	0.7149 (0.6457 to 0.9476)	
3	1.0000	
Histological Grade		0.5356
1	0.8268 (0.5818 to 1.1642)	
2	1.1113 (0.8647 to 1.4148)	
3	1.0000	
Subtype		0.6763
Clear cell	1.2126 (0.7934 to 1.8513)	
Endometrioid	0.8120 (0.5508 to 1.1914)	
Mucinous	1.0192 (0.5652 to 1.6841)	
Serous	1.0000	
Age		<0.0001**
	N/A*	
GPR54		0.0303**
Positive	0.5959 (0.3684 to 0.9523)	
Negative	1.0000	
Kisspeptin		0.0170**
Positive	0.4844 (0.2443 to 0.8841)	
Negative	1.0000	

*RR for age is not available because it is a continuous variable
** Represents statistical significance

3.3.3 Kisspeptin positivity correlates with GPR54 positive cases

Kisspeptin positive cases had a moderate correlation with GPR54 positivity as determined by Kendall's tau-b [41] (τ=0.3837, p<0.0001). There were 31 cases that were both kisspeptin and GPR54 positive, 90 cases that were kisspeptin negative and GPR54 positive, 12 cases with kisspeptin positivity and had loss of GPR54, 356 cases that had loss of both kisspeptin and GPR54, and the remaining 29 cases were uninterpretable. When kisspeptin-IR and GPR54-IR cases are grouped together (G+ K+), patients have a more favourable outcome than those that have loss of either one or both (G- K+, G+ K-, G- K-). There is a significant difference between survival for double positive patients (G+ K+) as compared to double negative patients (G- K-) in both disease specific (p=0.0067) and overall survival (p=0.0002, Figure 3.4).

3.3.4 Kisspeptin and GPR54 positive staining are significantly associated with clear cell carcinoma histo-pathological subtype

The percentage of kisspeptin and GPR54 positive cases within each histo-pathological subtype is listed in Table 3.4. The proportionality of primary histo-pathological cell type in the entire cohort, kisspeptin positive cases, and GPR54 positive cases are represented in Table 3.5. Testing for an association between ovarian carcinoma subtype and kisspeptin status, there was a highly significant positive association with clear cell carcinoma, and a significant negative association with serous carcinoma subtype ($\chi 2$, p<0.0001). GPR54 positive cases also had a significant positive association with clear cell carcinoma subtype and a negative association with the serous subtype ($\chi 2$, p<0.0001).

When disease specific survival and overall survival were analyzed within each ovarian carcinoma subtype, the Log-Rank test for kisspeptin-IR status failed to achieve significance (due to insufficient sample size), although for the clear cell cases statistical significance was approached (p=0.1042, p=0.0859, results not shown). Of note, none of the kisspeptin positive patients that were not clear cell subtype (16 cases) died from their disease. Similarly, when assessing GPR54 positivity within each ovarian cancer subtype, disease specific survival did not reach significance within the clear cell subtype (p=0.0656), although significance was achieved for overall survival (p=0.0102, Figure 3.5).

3.4 Conclusions

Although clear cell carcinomas comprise fewer than 5% of ovarian malignancies, they are notoriously difficult to treat due to their tendency to resist platinum based chemotherapy [42]. To date, clinical stage has been the only prognostic marker for clear cell ovarian carcinoma. Here we show for the first time, that kisspeptin and GPR54 immunoreactivity are distinctly favourable prognostic markers, with kisspeptin being independent of pathologic subtype, stage, grade, or age in both overall and disease specific survival, while GPR54 is an independent marker in overall survival. Within clear cell carcinomas, GPR54 expressers have a favourable prognosis and to our knowledge this is the first molecular marker of prognosis specifically applicable to clear cell ovarian cancer. Although several studies have suggested possible relationships between GPR54 and kisspeptin expression and clinical outcome [25, 26, 29-33, 43], these studies have consisted

Table 3.4 Percentage of kisspeptin and GPR54 positive cases within the histological subtypes

Histological Subtype	Kisspeptin Positive (%)	GPR54 Positive (%)
Clear Cell	21.88	66.41
Endometrioid	8.13	20.33
Mucinous	12.00	10.34
Serous	1.49	3.37

Table 3.5 Kisspeptin and GPR54 proportions within the cohort

Histological Subtype	Whole Cohort		Kisspeptin Positive		GPR54 Positive	
	Proportion	Count	Proportion	Count	Proportion	Count
Clear Cell	0.2640	132	*0.6364	28	**0.7131	87
Endometrioid	0.2500	125	0.2273	10	0.2049	25
Mucinous	0.0620	31	0.0682	3	0.0246	3
Serous	0.4240	212	*0.0682	3	**0.0574	7

*Chi square p-value<0.0001
**Chi square p-value<0.0001

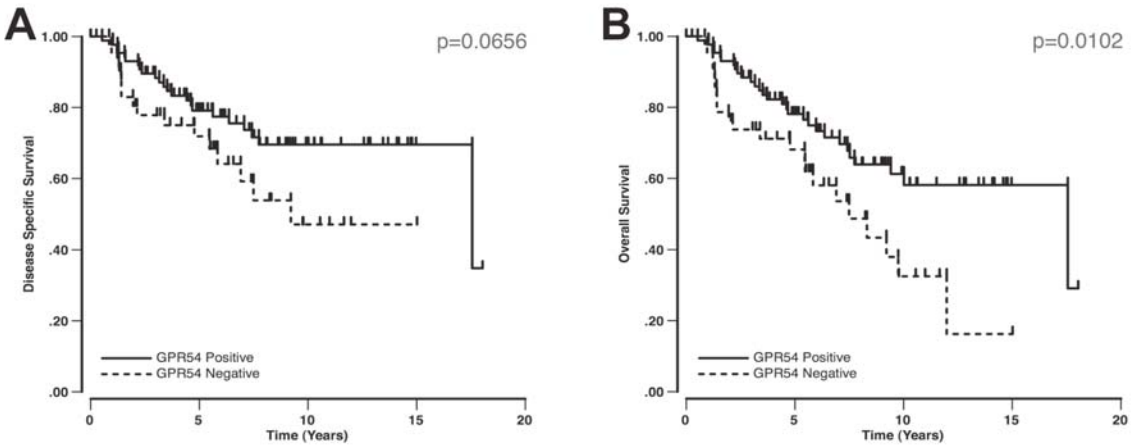

Figure 3.5 GPR54 is a favourable prognostic marker in the clear cell subtype

Kaplan-Meier survival curves for A) GPR54 expression in disease specific survival and B) GPR54 expression in overall survival. Cumulative survival on the y-axis; time in years on the x-axis.

of smaller cohorts and while some associations have been noted, some studies may not have been sufficiently powered to address possible prognostic or cell type specific effects with rigor. To date, the present study is the largest systematic analysis of GPR54 and kisspeptin expression determined by immunoreactivity for an epithelial malignancy. In part, this may be due to difficulties in obtaining sufficiently specific antisera and detection protocols, in that short peptides and GPCRs are notoriously difficult antibody targets. The antibodies and immunodetection protocols used in this study were verified by the use of either Western blotting and cell-type specific expression (GPR54), or cell-type specific expression and specific blocking peptides (kisspeptin). This is based on previous work showing differential expression of kisspeptin and GPR54 in human placental trophoblast cell types [3, 43]. The precise spectrum of immunoreactivity of the kisspeptin antiserum to kisspeptin fragments remains to be determined and, as with many antibodies, it remains possible that other proteins may be detected. Very recently a survey of 76 ovarian cancer patients using Q-PCR detection of GPR54 and kisspeptin transcripts [34] demonstrated a negative correlation between KiSS-1 and GPR54 mRNA levels with residual disease, although they showed no correlation with histopathological subtype (possibly due to the relatively small number of clear cell ovarian cancers in that cohort), however the overall correlation observed in this study is in agreement with our observations.

The mechanisms responsible for the association of kisspeptin and GPR54 expression with disease behaviour in ovarian cancer requires definitive studies,

however several possibilities arise. It is possible that expression of kisspeptins and/or GPR54 result in higher endogenous GPR54 signalling in malignant cells. Although no studies have directly addressed the degree of GPR54 signalling in epithelial malignancies in relation to clinical outcomes, the present study shows that both kisspeptin and GPR54 expression are associated with a better prognosis. Furthermore, patients with double positive tumours (G+ K+) have the most favourable prognosis (Figure 3.3). These observations together with previous evidence of the effects of GPR54 signalling on cell migration, suggest some form of autocrine or paracrine loop could exist in clear cell carcinomas. GPR54 is exquisitely sensitive to kisspeptin ligand stimulation [3, 5] and receptor overexpression alone may be enough to increase basal signalling through GPR54.

The interplay of mechanisms could be complicated by the major physiological role of GPR54, which is to regulate GnRH secretion at the hypothalamic level. Kisspeptins can cross from the peripheral circulation to act on the hypothalamus, as has been shown in numerous mammalian [21, 23, 44-46] and one human study [20]. It is possible that kisspeptin overexpressing tumours may result in stimulation of the hypothalamic-pituitary axis, resulting in the release of gonadotropins and other derived peptides with a possible paracrine/endocrine effect on tumour growth. Indeed Nash et al. [47], have shown that melanoma cells unable to signal on exposure to kisspeptins, can still be suppressed from metastasis by exogenous kisspeptin, suggesting that paracrine effects may operate in these cases. Finally, some evidence suggests

that kisspeptins and GPR54, which are expressed in ovarian epithelium and granulosa cells, may co-modulate the activity of gonadotropins in sex steroid release [40]. If such a mechanism were operational in clear cell ovarian cancers, it would imply that tumour behaviour is also linked to co-modulatory peptides.

Beyond the salient observation of prognostic significance in this study, the nature of the proteins involved suggests a number of possible areas for intervention. First, kisspeptins, the products of the KiSS-1 gene locus, are naturally occurring peptides that can be detected in human serum and other tissues [48-50]. It is possible that serum kisspeptide levels could be developed as a biomarker of disease activity in patients with clear cell carcinoma. Secondly, kisspeptins are naturally occurring peptide hormones that have activity in humans [20]. As such they are highly amenable to use as therapeutic agents, either alone or as modified peptides. We anticipate that the strong association of GPR54 and kisspeptin expression with outcome and clear cell type in ovarian carcinoma will stimulate fresh approaches to what is still a lethally intractable disease.

In conclusion, Kisspeptin and GPR54 are significantly associated with favourable prognosis in both disease specific and overall survival, as well as being significantly associated with the clear cell ovarian carcinoma subtype, thereby creating the first independent prognostic biomarkers specific for ovarian clear cell carcinomas.

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4 PLASMA KISSPEPTIN IS ELEVATED IN GYNAECOLOGICAL MALIGNANCY[3]

Objective: To analyze GPR54 and Kp IHC in an independent cohort of clear cell ovarian cancers and to determine if Kp can be detected in the plasma of ovarian cancer patients.

[3] A version of this chapter will be submitted for publication. Prentice LM, Murphy KG, Kalloger S, Köbel M, Mehl E, Miller D, Gilks CB, Bloom SR, Huntsman DG, and Aparicio SAJ. Plasma kisspeptin is elevated in gynaecological malignancy.

4.1 Introduction

Ovarian cancer has the highest number of cancer deaths of all the gynaecological cancers in North America [1, 2]. Many women unfortunately are diagnosed in the advanced stages of disease due to the lack of detectable symptoms in early stage cancer [3, 4]. Late stage directly correlates with poor prognosis [5] and early stage cancer is often treatable with surgery alone [3]. Although there is an ongoing search for biomarkers to reveal early stage ovarian cancer, few promising candidates have yet been unveiled.

The metastasis suppressor kisspeptin (Kp) and its receptor GPR54 have been shown to have anti-metastatic effects in *in vivo* systems and anti-invasive characteristics when overexpressed in *in vitro* models [6-14]. Similarly, when gene expression is examined in cancer tissues *KiSS1* and *GPR54* are associated with less malignant disease and a more favourable prognosis [10, 13, 15-20]. In normal physiology, Kp and GPR54 are thought to mediate controlled invasion in placental trophoblast during pregnancy [21]. Indeed, the secreted Kp peptide can be measured in plasma and is at 10,000 fold higher levels in pregnant women, reaching its highest levels by the third trimester of pregnancy [22, 23]. Normal physiological levels of Kp are barely measurable in men and non-pregnant women (ranges from 1.3±0.1-6.9±1.9 pmol/L; [22-26]).

Most recently, Kp was measured in the plasma of patients with malignant disease. Plasma Kp was elevated to near pregnant levels in women with gestational trophoblastic neoplasia (GTN); a malignant cancer of the placenta. Plasma Kp returned to barely detectable levels after surgery and chemotherapy

treatment of these patients; as is seen after the delivery of the placenta in normal physiology [22]. In pancreatic cancer, Kp was elevated in all patients compared to normal healthy volunteers, yet there was no correlation between plasma Kp and any prognostic markers such as: stage, grade, lymph node or distant metastasis [27]. However, clinical outcome as determined by survival analysis was not assessed. These initial studies suggest Kp may be a potential serum biomarker for cancer.

We have previously published Kp and GPR54 expression as being independent favourable prognostic markers in ovarian cancer, and specifically correlated expression to the clear cell cancer subtype [28]. Using a 445 case gynaecological cancer tissue microarray with matched plasma samples, we set out to determine Kp and GPR54 expression in these tumours and whether plasma Kp could also be measured. Here, we report that we have confirmed Kp and GPR54 specificity for the clear cell subtype in an independent cohort of ovarian cancers and for the first time determined that plasma Kp is elevated in gynaecological disease and cancer.

4.2 Material and Methods

4.2.1 Clear cell ovarian cancer arrays

Two tissue microarrays consisting solely of clear cell cancer subtype cases with linked clinical outcome data were used. One TMA was from the Memorial Sloan-Kettering Cancer Center, New York, NY, USA consisting of 39 cases initially diagnosed as clear cell carcinoma obtained from patients from 1980-2006 [29].

The second TMA was from the University of Northern Alberta, Edmonton, Canada, consisting of 42 cases dating back to 1989 [30]. Characteristics of these two cohorts are listed in Tables 4.1 and 4.2.

4.2.2 Gynaecological Cancer Tissue Bank and array

The Gynaecological Cancer Tissue Bank was formed to collect tissue from ovarian or uterine tumours from patients in British Columbia, Canada, and approval for the study was obtained from the ethics committee of the University of British Columbia. Patients undergoing oophorectomy or hysterectomy were asked to participate for one of the following reasons: (1) due to reasons unrelated to cancer, (2) due to disease which may be cancer, or (3) because the patient was at risk of developing cancer. After diagnostic pathological examination, remaining tissue was incorporated into the tissue bank, some of which had matched blood samples. Two gynaecological pathologists (Drs. Blake Gilks and Martin Köbel) performed a blinded full slide review of the cases to determine tumour cell type and grade (Silverberg). After review, 445 cases of gyneacological cancers were available in tissue blocks for tissue microarray construction. A representative area of each tumour was selected and a duplicate core tissue microarray (TMA) was constructed as previously described [31-33](Beecher Instruments, Silver Springs, MD); the cohort is described in Table 4.3. Serial 4 µm sections were cut for immunohistochemical (IHC) analysis.

Table 4.1 Characteristics of the New York clear cell array

Parameter	N
Histopathological Subtype	
Clear Cell	39
Grade	
3*	39
Stage	
1	18
2	8
3	9
4	2
Age (years)	
Mean (SD)	54.17 (11.47)
Median	54
Range (Min – Max)	31.0-82.0

*All clear cell ovarian cancers are Grade 3

Table 4.2 Characteristics of the Edmonton clear cell array

Parameter	N
Histopathological Subtype	
Clear Cell	42
Grade	
3*	42
Stage	
1	30
2	5
3	5
4	0
Age (years)	
Mean (SD)	55 (12.2)
Median	54
Range (Min – Max)	37.0-91.0

*All clear cell ovarian cancers are Grade 3

Table 4.3 Characteristics of the gynaecological tissue microarray

Parameter	N
Histopathological Subtype	**445**
Endometrial	**21**
Endometrial Clear Cell	1
Endometrial Endometrioid	17
Endometrial Mixed	2
Endometrial Serous	1
Ovarian	**337**
Adenocarcinoma NOS	13
Borderline Brenner Tumor	1
Brenner Tumour	3
Clear Cell	33
Dysgerminoma	2
Endometrioid	29
Endometrioid Borderline	2
Fibroma	1
Germinoma	1
Granulosa Cell Tumor	11
Gynandroblastoma	1
High-Grade Serous	218
Low-Grade Serous	9
Malignant Mixed Mullerian	10
Mucinous	8
Mucinous Borderline	2
Serous Borderline	17
Sertoli-Leydig Cell	1
Thecoma	2
Undifferentiated	10
Yolk sac tumor	3
Other	**47**
Fallopian Tube	3
Follicular Lymphoma	1
Leiomyosarcoma	4
Mesenchymal Neoplasia	1
Metastasis	32
Pleomorphic Sarcoma	2
Squamous Cell	2
Trophoblast Tumour	1
Vulvar Squamous	1

Parameter	N
Stage	**374**
1	78
2	51
3	199
4	46
Grade	**323**
1	43
2	66
3	214
Age (Years)	**445**
Mean (SD)	58.6 (14.5)
95%CI (LCI – UCI)	57.2 - 59.9
Follow Up (Years)	**402**
Mean (SD)	2.1 (1.6)
95%CI (LCI – UCI)	1.9 - 2.3

4.2.3 Blood sample collection

Blood samples were drawn from patients that had fasted for at least 8 hours prior to phlebotomy. Whole blood was drawn into a 6 ml BD Vacutainer tube containing 10.8 mg of K_2EDTA (BD Biosciences, Mississauga, ON) on the day of surgery prior to anesthetic induction. The blood was transferred to a 15 ml centrifuge tube and spun at 1000x g for 10 min at 4°C. The plasma was drawn off, snap frozen and stored at -140°C. Under optimal circumstances, the entire procedure took less than 30 min.

4.2.4 Kp detection in plasma

A well established radioimmunoassay (RIA) with 100% cross reactivity to Kp-54, Kp-14, and Kp-10 and with <0.01% reactivity to other human RFamide peptides was used [22, 24, 25, 34]. In brief, 100 μl of plasma Kp competitively bound to the Kp specific GQ2 antibody against ^{125}I-labeled Kp-54 in 700 μl of 0.07 M phosphate buffer, pH 7.2, containing 0.3% bovine serum albumin for 3 days at 4°C. Charcoal absorption was used to separate free and bound ^{125}I-labeled Kp-54 for Kp concentration determination. Each sample was measured in duplicate and concentrations yielding less than 10 pmol/l were considered as zero in statistical analysis.

4.2.5 Automated GPR54 and kisspeptin immunohistochemistry

All of the TMAs were stained using an automated system for both GPR54 and Kp IHC. TMA 4μm sections were processed using a Ventana Discovery XT

automated system (Ventana Medical Systems, Tucson, AZ) as per manufacturer's protocol with proprietary reagents. After slides were baked at 60°C for 1 h, they were deparaffinized on the automated system with EZ Prep solution (Ventana). Heat-induced antigen retrieval method was used in Cell Conditioning solution (CC1- Tris based EDTA buffer, pH 8.0, Ventana). The polyclonal rabbit GPR54 antibody was obtained from MBL International Corporation (Woburn, MA) specific for the N-terminal extracellular domain (catalogue number LS-A1929) and used with heat at a 1:25 concentration in Ventana antibody diluent. The polyclonal goat KiSS1 antibody from Santa Cruz (Santa Cuz, CA) was incubated for 2 h without heat at 1:10 dilution. For GPR54, the Ventana Universal Secondary Antibody was used for 32 min at 37°C, while a rabbit anti-goat (Jackson ImmunoResearch, West Grove, PA) at 1:500 dilution was used for KiSS1. The detection system used for GPR54 was the Ventana DABMap kit, while for KiSS1 the Ultra detection system (Ventana) was used. All slides were then counterstained with Hematoxylin and treated with a proprietary bluing agent (Ventana) and washes were conducted with the Ventana Reaction Buffer. Dehydration steps and coverslip procedure were completed manually as per manufacturer's recommendations. For the ovarian array with associated plasma, GPR54 was re-optimized and used at a 1:100 dilution for 2 h without using the Ultra detection system (Ventana).

Specificity was determined by Western blot for GPR54 and by using less than 10-week old human placenta as a positive control for both GPR54 and Kp.

Omission of primary antibody was used as a negative control for both antibodies as published previously [28].

4.2.6 Photomicrographs

The TMA was digitally scanned with a BLISS (Bacus Laboratories Inc., Slide Scanner) automated system (Bacus Laboratories, Lombard, IL) as previously described [28, 35]. These images are publically available on our webslide server: http://www.gpecimage.ubc.ca/tma/web/viewer.php.

4.2.7 Statistical Analysis

Univariable survival analysis was performed by the generation of Kaplan-Meier curves [36] and differences between the groups were assessed using Log-rank Statistic [37]. Contingency tables and the Pearson Chi-square statistic were used to test the change in the distribution of kisspeptin and GPR54 expression across diagnostic group and IHC score [38]. Comparisons between groups for plasma Kp analysis were performed using ANOVA (analysis of variation) followed by a Tukey's test to account for multiple comparisons. All analyses were performed using JMP version 6.0.3 (SAS Institute, Cary NC, U.S.A.).

4.3 Results

4.3.1 Kp as a favourable marker for prognosis is variable between tissue microarrays

Based on our initial findings that Kp and GPR54 are specific to the clear cell subtype of ovarian cancer, we used two additional clear cell cancer specific arrays with linked clinical outcome data to validate Kp and GPR54 as markers for favourable prognosis within this subtype. However, the results differed quite dramatically between the two clear cell cohorts. For the New York array Kp cases with an IHC score of 0 or +1 were grouped as negative and cases that had the most intense staining at a score of +2 were considered positive. Based on this grouping, Kp was determined to be a significant marker for favourable prognosis in disease specific survival as only one Kp positive patient succumbed to their disease ($p=0.0383$, Figure 4.1 A). Conversely the Edmonton array, regardless of how Kp IHC scoring was grouped, had no significant association with survival (Figure 4.1 C). As the majority of cases were positive for GPR54 in both cohorts, the prognostic significance could not be delineated in either TMA (Figure 4.1 B, D).

4.3.2 Kp and GPR54 are associated with clear cell subtype in an independent tissue microarray

Using a gynaecological cancer tissue microarray consisting primarily of ovarian cancers (377/445) varying in subtype, Kp and GPR54 expression correlated with the clear cell subtype ($p \leq 0.0001$, Tables 4.4 and 4.5), validating our previous report [28]. For this analysis GPR54 IHC scores 0 and +1 were grouped as

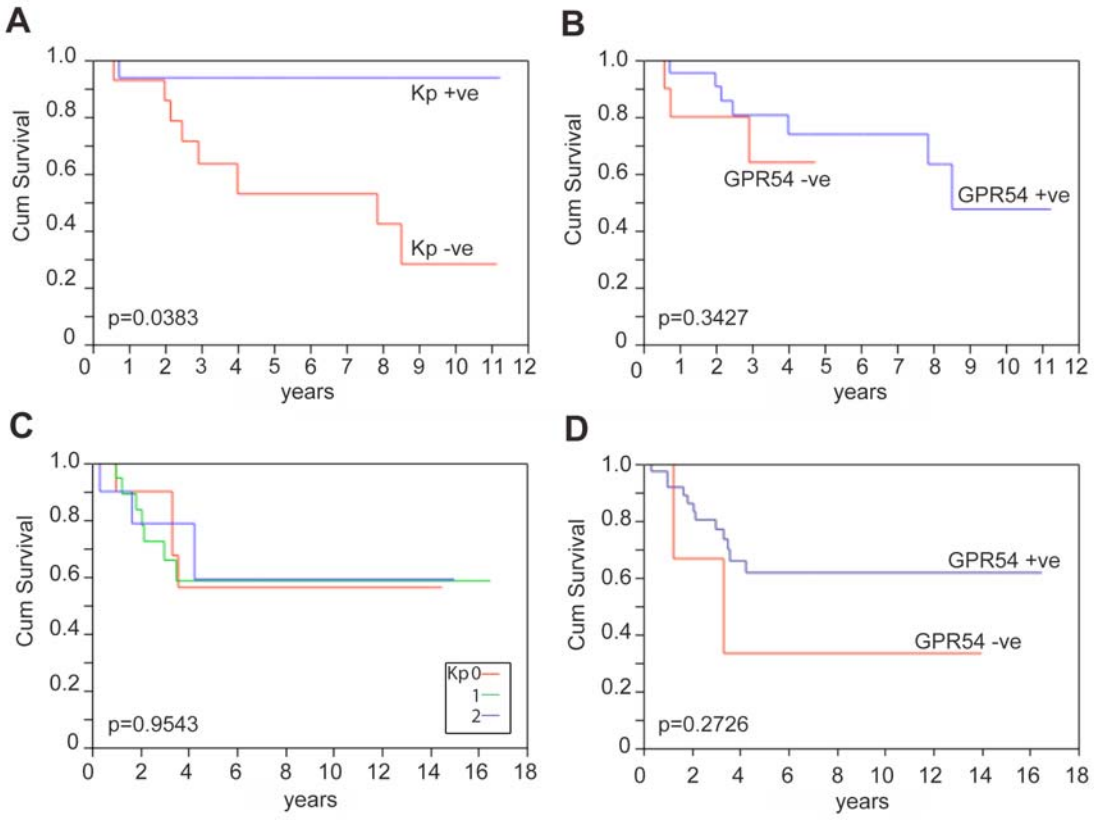

Figure 4.1 Disease specific survival curves for Kp and GPR54 in ovarian cancer clear cell subtype

A) Kp positivity is associated with favourable prognosis in disease specific survival in the New York array (18 +ve cases, 14 -ve cases). B) GPR54 expression in the New York array (24 +ve cases, 10 -ve cases). C) Kp expression in the Edmonton array (10 cases for 0 group, 20 cases for 1 group, 10 cases for 2 group). D) GPR54 expression in the Edmonton array (38 +ve cases, 3 -ve cases). Cumulative survival is on the y-axis and time in years is on the x-axis.

Table 4.4 Percentage of kisspeptin and GPR54 positive cases within the histological subtypes.

Histological Subtype	Kisspeptin Positive (%)	GPR54 Positive (%)
Clear Cell	46.15	78.57
Endometrioid	10.71	3.33
Mucinous	11.92	37.5
HG Serous	12.5	11.96
LG Serous	0	25

Table 4.5 Kisspeptin and GPR54 proportions within the cohort

Histological Subtype	Whole Cohort		Kp Positive		GPR54 Positive	
	Proportion	Count	Proportion	Count	Proportion	Count
Clear Cell	0.0989	28	0.3077*	12	0.4151*	22
Endometrioid	0.1060	30	0.0769	3	0.0189	1
Mucinous	0.0283	8	0.0000	0	0.0566	3
HG Serous	0.7385	209	0.5897	23	0.4717	25
LG Serous	0.0283	8	0.0256	1	0.0377	2

* Chi square p-value<0.0001

negative to represent GPR54 loss, and +2 cases were considered as positive as in our initial study. Kp was also grouped as previously described with +2 considered as Kp overexpression and IHC scores of 0 and +1 were grouped as negative. However, as this cohort of patients is relatively new in terms of clinical outcome, no significant correlations were made with survival due to limited follow-up time (95% confidence interval for follow-up time is 1.9-2.3 years).

4.3.3 Kp plasma concentration is elevated in women with ovarian cancer, endometrial cancer and benign gynaecological disease

From the gynaecological tissue bank, 225 plasma samples were available for Kp analysis. As Kp has a half-life of 27.6 ± 1.1 minutes [24], and it has been previously established that samples processed within an hour of venipuncture provide optimal results with this assay [34] only samples that had been processed within an hour were used (184/225). Patients were categorized based on the state of their disease at the time of blood draw. These categories were: benign, history of cancer, metastasis, malignant, low malignant potential (LMP), and no disease. The benign group consisted of patients that had benign disease at the time of the blood draw, while patients who had previously been treated for cancer but did not have cancer at the time of blood draw were classified as having had a history of cancer, and patients that were negative for any disease were classified as no disease. Patients presenting with a metastasis to the ovary from a primary cancer outside of the gynaecological malignancies were grouped as metastasis and the rest of the patients were grouped as either malignant or low malignant potential to describe their cancer. A summary of the patients within

each group is listed in Table 4.6. Based on this classification system, women presenting with a metastasis to the ovary had significantly higher levels of plasma Kp than did patients that had been previously treated for cancer (Figure 4.2 A).

As we have previously reported significant association of Kp expression with the clear cell subtype of ovarian cancer, the malignant group of patients was further divided into the five main subtypes of ovarian cancer: endometrioid, clear cell, mucinous, high-grade serous, and low-grade serous [39]. Although there was no significant association of Kp concentration with any of the subtypes the clear cell cases had the highest mean concentration (Figure 4.2 B). More cases would be required to reach statistical significance between subtypes, as there are currently only 7 clear cell cases. Nor was there a significant association with plasma Kp when correlated with age at the time of surgery (p=0.3889).

Of the 445 cases on the gynaecological cancer tissue microarray, 190 cases had matched plasma. Of these, 79 samples were measured for plasma Kp concentration, resulting in 71/79 cases that had matched Kp results from the IHC analysis, and 76/79 cases that had GPR54 IHC results. There was not a significant association between Kp plasma concentrations and Kp IHC expression (p=0.2847), nor was there any correlation between Kp plasma concentration and GPR54 IHC expression (p=0.1422, Figure 4.3).

Table 4.6 Characteristics of plasma samples by group

Classification	N
No Disease	**10**
BRCA Mutation Carrier	10
Benign	**43**
Brenner Tumour	2
Endometriosis	10
Fibroma	6
Hyperplasia	2
Leiomyoma	4
Mature Teratoma	5
Mucinous Cystadenoma	4
Serous Cystadenoma	8
Struma Ovarii	1
Thecoma	1
Low Malignant Potential	**9**
Mucinous Borderline	3
Serous Borderline	6
Malignant	**125**
Adenocarcinoma NOS	3
Clear Cell	7
Dysgerminoma	2
Endodermal Sinus	1
Endometrial	44
Endometrioid	13
High-Grade Serous	33
Immature Teratoma	1
Leiomyosarcoma	1
Low-Grade Serous	6
Malignant Mixed Mullerian	7
Mucinous	4
Squamous Cell	2
Undifferentiated	1
Metastasis (Primary)	**15**
Breast	4
Carcinoid	1
Krukenberg	5
Lymphoma	2
Pseudomyxoma Peritoneii	1
Renal Clear Cell	1
Lung Small Cell	1
History of Cancer	**18**
Breast	13
Endometrial	4
Colon	1

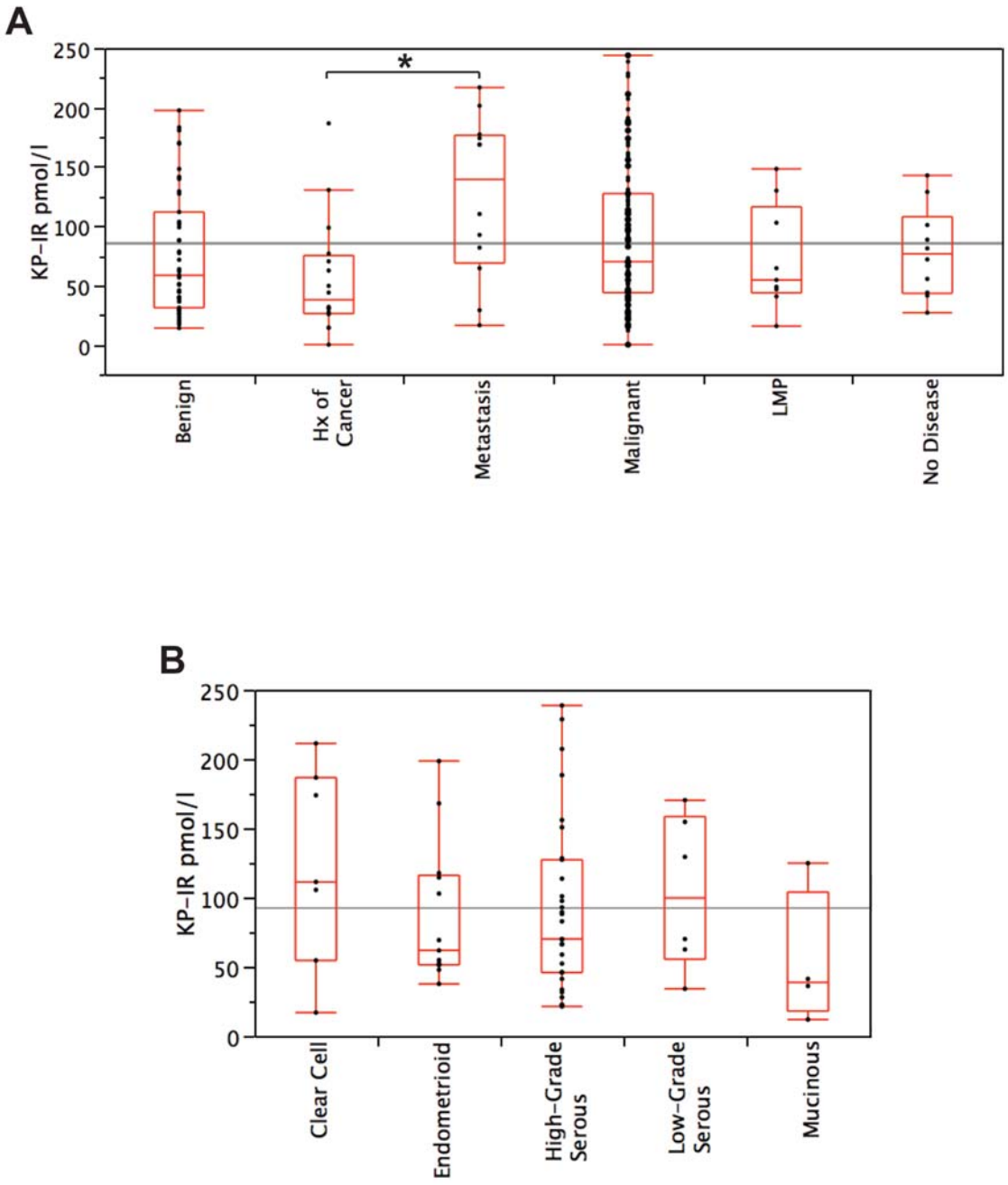

Figure 4.2 Plasma Kp correlation with malignant potential and subtype

A) Kp concentration in patients that have had a history of cancer is significantly different than patients who have developed a metastasis to the ovary (p=0.0258). B) Plasma Kp by ovarian cancer subtype. Description of groups is in Table 4.6. Hx=history, LMP=low malignant potential.

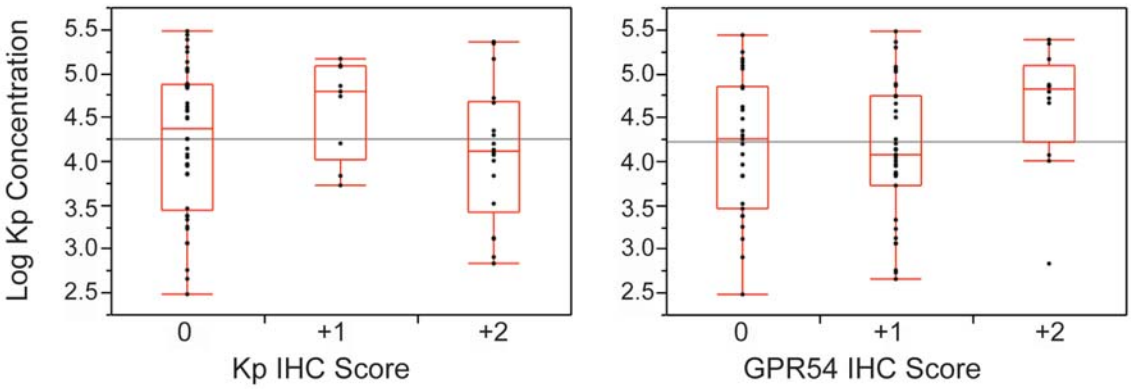

Figure 4.3 Plasma Kp compared to IHC expression

Boxplots showing relationship between IHC score on the x-axis and plasma Kp concentration on the y-axis for Kp IHC scores (left) and GPR54 IHC scores (right).

4.4 Conclusions

In our study we have determined in an independent cohort of patients that Kp and GPR54 are indeed markers for the ovarian cancer clear cell subtype. Furthermore, we have been able to measure plasma Kp in a variety of gyneacological diseases including malignancy. Although Kp has been measured in the plasma of gestational trophoblastic neoplasia [22] and pancreatic cancer patients [27], this is the first report of plasma Kp measurements in gynaecological disease and malignancy.

We have also determined that Kp protein expression varies across tissue microarrays originating from different institutions, though these TMAs are all comprised of the clear cell cancer subtype. We previously reported that Kp overexpression in ovarian cancer is an independent marker for favourable prognosis and correlated to the clear cell subtype [28]. This was established by classifying the highest Kp expressors (IHC score of +2) as positive. Using this same classification system, we have determined that the highest Kp expressors are a marker for favourable prognosis in disease specific survival for a clear cell specific TMA. Curiously, in a second cohort of clear cell patients, regardless of Kp IHC grouping, there was no significance associated with survival. This may be due to the differences in tissue preservation that may affect the stability of Kp in paraffin. However, as the preservation methods utilized by these institutions are unknown, we cannot explore this theory further.

We have also determined that in the gynaecological array, the Kp IHC results were not consistent with the plasma Kp levels. This may suggest that the

tissue is creating more Kp than is being maintained in the cells. This may be possible if Kp is being rapidly secreted into the bloodstream directly after translation. Indeed this hypothesis was first proposed by Bilban et al. [21] who were able to detect the 145aa KiSS1 pro-peptide in cell lysates, but unable to detect the mature Kp54 peptide. Assuming this hypothesis, the cells should have Kp mRNA levels that match the plasma Kp concentrations. However, Torricelli et al. [40] compared *KiSS1* mRNA expression in term placenta, pre-term placenta and placenta from term non-labour deliveries (caesarean section) and determined that there was significantly more *KiSS1* mRNA in placentas that had been delivered vaginally. The highest *KiSS1* mRNA levels were seen in the pre-term placentas, though all three types of deliveries had similar plasma Kp detected in the maternal blood. Unfortunately the antibody used in the EIA assay to measure the maternal plasma has since been shown to cross-react with neuropeptide FF [41] that is also secreted into the bloodstream [42].

Curiously, it is the metastasis group of patients that have a significantly higher level of plasma Kp as compared to those that had been previously treated for cancer. It is important to distinguish that the metastasis group does not consist of patients whose gynaecological cancer had metastasized, but of patients who had a primary non-gynaecological cancer that had metastasized to the ovary. It has been established that the ovary expresses Kp in rodents, and overexpresses Kp in cancer [28, 43]. Therefore this result could arise from the ovary or surrounding tissues expressing Kp as a means to control the invasiveness of the metastasis.

Currently CA125 is the most promising candidate as a serum biomarker for ovarian cancer. However, CA125 is only elevated in half of stage I cancers [44] and elevated in benign disease and normal physiological conditions such as pregnancy and menstruation [3, 45]. These characteristics make it a poor marker for early stage disease and have prevented its use as a diagnostic marker. However it is currently used to monitor disease progression as CA125 levels increase upon a secondary recurrence and remain stable in the absence of malignancy [3]. Unlike CA125, Kp is not elevated during menstruation, as Kp is barely detectable through all phases of the human female menstrual cycle [25]. It has also been established that after treatment and eradication of the initial cancer, plasma Kp rapidly drops back to barely detectable levels [22], demonstrating that in gestational trophoblastic disease Kp is highly specific for the disease. Furthermore, Kp is barely detectable in normal human male and female physiology outside of pregnancy [22-26]. However, similar to CA125, Kp is elevated in benign disease and pregnancy.

The current trend is to assess a panel of serum biomarkers to be used in conjunction with CA125 to monitor ovarian cancer progression to achieve higher specificity and sensitivity [46, 47]. We propose that Kp, although perhaps not a valid serum biomarker in itself, with further validation may be a valuable addition in a serum biomarker panel.

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5 ENDOGENOUS GPR54 IN CANCER CELL LINES IS INTRACELLULAR AND DOES NOT SIGNAL THROUGH THE CANONICAL Gαq PATHWAY[4]

Objective: To assess Kp-GPR54 signalling in malignant cells that endogenously express GPR54 by creating a stably transfected GPR54 knockdown cell line model.

[4] A version of this chapter will be submitted for publication. Prentice LM, Huntsman DG, and Aparicio SAJ. Endogenous GPR54 in cancer cell lines is intracellular and does not signal through the canonical Gαq pathway.

5.1 Introduction

KiSS1 was so named due to the metastatic *S*uppressor *S*equence that it encodes as well as a tribute to the city in which it was discovered; Hershey, Pennsylvania [1]. The translated peptide, originally named metastin, was first discovered to have anti-metastatic properties in a human melanoma cell line [1]. Since this discovery, there have been several *in vitro* cell line models created overexpressing either *KiSS1* or *GPR54* as a means to dissect the intracellular signalling pathway [1-10]. Though these cell line models have re-created the anti-metastatic properties originally observed, few studies have been able to determine the precise mechanism by which GPR54 signalling induces anti-metastatic behaviour.

Two of the original discovery papers attributed the anti-invasive effects of Kp to formation of focal adhesions and stress fibers; this has since been confirmed by a third group [5, 8, 11]. Kisspeptin expression and activation of GPR54 has also been linked to decreased matrix metalloproteinase activity, suggesting that a metastatically favourable environment created by matrix metalloproteinases is prevented [10, 12, 13]. Finally, Kp stimulated GPR54 has been suggested to inhibit chemotaxis, specifically through the prometastatic G protein-coupled receptor CXCR4, as CXCR4 was unable to respond to its ligand, SDF-1, after treatment with Kp-10 [8, 14].

The detection of intracellular calcium release upon Kp activation of GPR54 has been consistent amongst research groups, suggesting that Kp-GPR54 signalling is mediated through a $G\alpha_q$ coupled pathway [5, 7, 8]. Similarly, many of

these same groups have seen mitogen-activated protein kinase (MAPK) activation through the increased phosphorylation of the extracellular signal-regulated kinase (pERK) [5, 9, 15-17]. Some studies have demonstrated that phosphorylation of Akt is a downstream effect of GPR54 signalling [9, 16, 18], however this is less well established than MAPK activity.

Though there have been many studies overexpressing either *KiSS1* [4, 6, 10] or *GPR54* [2, 3, 5, 7-9]; no studies have yet been published reporting the impact of down-regulation of either ligand or receptor. Furthermore, the few studies based on cancer cell lines with endogenous GPR54 are renal [11, 13], pancreatic [15], and thyroid [16]. Our group is the first to assess endogenous GPR54 in ovarian and breast cancer cell lines, and to establish a successful receptor knockdown model to assess intracellular GPR54 signalling upon Kp stimulation.

5.2 Material and Methods

5.2.1 Cell lines

The MCF-7 breast cancer cell line (ATCC; HTB-22) was grown in Dubellco's modified Eagle's medium (DMEM; Stemcell Technologies, Vancouver, BC) supplemented with 15% fetal bovine serum (FBS; Gibco Invitrogen, Carlsbad, CA) while the MDA-MB-231 breast cancer cell line (ATCC; HTB-26) was grown in DMEM supplemented with 10% FBS. The ovarian cell lines TOV-21G (ATCC; CRL-11730, clear cell subtype), TOV-112D (ATCC; CRL-11731, endometrioid subtype), and OV90 (ATCC; CRL-11732, serous subtype) were all grown in a 1:1

mixture of MCDB 105 with 1.5 g/l sodium bicarbonate (Sigma-Aldrich, St. Louis, MA) and Medium199 (Stemcell Technologies, Vancouver, BC) supplemented with 15% FBS (Gibco Invitrogen, Carlsbad, CA). The 184 h-Tert cell line was an immortalized human breast epithelial cell line that was a generous gift from Dr. Sandra Dunn (Departments of Pediatrics, Experimental Medicine and Medical Genetics, University of British Columbia, Canada) and were grown in MEBM medium (Clonetics, San Diego, CA) supplemented with SingleQuots (Clonetics, San Diego, CA), 400 μg/ml neomycin (Gibco Invitrogen, Carlsbad, CA), 1 μg/ml transferrin (VWR, Mississauga, ON), and 1.25 mg/ml isoproteranol (Sigma-Aldrich, Oakville, ON). All cell lines were incubated at 37°C with 5% CO_2. Except for the 184 h-Tert cells, all cells were purchased from the American Type Culture Collection (ATCC; Manassas, VA).

5.2.2 RNA extraction

RNA was extracted from cell lines using Trizol (Invitrogen, Carlsbad, CA) as per manufacturer's recommendations. After a phosphate buffered saline (PBS) wash, Trizol was added to the tissue culture plate and cells were incubated at room temperature (RT) for 5 min. Chloroform (Sigma-Aldrich, Oakville, ON) was added to the Trizol treated sample at 20% of the volume and mixed vigorously for 15 sec before a second RT incubation of 2-3 m. Samples were then centrifuged at maximum speed for 30 min at 4°C. The aqueous layer was pipetted into a new eppendorf tube without disruption of the organic layer. RNA was precipitated from the aqueous layer with an equal volume of isopropanol and incubated at RT

for 10 min. The samples were centrifuged at maximum speed for 10 min at 4°C. The RNA pellets were washed with 75% ethanol and centrifuged at half maximum speed for 5 min at 4°C. The RNA pellets were air dried before being resuspended in an appropriate volume of DNAse/RNAse free water. RNA concentrations and purity were measured with a NanoDrop spectrophotometer (Thermo Fisher Scientific, Wilmington, DE).

5.2.3 Reverse transcription

One microgram of RNA was DNAse treated prior to reverse transcription to eliminate any contaminating genomic DNA from the sample. Samples were incubated at RT with DNAse I (Invitrogen, Carlsbad, CA) for 15 min before the addition of 25 mM EDTA (Invitrogen, Carlsbad, CA) and heated to 65°C for 10 min to stop the reaction as per manufacturer's recommendation. Samples were then immediately put on ice to cool. Random hexamers at 250 ng/μl (Invitrogen, Carlsbad, CA), 10 μM dNTPs (Invitrogen, Carlsbad, CA), 400 U MMLV-Reverse transcriptase enzyme (Invitrogen, Carlsbad, CA), 0.1 M DDT (Invitrogen, Carlsbad, CA), reverse transcriptase buffer (250 mM Tris-HCl pH 8.3, 375 mM KCl, 15 mM $MgCl_2$), 8 U RNase OUT enzyme, and water were added to the DNAse treated samples. Samples were incubated at 23°C for 10 min then incubated at 37°C for 1 h. The reaction was stopped by a 10 min 65°C incubation step.

5.2.4 Quantitative real-time PCR (QPCR)

The Applied Biosystems (ABI) 7900HT Fast Real-Time System was used to amplify and detect *KiSS1* and *GPR54* mRNA transcripts (Applied Biosystems, Foster City, CA). Gene specific probe and primer sets were ordered as 20x target assays from ABI specifically covering exon boundaries for human *KiSS1* (Hs00158486_m1) and *GPR54* (Hs00261399_m1). The 18s rRNA subunit (Hs99999901_s1) expression levels at 1/250 dilution were used as an endogenous loading control before comparative normalization to one of the samples included in the analysis. The ABI software calculated the gene expression values using relative quantification ($RQ=2^{-\Delta\Delta Ct}$).

5.2.5 Protein extraction and detection

Cells at 70-85% confluency were washed 2x with PBS before addition of radioimmunoprecipitation assay (RIPA) lysis buffer (150 mM NaCl, 50 mM Tris HCl pH 7.5, 1 mM EDTA, 0.1% SDS, 1% Sodium deoxycholate (NaDOC), 1% Triton-X 100) with protease inhibitors (Roche, Laval, QC) and incubated on ice for 20 min. Cells were then scraped from the tissue culture dish and centrifuged at maximum speed for 10 min at 4°C. The pelleted cell debris was then removed from the supernatant. Protein concentrations were assessed using either the Bradford assay (Bio-Rad, Hercules, CA) or the BCA Protein Assay Kit (Pierce, Rockford, IL) using the microplate procedure. Protein was detected by one of two methods. The first method consisted of running samples on a discontinuous SDS-polyacrylamide gel after heating with 10x sample buffer (60 mM Tris-Cl pH

6.8, 2% SDS, 10% glycerol, 0.025% bromophenol blue) at 95°C for 5 min, followed by wet transfer to a polyvinylidene fluoride (PVDF) membrane (Millipore, Billerica, MA) that was blocked with Tris buffered saline with 0.5% Tween (TBST) containing 5% fat-free milk powder or 5% bovine serum albumin (BSA; pERK detection) at RT for 1 h. The membrane was then incubated overnight in blocking buffer with the primary antibody at 4°C followed by incubation with a horseradish peroxidase (HRP) conjugated secondary antibody at RT for 1 h prior to enhanced chemiluminescent (ECL) detection (Amersham Bioscience, Buckinghamshire, UK) or SuperSignal detection (Pierce, Rockford, IL) onto x-ray film (Kodak, Burnaby, BC). Alternatively, the ovarian cell protein lysates, were run on a 4-12% gradient NuPage Bis-Tris precast gel (Invitrogen, Carlsbad, CA) after protein lysate was incubated with 4x NuPage LDS sample loading buffer and 10x Reducing Agent (Invitrogen, Carlsbad, CA) at 70°C for 10 min followed by semi-dry transfer onto nitrocellulose using the iBlot system (Invitrogen, Carlsbad, CA). Similarly, these membranes were blocked at RT for 1 h in Odyssey blocking buffer (LI-COR Biotechnology, Lincoln, NB) and incubated overnight with the primary antibody in blocking buffer at 4°C, followed by incubation with either goat anti-mouse or goat anti-rabbit secondary antibody conjugated to an infrared (IR)-dye before being visualized with the LI-COR Odyssey infrared detection system (LI-COR Biotechnology, Lincoln, NB). Protein levels were quantified using intensity values provided by the LI-COR software. A ratio was obtained by comparing the intensity of the protein of interest to the loading control before being normalized to a control sample on the same blot.

5.2.6 Antibodies and peptides

For the kisspeptins, two peptides were alternately used depending on the results of the assay. The literature argues that GPR54 has higher affinity for the smallest kisspeptin, Kp10, however there are many articles that have only found metastin as the biologically active peptide for GPR54 as discussed in Chapter 1. Consequently, if initial studies using one peptide were negative, then the second peptide was attempted. There were no differences between the two peptides throughout this chapter. Peptides used to stimulate the mitogen-activated protein kinase (MAPK) pathway were either; metastin (Kp-54 amide; Phoenix Pharmaceuticals, Burlingame, CA), kisspeptin-10 (Kp-10 amide; Phoenix Pharmaceuticals, Burlingame, CA) or epidermal growth factor (EGF: Sigma-Aldrich, Oakville, ON) as a positive control. Reagents used to stimulate the GPCR $G\alpha_q$ pathway were Kp-10 and adenosine triphosphate (ATP; Sigma-Aldrich, Oakville, ON) as a positive control. Cells were stimulated with either Kp-10 or Forskolin as a positive control for the cAMP assay. The antibodies that were used including their characteristics and concentrations are listed in summary Table 5.1.

5.2.7 Calcium mobilization assay

Intracellular calcium was measured by a single-wavelength indicator (Fluor-4) to assess any rapid release of calcium, and also measured by a dual-wavelength indicator (Fura-2 AM) to account for any uneven indicator distribution and/or concentrations. For Fluor-4 (Molecular Probes, Eugene, OR) experiments, 2 $x10^4$

cells were plated into an Opti-well polysterene 96-well plate (Nalge Nunc, Rochester, NY) after 1:100 poly-L-lysine treatment, and incubated overnight at 37°C with 5% CO_2. The cells were loaded with indicator and measured at 518 nm after a 444 nm excitation as per manufacturer's protocol. Relative fluorescent units (RFU) were determined using the equation: $RFU = F_t–F_o/F_o$

Where F_t was the averaged fluorescence emitted after treatment and F_o was the averaged fluorescence emitted before treatment. The RFU value for each cell type was averaged across three experiments.

For dual-wavelength experiments, cells were plated at 40,000 cells/well in a glass-bottomed black-walled 96-well sterile tissue culture plate (Nalge Nunc, Rochester, NY). After a physiological saline solution (PSS; 10 mM HEPES, 5.9 mM KCl, 1.4 mM $MgCl_2$, 1.2 mM NaH_2PO_4, 5 mM $NaHCO_3$, 140 mM NaCl, 11.5 mM glucose, 18 mM $CaCl_2$) wash, the cells were loaded with 4 μM Fura-2 AM (Invitrogen, Carlsbad, CA) at RT for 30 min. Cells were then washed and incubated in PSS at RT for an additional 30 min in the dark. Dual kinetic measurements were obtained at 340 nm (excitation 340 nm, emission 518 nm) and 380 nm (excitation 390 nm; emission 518 nm) using a Fluoroskan Ascent FL (ThermoLabsystem, Helsinki, Finland) for ratiometric analysis of calcium release. Background was measured from empty wells containing only PSS and Fura-2 AM and was subtracted from the 340 nm and 380 nm measurements before obtaining the 340/380 ratios. To determine basal calcium levels, five dual-kinetic measurements were made before the addition of 1 μM Kp10 or 1 μM ATP by injection. Twenty to sixty measurements with five-second intervals were

conducted after the injection of the stimulus. To calculate concentrations of released calcium, 10 μM ionomycin with 40 μM EGTA was added to the cells after the 60 measurements and the cells were measured again 80 times at 20 sec intervals. These final, stabilized EGTA measurements were considered the calcium-free measurement while addition of 100 mM $CaCl_2$ in 10 μM ionomycin measured 60 times with 20 sec intervals was considered the calcium-bound measurement. Finally, 2 mM $MnCl_2$ in 10 μM ionomycin was added to the cells and measured 60 times at 20 sec intervals for the background measurements to be subtracted from all previous measurements. The internal calcium concentration was calculated as: $[Ca^{++}]i\ nM = 135((R-R_{min})/(R_{max}-R))(F_{380}/B_{380})$. Where 135 is the disassociation constant for Fura-2 at RT, and R is the ratio of the treatment (Kp-10 or ATP) being assessed. R_{min} is the averaged ratio of the final three calcium-free measurements, while R_{max} is the averaged ratio of the final three calcium-bound measurements. F_{380} is the averaged calcium-free 380 nm measurement and B_{380} is the averaged calcium-bound 380 nm measurement. For basal calcium concentrations, ratios were obtained prior to addition of stimulus, while max calcium concentrations where obtained after the addition of Kp or ATP. Each basal and max calcium concentration is the average of three individual experiments.

5.2.8 Cyclic AMP assay

2×10^4 cells per well were plated out into a 96-well plate and incubated overnight at 37°C with 5% CO_2. Cells were stimulated with either Kp-10 (0 nM, 1 nM, 10

nM, 100 nM, 1 μM, 10 μM) or 10 μM Forskolin, as a positive control, in a solution containing 50 μM isobutylmethylxanthine (IBMX). After a 30 min incubation at 37°C with the stimulus, cells were lysed and intracellular cAMP levels were measured by Biotrak competitive binding enzymeimmunoassay (EIA) as per manufacturer's protocol (Amersham, Piscataway, NJ). Control wells that were not carried forward with the cAMP assay were assessed for complete cell lysis by trypan blue staining.

5.2.9 Flow cytometry

Cells at 70-85% confluency were washed with PBS before a 3 min incubation at 37°C with 0.05% trypsin-EDTA (Gibco Invitrogen, Carlsbad, CA) to detach the adherent cells from the tissue culture plate. Detached cells were placed into cold Hank's Balanced Salt Solution (Gibco Invitrogen, Carlsbad, CA) with 2% FBS (HF) and centrifuged at 1200 rpm for 5 min. Cells were then resuspended in 10 ml of HF and filtered using a 40 μm filter (BD Biosciences, Mississauga, ON) before counting live cells. Each treatment and control used $7x10^5$ cells. The permeabilized cells were incubated on ice in 4% paraformaldehyde (Electron Microscopy Sciences, Hatfield, PA) for 20 min before addition of Trition-X 100 (Sigma-Aldrich, Oakville, ON) to a final concentration of 0.1% and incubated for a further 10 min. All cells were incubated with 10 μg of human IgG anti-CD32 (ATCC; Manassas, VA) in HF as a blocking solution for 30 min on ice. Cells were then incubated with 1:50 GPR54 MBL#29 antibody (Ab) on ice for 30 min prior to incubation with the FITC conjugated anti-rabbit secondary Ab (Jackson

ImmunoResearch, West Grove, PA) at 1:200 for 15 min on ice. Finally, cells were incubated with 1:1000 propidium iodide (PI) for 5 min before assessment by a FACsort cell sorter (BD Bioscience, San Jose, CA). Negative controls were: no primary Ab, GFP Ab as an isotype control, and PI only. Cells were gated by shape using side scatter versus forward scatter then assessed for PI levels and FITC levels to determine dead versus live cells and GPR54 expressors versus non-expressors.

5.2.10 Immunofluorescence

Cells were plated onto sterile coverslips in a 6-well plate in normal growth media and incubated at 37°C with 5% CO_2 overnight. Cells were washed 2x with PBS before incubation with 4% paraformaldehyde (Electron Microscopy Sciences, Hatfield, PA) at RT for 20 min. Cells were then washed 3x with PBS before permeabilization with 0.1% Triton-X (Sigma-Aldrich, Oakville, ON) for 5 min at RT. A 5 min RT incubation with 0.1 M Glycine was used to decreased background fluorescence, prior to 1 h RT incubation with blocking buffer (8% BSA, 5% human serum, 5% donkey serum in PBS). Coverslips were incubated overnight at 4°C with 1:100 GPR54 MBL#29 in blocking buffer before 1 h RT incubation with 1:200 FITC-conjugated donkey anti-rabbit (Jackson ImmunoResearch, West Grove, PA) in PBS with 1% BSA. 1:5000 Draq5 (Alexis Biochemicals, San Diego, CA) or 1:1000 PI was used as a nuclear stain and omission of the primary antibody was used as a negative control. Images were acquired using a fluorescent confocal microscope (Nikon Instruments,

Mississauga, ON) and accompanying software. For β-arrestin co-localization studies, 1:100 β-arrestin was used with 1:100 GPR54 MBL#29. Secondary antibody contained 1:200 of both donkey anti-rabbit conjugated with Cy-5 and donkey anti-goat conjugated to FITC. For co-localization of GPR54 with actin, an Oregon Green 488-conjugated phalloidin molecule was used (Molecular Probes, Invitrogen, Carlsbad, CA).

5.2.11 *GPR54* shRNA knockdown

After attempts at transient siRNA knockdown of GPR54 failed, a more stable knockdown method was used. Short hairpin RNAs for human GPR54 in pLKO.1 vectors were used to create stably transfected cell lines (shRNA11605, shRNA11606, shRNA1108, shRNA1109; Open Biosystems Thermo Fisher Scientific, Huntsville, AL). A pLKO.1 empty vector (Origene, Rockville, MD) and a pLKO.1 vector containing a non-specific scramble sequence (Addgene, Cambridge, MA) were used as negative controls. Hairpin containing vectors were purified from ampicillin resistant bacteria by an endotoxin free Maxi Prep kit as per manufacturer's recommendations (Qiagen, Mississauga, ON). Vectors were restriction digested and sequenced by Sanger method to verify for accuracy. Cells were transfected using Lipofectamine 2000 (L2K; Invitrogen, Carlsbad, CA) as per manufacturer's recommendations. Due to the variety of the cell lines used in this experiment (MCF-7, TOV21G, TOV112D, OV90), transfection and puromycin selection conditions were optimized for each cell line prior to knockdown experiments. Based on these optimizations each cell line was

transfected using at least two different L2K to DNA concentrations and assessed for successful GPR54 knockdown by western blot assay. The summary of these experiments is listed in Table 5.2. For transfected cells that successfully formed puromycin resistant clones, these colonies were pipetted from the plate into a new 24-well plate.

5.2.12 *KiSS1* siRNA knockdown

Transient knockdown of *KiSS1* was performed as per manufacturer's recommendations using three separate siRNAs (siRNA08, siRNA09, siRNA10; Dharmacon, Chicago, IL) in the TOV21G cells. Transfection methods for this cell line were based on successful lamin knockdown using Dharmacon reagents. Cells at 70% confluency in a 6-well plate were treated with either 50 nM DharmaFECT reagent 2 alone as a transfecting reagent control, 50 nM with siControl #2 as a non-specific target control, 50 nM of either of the *KiSS1* siRNAs (08, 09, 10), and the last remaining well was left untreated. This experiment was done twice; once in eightfold for four time points (24 h, 48 h, 72 h, 96 h) to determine GPR54 localization by immunofluorescence (IF) with matched RNA samples to determine *KiSS1* knockdown, and once in six fold for three time points (12 h, 36 h, 60 h) to determine GPR54 localization by flow cytometry with matched RNA samples to verify *KiSS1* knockdown.

Table 5.1 Summary of antibodies used

Antibody	Target	Host	Clonality	Supplier	Use	Dilution
β-arrestin-1 (K-16)	near C-terminus	Goat	Polyclonal	Santa Cruz	IF	1/100
GPR54 LS-A1929	N-terminus	Rabbit	Polyclonal	MBL International	IF	1/100
					WB	1/1000
					FC	1/50
pERK	p44/p42 MAPK	Rabbit	Polyclonal	Cell Signalling	WB	1/1000
tERK (44/42 MAPK)	clone 1B3B9	Mouse	Monoclonal	Upstate Biotechnology	WB	1/5000
β-actin	N-terminus	Mouse	Monoclonal	Sigma-Aldrich	WB	1/5000
Vinculin	entire protein	Mouse	Monoclonal	Sigma-Aldrich	WB	1/10000

IF=immunofluorescence, WB=western blot, FC=flow cytometry

Table 5.2 Summary of transfection conditions

Cell Line	Puromycin	L2K (ug/ml)	Vector (ug/ml)	Clones	GPR54 KD
MCF-7	400ng/ml	3.5	3.5	No	No (p1)
		4.5	3	No	No (p1)
		3.5	3	No	ND
TOV112D	600ng/ml	3.5	3.5	No	No (p1)
		4.5	3	No	ND
		6	3	Mixed	No (p2)
				C1	Yes (p3)
TOV21G	2ug/ml	4.5	3.5	No	No (p1)
		6	3	Mixed	No (p2)
				C1	Unclear (p3)
				C2	Yes (p3)
OV90	1ug/ml	7	3.5	No	No (p1)
		8	4	No	Yes (p2)

L2K=lipofectamine 2000, KD=knockdown, p=passage number, ND=not determined

5.3 Results

5.3.1 GPR54 and Kp expression varies across a panel of cell lines

To determine which cell lines would be best suited for *in vitro* functional studies of GPR54 signalling, it was imperative to determine endogenous expression levels of both the receptor and ligand across a panel of cell lines. In the ovarian cell lines, GPR54 protein was most highly expressed in the OV90 serous cell type, and least expressed in the TOV21G clear cell line (Figure 5.1 A). *KiSS1* expression did not vary dramatically across the ovarian cell line subtypes and was most highly expressed in the MDA-MB-231 breast cancer cell line (Figure 5.1 B). Of the breast cell lines, GPR54 was most highly expressed in the MCF-7 cell line and the MDA-MB-231 cells had low expression (Figure 5.1 A). Of note, the immortalized normal epithelial mammary cell line 184 h-Tert did not express either GPR54 or *KiSS1* and was used as an endogenous negative control for some of the studies.

RNA expression of *GPR54* as determined by real-time PCR correlated well with the protein levels determined by western blot analysis (linear regression r^2=0.94471). Only RNA expression of *KiSS1* could be determined for the cell lines as a specific antibody that works for western blotting has not yet been made available.

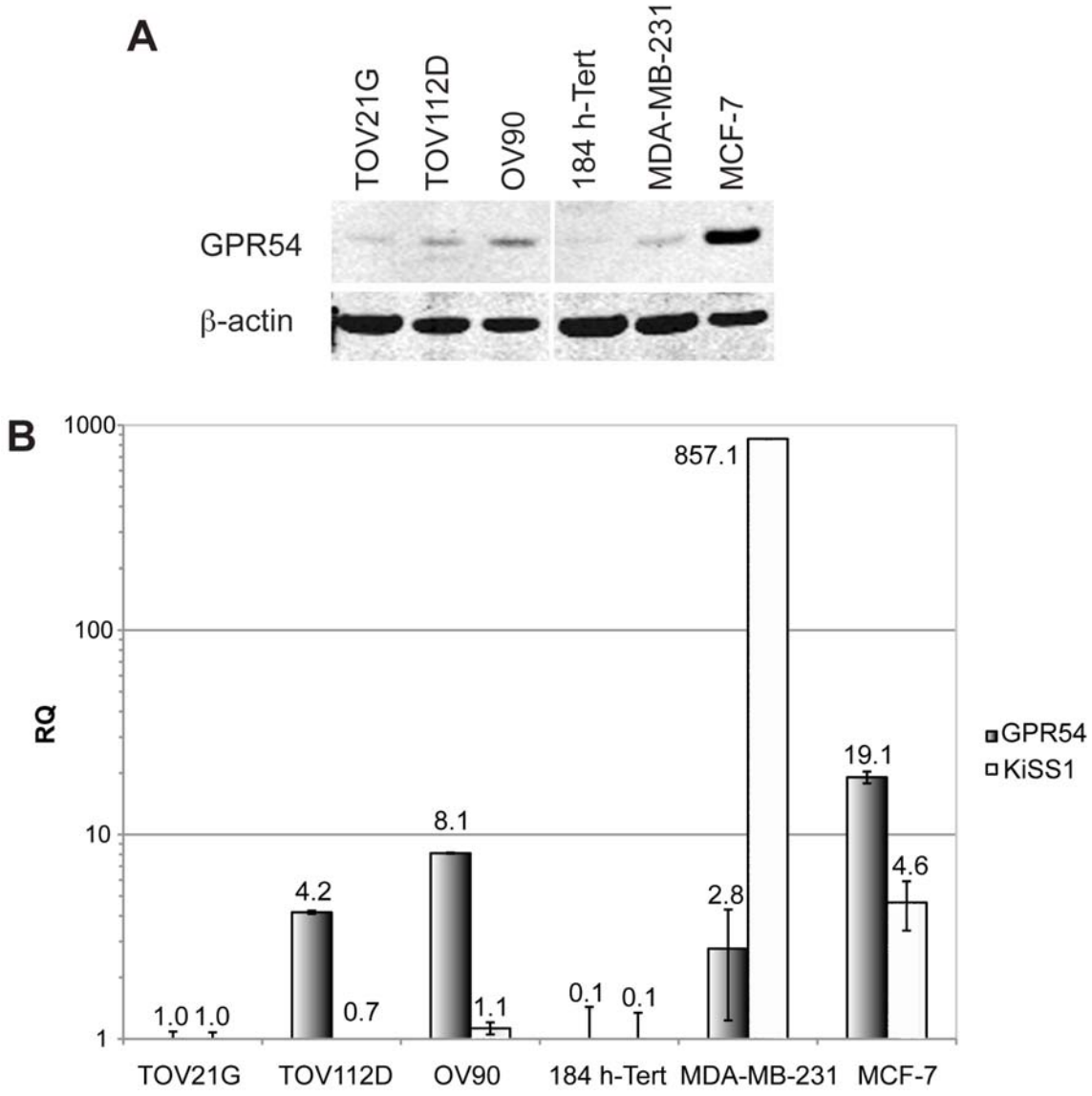


Figure 5.1 Comparison of *GPR54* and *KiSS1* expression across cell lines
A) Protein levels of GPR54 in the ovarian cell lines TOV21G, TOV112D, and OV90 as well as in breast cell lines, 184 h-Tert, MDA-MB-231, and MCF-7 by immunoblot analysis. B) Relative quantification of RNA levels of *GPR54* and *KiSS1* in the cell lines shown in (A), all normalized to the ovarian clear cell line TOV21G. Error bars represent standard error of the mean (SEM) across three replicates.

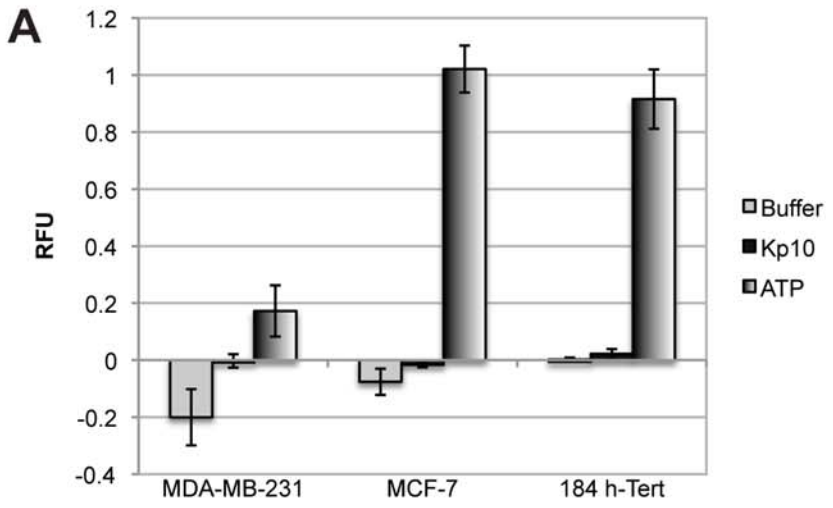
5.3.2 GPR54 does not signal through the $G\alpha_q$ or the $G\alpha_s$ signalling pathways upon Kp stimulation in breast cancer cell lines

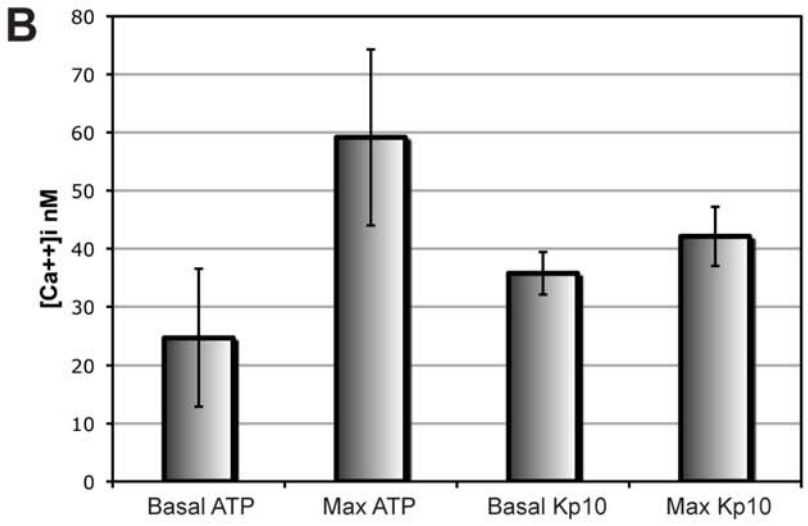
It has been previously reported that GPR54 signals through the $G\alpha_q$ pathway that involves intracellular calcium release upon ligand binding. In order to assess receptor function in GPR54 expressing cell lines, we stimulated cells with either 1 μM Kp-10 or 1 μM Kp-54 and measured calcium release; as Kp-10 and Kp-54 have been previously shown to have equivalent effects on GPR54. Initial studies conducted in the MDA-MB-231, MCF-7, and 184 h-Tert breast cell lines showed no response to Kp-10 although the MCF-7 and 184 h-Tert cells responded strongly to 1 μM ATP (Figure 5.2 A). Confirming the presence of calcium stores capable of large release, the MCF-7 cells were responsive to 1 μM ATP and released 34.4 ± 3.3 nM of intracellular calcium. However, the addition of 1 μM Kp-10 did not significantly increase calcium levels above basal levels (Figure 5.2 B).

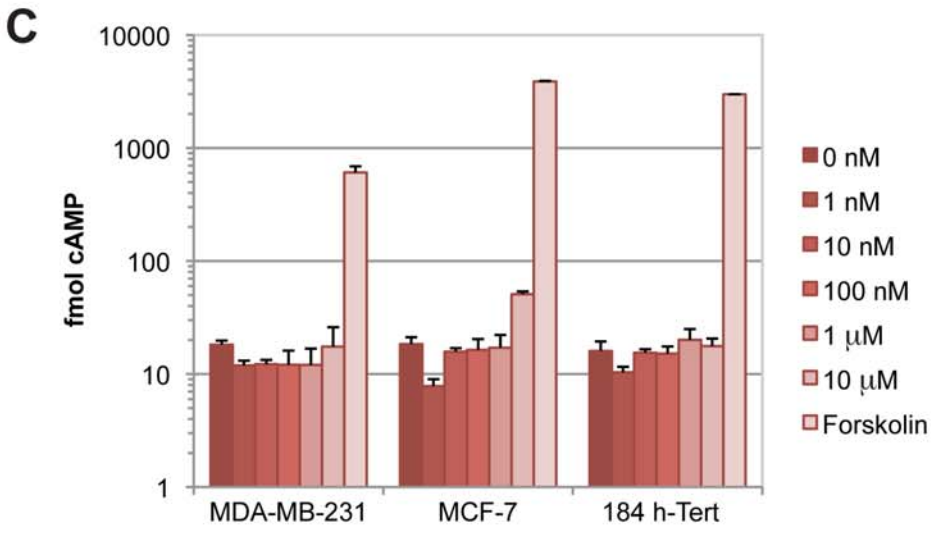
To ensure that GPR54 was not signalling through an alternative G-protein coupled pathway, cyclic-AMP levels were measured as an indicator of $G\alpha_s$ activation. Intracellular cAMP did not increase with 1 μM Kp-10 stimulation of the MDA-MB-231, MCF-7, or 184 h-Tert cells. At 10 μM of Kp-10, levels of cAMP increased in the MCF-7 cells from 18 fmol (at 0 nM Kp-10) to 51 fmol suggesting that super-physiological levels of Kp-10 could force signalling through the $G\alpha_s$ pathway (Figure 5.2 C).

Figure 5.2 GPR54 does not signal through $G\alpha_q$ or $G\alpha_s$ in mammary cell lines

A) 1 μM Kp-10 did not elicit a calcium flux from the MDA-MB-231, 184 h-Tert, or MCF-7 cell lines, although the 184 h-Tert and MCF-7 cells responded readily to 1 μM ATP as shown by increased relative fluorescence (RFU on the y-axis). B) The intracellular release of calcium ($[Ca^{++}]i$ on the y-axis) could be calculated above basal levels in the MCF-7 cells when stimulated with 1 μM ATP, but there was no detectable difference when stimulated with 1 μM Kp-10. C) Kp-10 stimulation of GPR54 did not signal through an alternate $G\alpha$ pathway as determined by cAMP assessment (fmol cAMP on the y-axis) in the breast cell lines. Error bars represent ±SEM and are representative of three experiments in the calcium release assays (A and B), while they are indicative of duplicates in the cAMP study (C).


A
1.2
1
0.8
0.6
0.4
0.2
0
-0.2
-0.4
RFU
MDA-MB-231
MCF-7
184 h-Tert
Buffer
Kp10
ATP


B
80
70
60
50
40
30
20
10
0
[Ca++]i nM
Basal ATP
Max ATP
Basal Kp10
Max Kp10


C
10000
1000
100
10
1
fmol cAMP
MDA-MB-231
MCF-7
184 h-Tert
0 nM
1 nM
10 nM
100 nM
1 μM
10 μM
Forskolin

5.3.3 The MAP Kinase pathway is activated upon Kp stimulation of GPR54 expressing breast and ovarian cells

Although the downstream effect of GPR54 $G\alpha_q$ signalling is to activate the mitogen-activated protein kinase (MAPK) pathway, we asked whether MAPK activation could occur in the absence of $G\alpha_q$ mediated calcium release. To measure MAPK activity, phosphorylated extracellular signal-regulated kinase (pERK) levels were determined by western blot after stimulation with Kp. The MCF-7 cells had a rapid increase of pERK (5 min) after stimulation with 1 μM Kp-10, and this was dose dependent (Figure 5.3 A). Similar results were seen with the addition of 1 μM Kp-54 (results not shown). As 1 μM Kp gave reproducible results and was most often cited in the literature, this concentration was used for the rest of the MAPK activation experiments.

The MDA-MB-231 cells have increased endogenous levels of pERK due to a K-ras mutation that constitutively activates the MAPK pathway, thus GPR54 activity could not be determined upon the addition of 1 μM Kp-10 or 1 μM Kp-54, even under serum starved conditions (Figure 5.3 B). As expected, the GPR54 non-expressing 184 h-Tert cell line has no discernable levels of pERK after 1 μM Kp-10 or 1 μM Kp-54 stimulation (Figure 5.3 B).

For the ovarian cancer cell lines, which all express GPR54, there were increased levels of pERK upon 1 μM Kp-54 stimulation (Figure 5.3 C). Although the ovarian TOV21G clear cells express the lowest levels of GPR54, they had the highest upregulation of pERK upon Kp-54 stimulation; the highest fold

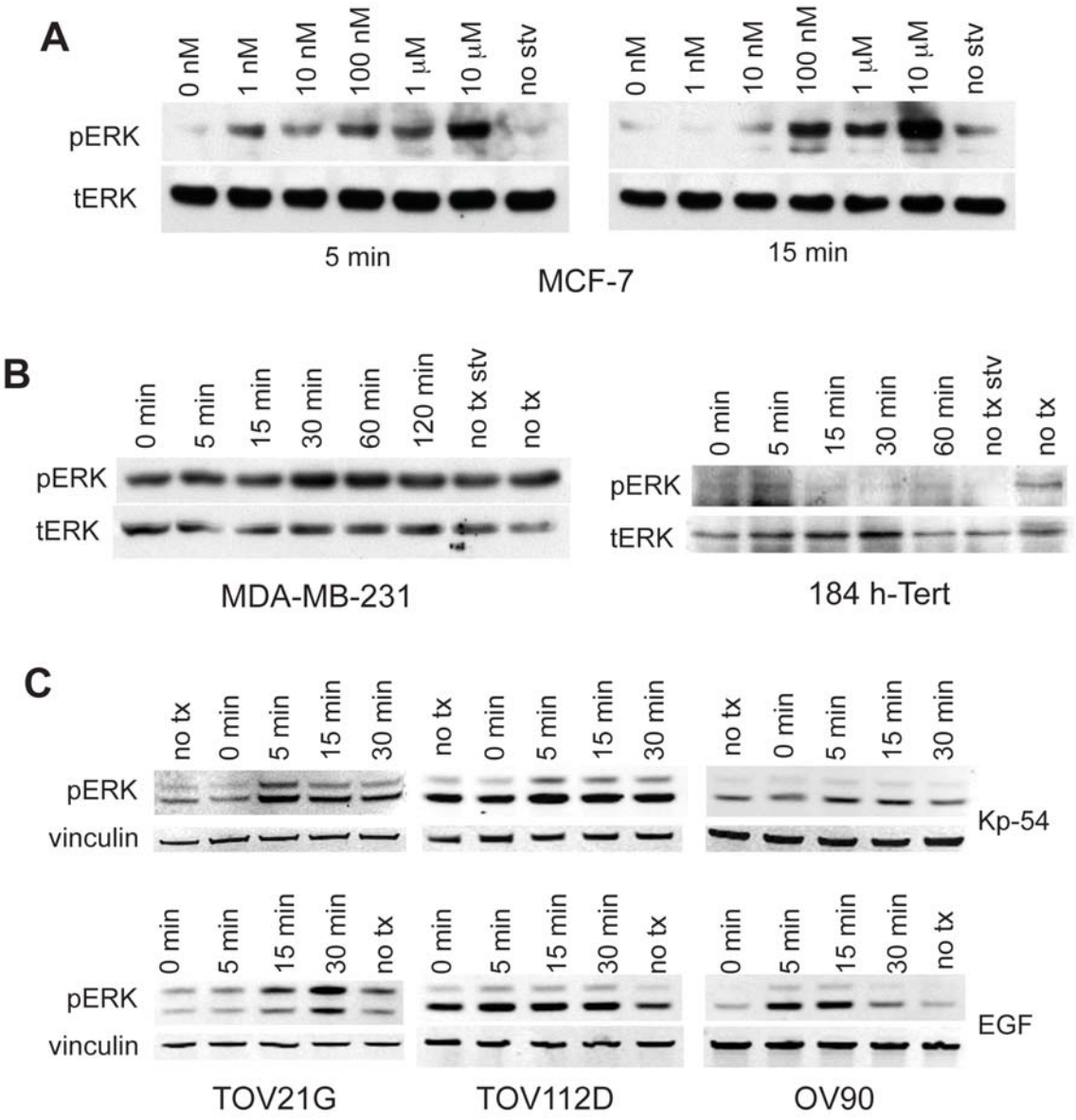


Figure 5.3 MAPK activation by Kp stimulation of GPR54

A) MAPK activation in MCF-7 cells at 5 min (left) and 15 min (right) after Kp-10 stimulation in serum starved conditions. The last lane shows pERK levels in normal medium without Kp-10 stimulation (no stv). B) MAPK activation in MDA-MB-231 cells (right) and 184 h-Tert cells (left) after stimulation with 1 μM Kp-10 in serum starved conditions. Last two lanes are Kp-10 untreated cells in either serum starved conditions (no tx stv) or in normal medium (no tx). C) MAPK

activation in ovarian cancer cell lines treated with 1 μM Kp-54 in serum starved conditions (top) compared to 10 ng/ml EGF used as a positive control. Vinculin is used as the loading control as tERK could not be determined due to a different blotting technique used in this assay, however tERK and vinculin levels were similar in these cell lines when assessed together in a separate experiment.

change was 2.33 5 min after treatment when compared to untreated cells. Comparatively, the TOV112D cells had a maximum fold increase of 1.63 at 5 min after treatment and the OV90 cells had a maximum fold increase of 1.82 at 10 min after treatment. As reproducible results were achieved with Kp-54 stimulation of the ovarian cancer cell lines, stimulation with Kp-10 was not performed. Cells were treated with 10 ng/ml EGF as a positive control.

Of note, p42 and p44 ERK can be distinguished in the ovarian cancer cells, and were not obvious in the breast cells. This is most likely due to the infrared detection system used for the ovarian cells whereas chemiluminescence was used to detect pERK signal in the breast cells. However, due to the infrared detection system, tERK could not be measured on the same blot as pERK and vinculin was used as a replacement. Vinculin and tERK were analyzed on the same immunoblot to ensure vinculin was an adequate substitute.

5.3.4 Knockdown of GPR54 is a temporary effect and does not affect pERK activation upon Kp10 stimulation

After several unsuccessful attempts to knockdown GPR54 by transient short interfering RNAs (siRNA), a more permanent short hairpin (shRNA) method was used. Of the MCF-7, TOV21G, TOV112D, and OV90 cells transfected with the RNA hairpins, only the ovarian cell lines had successful knockdown of GPR54. All cell lines were transfected with three different hairpins (shRNA05, shRNA06, shRNA07) and at least two separate transfection parameters were used after lipofectamine:vector ratio was optimized. Of these four cell lines, only the three ovarian lines had detectable knockdown of GPR54 by western blot. Of these

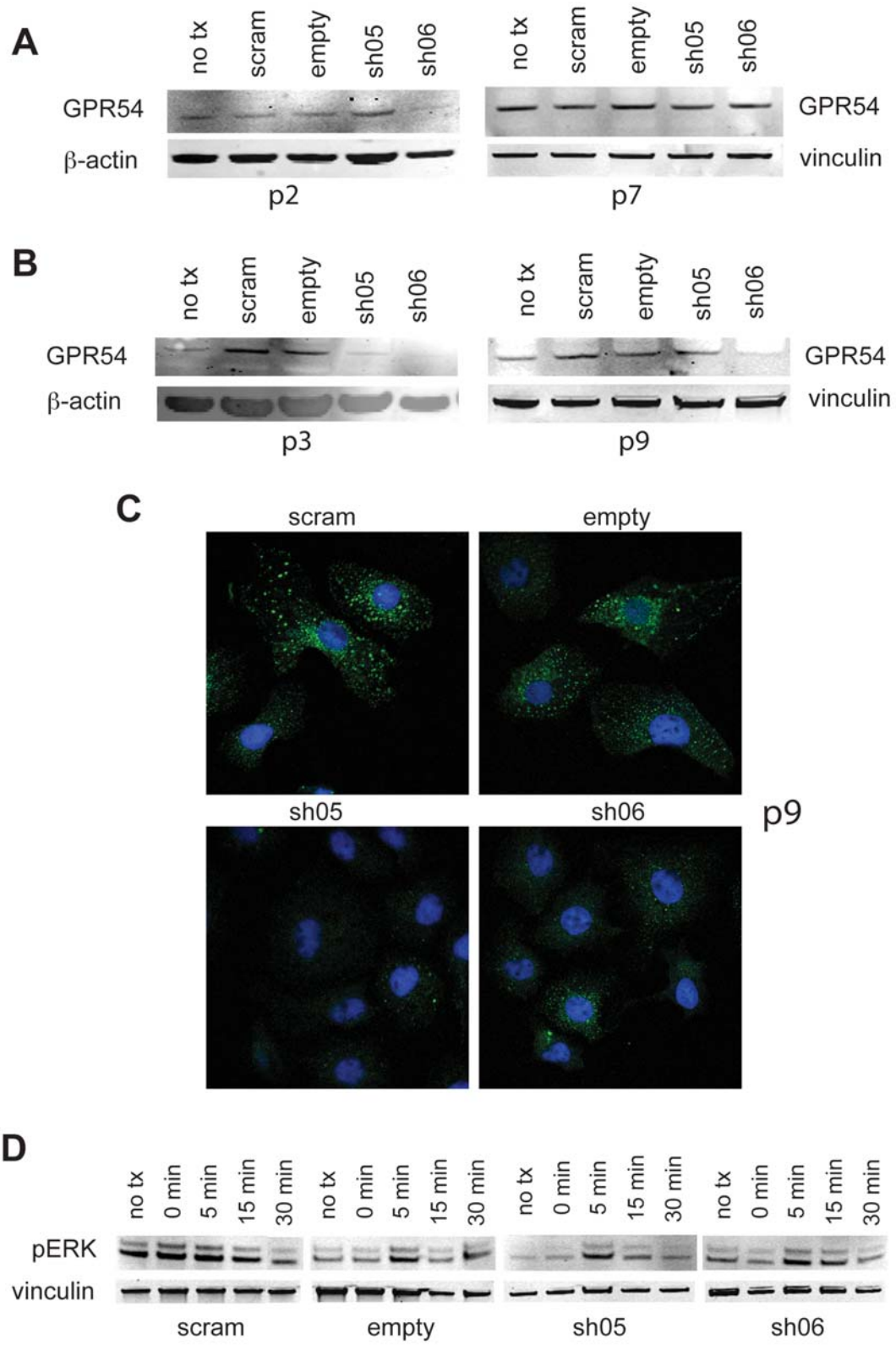
three cell lines only the TOV21G and TOV112D successfully formed puromycin resistant clones. All the transfection parameters and results for each cell line are summarized in Table 5.2.

For the OV90 cells, at passage 2 (p2) after transfection, GPR54 was successfully knocked-down by 85% using shRNA06 as compared to untreated cells. However by p7, GPR54 protein levels only had 5% knockdown in the shRNA06 treated cells (Figure 5.4 A). For the TOV21G cells, shRNA05 and shRNA06 successfully knocked down GPR54 by p3 after transfection, and by p9 shRNA06 had maintained GPR54 knockdown by 88% as compared to untreated cells (Figure 5.4 B).

IF detection of GPR54 in the TOV21G cells confirmed that GPR54 protein was decreased in the shRNA05 and shRNA06 hairpin expressing cells at p9 (Figure 5.4 C). Having established prolonged knockdown of GPR54 in TOV21G cells, we investigated the consequences for pERK signalling at 1 μM Kp-10. Despite knockdown of GPR54, the TOV21G shRNA expressing cells and their controls all had upregulation of pERK upon 1 μM Kp-10 stimulation at p8 and again at p13 (Figure 5.4 D). Specifically the shRNA05 expressing cells had a maximum fold increase of 1.98 at 5 min after treatment while the shRNA06 expressing cells had a maximum fold increase of 1.94 at 5 min, compared to untreated cells. The TOV21G shRNA scramble control cells proliferated more quickly than the other transfected cells and increased cell confluency may account for raised basal levels of pERK seen in no tx, and 0 min control lanes.

Figure 5.4 Stable knockdown of GPR54 in TOV21G but not OV90 ovarian cells

A) Immunoblot showing GPR54 expression in OV90 cells at passage 2 (p2; left) and p7 (right). Cells either received no treatment (no tx), scramble vector control (scram), empty vector control (empty), or a RNA hairpin specific for GPR54 (sh05 and sh06). B). Same experiment as described in (A) using the TOV21G ovarian cells at p3 (left) and p9 (right). β-actin was used as a loading control in early passage (left) and vinculin was used as a loading control in later passage (right). C) FITC-labeled GPR54 (green) confirms knockdown in the shRNA05 and shRNA06 treated TOV21G cells as compared to the scramble and empty vector controls at passage 9. D) MAPK activation as determined by pERK in stably transfected TOV21G cells at p13. Cells are treated with 1 μM Kp-10 except for the no tx lanes; each lane represents time after Kp stimulation. p=passage, sh=short hairpin RNA


A
no tx
scram
empty
sh05
sh06
GPR54
β-actin
p2
p7
vinculin
B
p3
p9
C
scram
empty
sh05
sh06
p9
D
0 min
5 min
15 min
30 min
pERK
vinculin

5.3.5 GPR54 is almost entirely cytoplasmic in MCF-7, TOV21G, TOV112D, and OV90 cells

Although knockdown of GPR54 protein was observed in the TOV21G cells, we noticed that the GPR54 immunoreactivity was mostly cytoplasmic. Since GPR54 signalling through exogenous peptide normally requires a membranous receptor, we investigated the localization of GPR54 in the cancer cell lines. We used flow cytometry to determine if GPR54 was on the surface of the four cell lines, MCF-7, TOV21G, TOV112D, and OV90. We compared GPR54 expression of live intact cells to permeabilized cells, as live cells would only provide a signal for extracellular (membranous) GPR54 and permeabilized cells would reveal intracellular (cytoplasmic) GPR54.

The intact cells had low GPR54-FITC labeled signal indicative of minimal cell surface protein. Approximately one percent of the OV90 and theTOV21G cells had surface GPR54, while only 0.5% of the TOV112D and MCF-7 cells had plasma membrane GPR54 when compared to cells that had the GPR54 antibody omitted as a negative control (Figure 5.5). Of note, we were unable to locate a cell line with confirmed plasma membrane GPR54 to use as a positive control.

Comparatively, all cells had an approximately 10-fold increase in GPR54 signal after permeablization when compared to negative controls lacking the primary antibody; indicating a strong cytoplasmic signal for GPR54. Cytoplasmic GPR54 expression in ovarian cells compared to membranous expression is depicted in flow cytometry histograms in Figure 5.6 B.

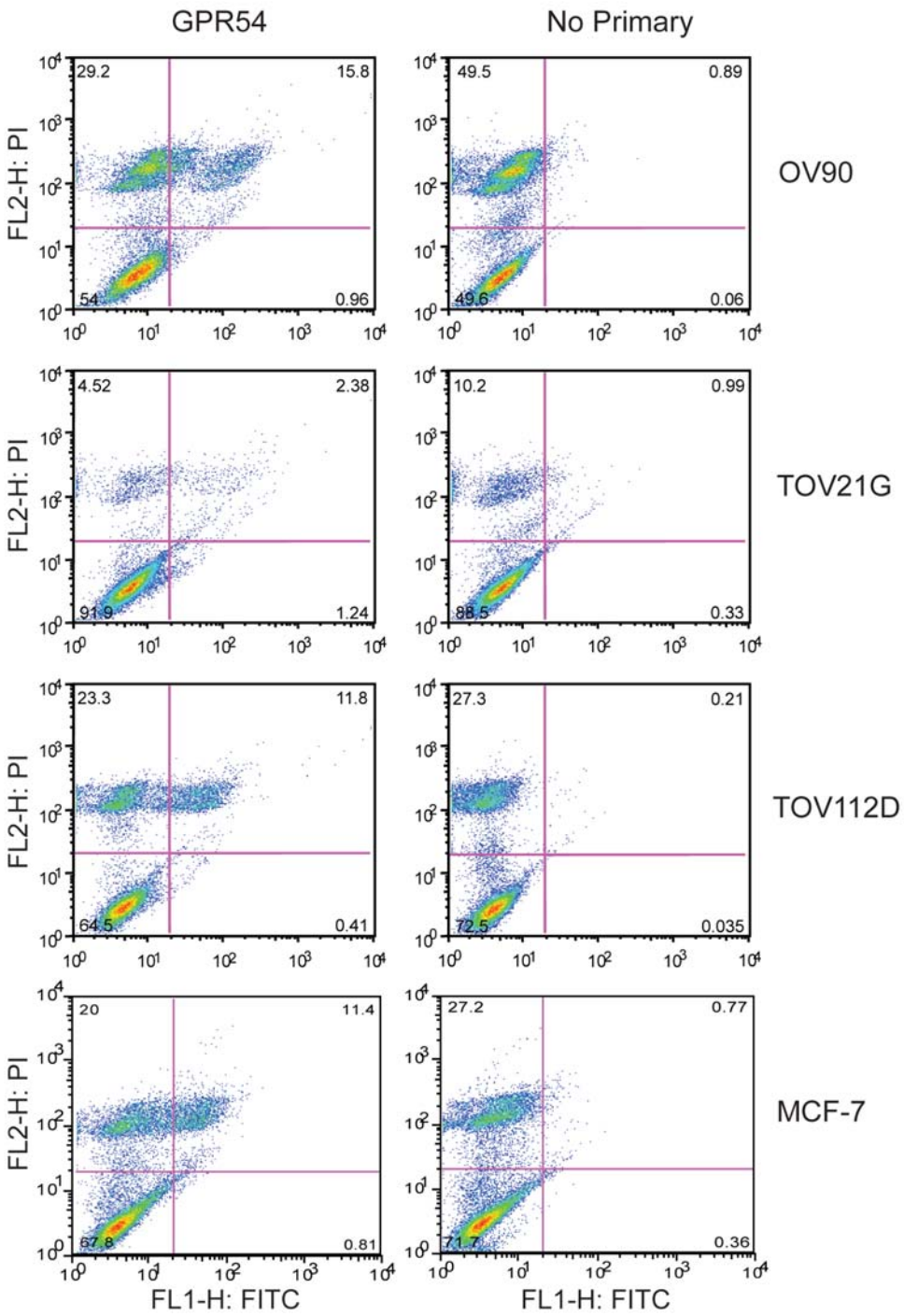


Figure 5.5 Cell line expression of GPR54 on the plasma membrane as determined by flow cytometry

Vertical axis measures propidium iodide (PI) signal indicative of cell death, while the horizontal axis measures FITC indicative of GPR54 signal. Percent of live cells expressing GPR54 are displayed in the lower right quadrant of each graph.

5.3.6 Intracellular GPR54 co-localizes with β-arrestin but does not return to the membrane after knockdown of endogenous Kp in TOV21G cells

G-protein coupled receptors may become internalized through phosphorylation of the intracellular C-terminus and subsequent β-arrestin binding to the phosphorylated site. β-arrestin can then signal for endocytosis of the receptor; either sequestering it to the cytoplasm or sending the receptor for protein degradation (as reviewed in [19, 20]). To determine whether GPR54 was co-localized with β-arrestin, we performed dual IF in TOV21G cells (Figure 5.6 C). GPR54 was confirmed to be intracellular in TOV21G cells by IF when labeled actin revealed that the cell membrane did not co-localize with GPR54. However, cytoplasmic GPR54 could be detected (Figure 5.6 A).

G-protein coupled receptors may become desensitized through continuous ligand binding which can lead to receptor internalization [20]. Since all four of the cell lines studied have *KiSS1* expression (Figure 5.1), we hypothesized that endogenous Kp may be desensitizing GPR54. Therefore, we decreased *KiSS1* transcripts by siRNA in the TOV21G cells to assess if GPR54 was being desensitized by autocrine Kp expression. However, the knockdown of *KiSS1* in TOV21G cells did not shift GPR54 from the cytoplasm back to the membrane as determined by flow cytometry and IF (Figure 5.7).

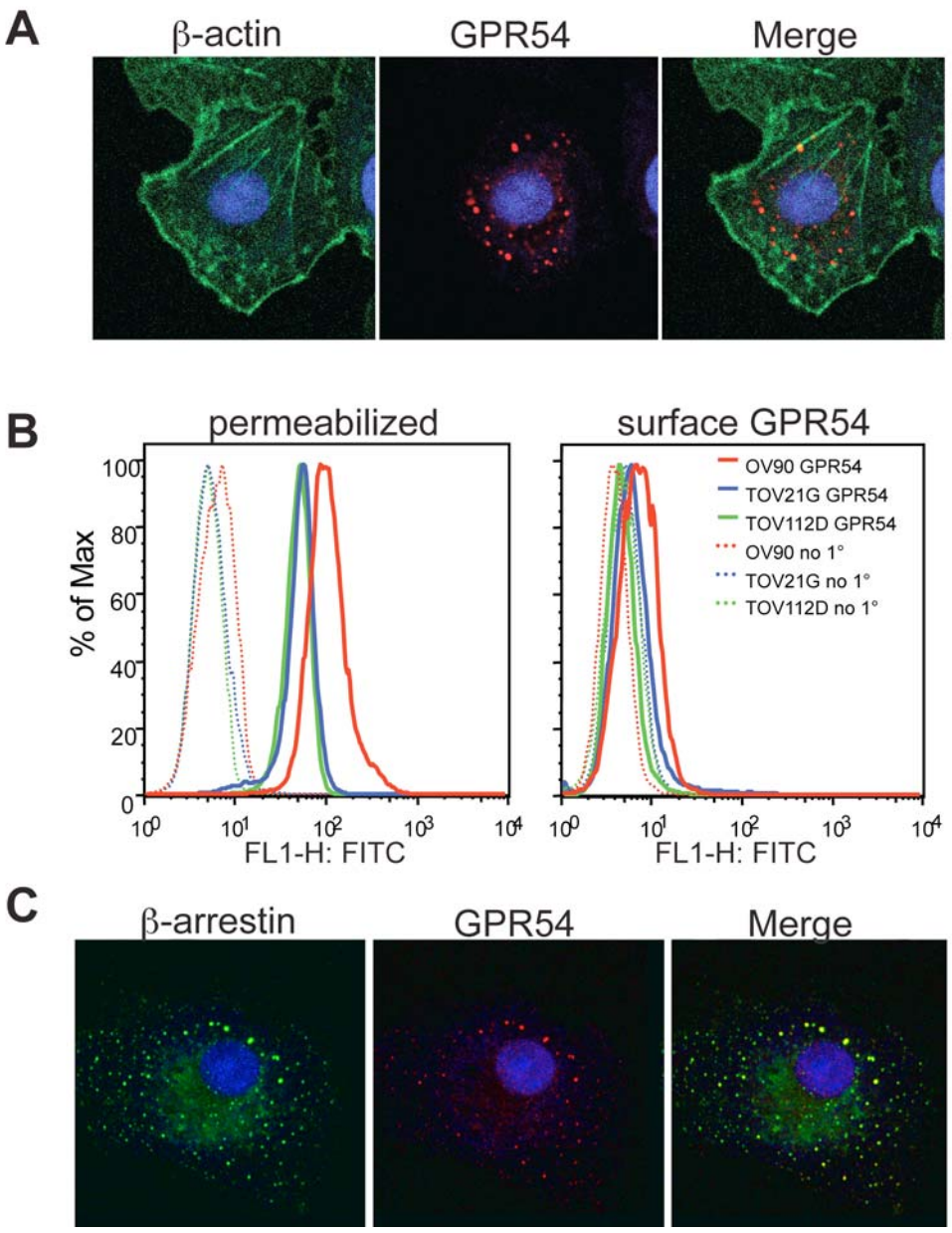


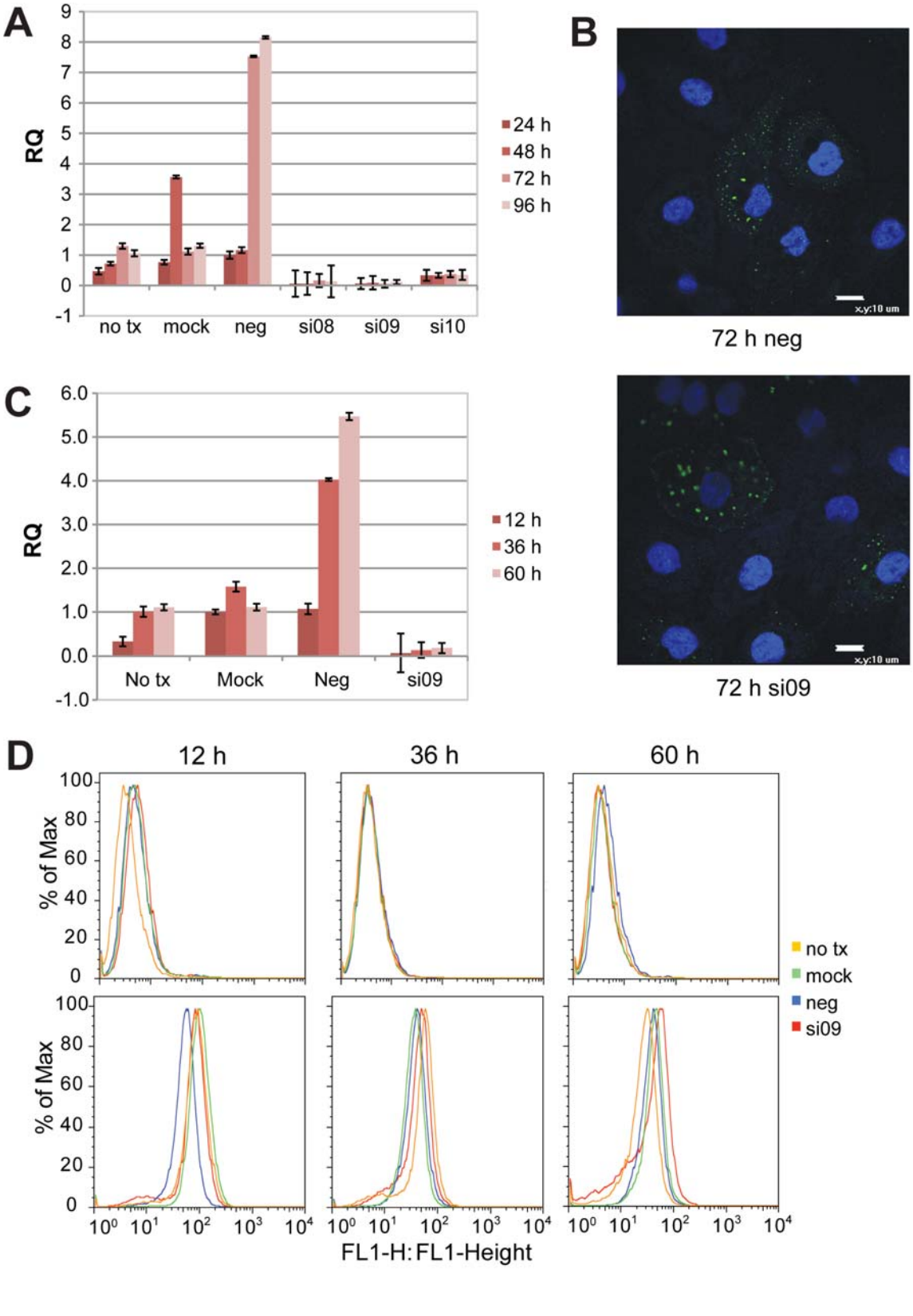
Figure 5.6 GPR54 is intracellular and co-localizes with β-arrestin in TOV21G cells

A) β-actin outlines the membrane of the TOV21G cell (green); GPR54 is shown in red. B) Histogram depicting flow cytometry results for GPR54 signal (solid line) in ovarian cancer cells as compared to negative control without GPR54 primary

antibody (broken line). FITC signal representative of GPR54 expression is on the x-axis. C) Dual IF labeling of β-arrestin in green and GPR54 in red in TOV21G cells. Co-localization is determined by a yellow signal resulting from the overlap of the two proteins depicted in the merge panel.

Figure 5.7 Localization of GPR54 is not altered by knockdown of endogenous Kp

A) *KiSS1* expression as determined by QPCR analysis in untreated cells (no tx), L2K only treated cells (mock), negative siRNA control treated cells (neg) and Kp siRNA treated cells (si06, si09, si10); error bars represent ±SEM across three technical replicates. Expression was measured at four timepoints from treatment (24 h, 48 h, 72 h, and 96 h) and is measured as relative quantification (RQ; y-axis). B) GPR54 protein is shown in green as determined by IF at 72 h after treatment with the negative siRNA control (neg; top) and siRNA 09 (bottom). C) Similar experiment as in (A) only with three timepoints (12 h, 36 h, and 60 h) and using only one siRNA. D) Determination of GPR54 localization by flow cytometry for cells treated in (C). Top three timepoints demonstrate GPR54 membranous signal as compared to cytoplasmic GPR54 (bottom). FITC signal that is specific to GPR54 is measured on the x-axis.


A
RQ
9
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1
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-1
no tx
mock
neg
si08
si09
si10
24 h
48 h
72 h
96 h
B
x,y:10 um
72 h neg
C
RQ
6.0
5.0
4.0
3.0
2.0
1.0
0.0
-1.0
No tx
Mock
Neg
si09
12 h
36 h
60 h
x,y:10 um
72 h si09
D
12 h
36 h
60 h
% of Max
100
80
60
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20
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10^0 10^1 10^2 10^3 10^4
FL1-H: FL1-Height
no tx
mock
neg
si09

5.4 Conclusions

The initial objective was to find breast and/or ovarian cancer cell lines expressing GPR54 to investigate the effect of abrogating GPR54 signalling. While the effects of kisspeptin signalling through endogenous GPR54 has been explored in trophoblast cells [12], renal cancer cells [11, 13], pancreatic cancer cells [15], and thyroid cancer cells [16] most have been engineered to overexpress GPR54 [2, 3, 5, 7-9]. Potent small molecule inhibitors of GPR54 have yet to be discovered, necessitating the use of RNA interference to knock down the expression of GPR54. After screening several cell lines, we found expression of GPR54 detectable by western blot, in two breast and three ovarian cancer cell lines.

Several studies have shown that GPR54 signals through the $G\alpha_q$ pathway [5, 7, 8, 18, 21, 22]. Canonical GPCR signalling through G-proteins coupled to the $G\alpha_q$ subunit results in intracellular calcium release and this was used to assay the responses of the cell lines to Kp-10 and Kp-54 (metastin) peptides. It takes as little as 6.4 nmol/kg Kp-54 to elicit significant release of FSH and LH in women *in vivo* [23]. Although most *in vitro* studies use 100 nM to1 μM of Kp when assessing GPR54 function in cell lines [3, 13, 21], concentrations as low as 10 nM have shown to elicit a response [24]. Engineered GPR54 overexpressing cell lines have exhibited an EC50 as low as 0.1 nM of Kp [5, 7, 8]. None of the breast cell lines investigated in our study manifest a calcium flux response, even when stimulated in the micro molar range, well above the physiological level at

which kisspeptins are thought to act, although all were capable of releasing calcium in response to ATP.

In the absence of $G\alpha_q$-protein mediated signalling, we next asked whether activation of the MAPK component ERK could be observed. GPCR mediated ERK activation can occur through $G\alpha_s$-proteins, $G\beta\gamma$ heterodimers, or through the non G-protein β-arrestin pathway [19, 20, 25-27]. After Kp stimulation of the breast cell lines, we found dose dependent ERK phosphorylation in MCF-7 cells; the highest of the GPR54 expressing cells. The malignant MDA-MB-231 breast cell line showed high endogenous pERK even after serum starvation and careful washing to remove any potential mitogens, and thus could not be assessed further. The ovarian cancer cell line TOV21G exhibited measurable transient pERK1/2 increase at 1 μM Kp-54, despite having the lowest apparent level of expression of GPR54 by western blot. The OV90 cell line expressed the greatest amount of GPR54 amongst the ovarian cell lines, and showed an intermediate pERK1/2 response.

Having shown that GPR54 was expressed in these lines and that a pERK1/2 response could be seen, albeit at super-physiological levels in the ovarian cell lines, we attempted to knockdown GPR54 using RNA mediated interference. We screened several independent shRNA hairpins in each of the MCF-7 and ovarian cell lines and were able to obtain knockdown of GPR54 protein in the ovarian cells. The shRNA06 hairpin was capable of mediating stable knockdown of GPR54 in the TOV21G cells, by 88% compared to untreated cells, and transiently stable knockdown in OV90. Though there was

evidence of GPR54 knockdown in the TOV112D cells, they were not suitable for IF and were not carried forward for further analysis. No measurable knockdown of GPR54 was observed in MCF-7 cells with the same shRNAs.

We investigated whether the knockdown in the TOV21G cell lines resulted in abrogation of signalling. Despite measurable knockdown of protein, no effect on pERK1/2 response to Kp-10 was observed. Since we could not achieve sufficient stable knockdown of GPR54 to affect any change in pERK1/2 responses to Kp, and since Ca^{++} signalling was also not observed, we concluded that the original aim of altering endogenous GPR54 signalling to observe responses could not be assayed with the breast and ovarian cancer cells. The lack of knockdown effect on signalling could be due to a small number of remaining GPR54 receptors or due to Kp-10 acting through other receptors in the cells. The possibility of additional kisspeptin receptors has been suggested in the literature [28, 29], but to date without a candidate having been identified.

During screening of the cell lines for GPR54 expression by IF, we noticed that the predominant localization of expression in TOV21G cells appeared to be cytoplasmic. We confirmed this in four cell lines (TOV21G, TOV112D, OV90, and MCF-7) by using flow cytometry to estimate the relative levels of GPR54 immunoreactivity. Fewer than 1% of live cells showed surface immunoreactivity for GPR54 whereas intracellular GPR54 immunoreactivity was approximately 10 fold above background.

The cytoplasmic localization of GPR54 prompted us to ask whether GPR54 was trapped with β-arrestin, a known alterative signalling and

sequestration compartment for GPCRs [19, 20]. Dual IF of GPR54 and β-arrestin showed co-localization of the two proteins in the cytoplasm. Arrestins can sequester GPCRs in the cytoplasm and thereby downregulate activity of a signalling pathway [19, 20]. This can occur as a physiological response to chronic ligand stimulation of GPCRs, resulting in internalization. In fact, this process is exploited by GnRH agonists that operate on the GnRH receptor, through desensitization and downregulation of surface expression by chronic stimulation [30].

Finally, we decided to test the hypothesis that autocrine Kp released by the cell lines might be causing desensitization of the cells by β-arrestin mediated trapping of internalized receptor. A siRNA construct capable of knocking down endogenous *Kiss1* transcript by 90% as compared to controls resulted in no effect on co-localization of GPR54. In Chapter 2 of this dissertation it was observed that *Kiss1* transcripts do not always match protein Kp as determined by IHC. We concede that *Kiss1* knockdown may not reflect true Kp protein levels, yet as no antibody suitable for immunoblot detection of Kp exists, we are unable to verify downregulation of protein Kp.

Although signalling through the MAPK cascade can be observed in the breast and ovarian cells, it is not clear whether this is through an alternative GPR54 mediated β-arrestin pathway or through a different receptor. The predominantly cytoplasmic expression of GPR54 suggests that the canonical membrane signalling arrangement does not occur in these cells. However, there are reports of endogenously internalized GPCRs that are still responsive to their

agonists, albeit the literature reports mutations in these GPCRs [31, 32]. There has yet to be a report of GPR54 mutations in cancer and we were unable to successfully sequence GPR54 in our cell lines. Thus, we acknowledge that an as yet unknown GPR54 mutation may exist and these results should be interpreted with caution.

This proposed mechanism of GPR54 activation bypassing the canonical $G\alpha_q$ calcium release may be specific for endogenously expressing GPR54 cancer cell lines. Indeed, there has been the recent discovery of cell specific GPR54 signalling in neurons. Inhibition of ERK1 and ERK2 in the hippocampus prevented neuronal activity [33], while this same inhibitor did not affect neuronal activity in the hypothalamus after Kp treatment [34]. Calcium flux has been determined in endogenous GPR54 expressing primary trophoblast cells [12] and many *in vitro* overexpressing models [2, 3, 5, 8, 9], but this is the first reported case of an endogenously expressing cancer cell line that is unable to release calcium upon Kp stimulation. Other studies that have used endogenous GPR54 expression in cancer cell lines did not measure intracellular calcium release or determine GPR54 localization [11, 15, 16].

The major limitations of this study are specific to the functional experiments used in assessing GPR54 signallling. Firstly, a cell line expressing GPR54 on the plasma membrane would be a more appropriate positive control in the flow cytometry and the activation of MAPK studies. Similarly such a cell line would have been an appropriate control for the calcium flux analysis. If there was calcium flux using a membrane bound GPR54 cell line, we could infer that the

lack of calcium flux in the MCF-7 cell lines is specific to GPR54 localization. Unfortunately we did not have access to such a cell line. The second limitation of this study is the lack of a negative control specific to the Kp ligand. Although the Kp peptides used in these studies had the required amidation group at the N-terminus, by modifying the Kp peptide through removal of the amidation group, we could have determined that the Kp used in our studies was indeed active.

This research demonstrates for the first time the endogenous GPR54 intracellular localization in four individual cancer cell lines. Arguably these results question the significance of relating GPR54 alone, to physiological effects or tumour cell behaviour. Clearly the membrane location and ability to signal should be taken into account in future studies.

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6 DIFFERENTIAL TRANSCRIPTION IN THE HYPOTHALAMUS OF *GPR54* AND *KISS1* KNOCKOUT MICE REVEALED BY ONE MILLION EXON ARRAY ANALYSIS[5]

Objective: To analyze differential gene expression between *Gpr54* and *Kiss1* knockout mice compared to wildtype animals to elucidate novel genes involved in the regulation of the ligand-receptor pair.

[5] A version of this chapter will be submitted for publication. Prentice LM, d'Anglemont de Tassigny X, McKinney S, Fee J, Ruiz de Algara T, Huntsman DG, Colledge WH, and Aparicio SAJ. Differential transcription in the hypothalamus of *Gpr54* and *Kiss1* knockout mice revealed by one million exon array analysis.

6.1 Introduction

In 2003 it was discovered that the kisspeptin-GPR54 ligand-receptor pair are significant players in the endocrine regulation of mammalian reproduction [1, 2]. It has been established subsequently that Kp binding to GPR54 releases gonadotropin releasing hormone (GnRH) from the hypothalamus to stimulate the pituitary-gonadal axis in a multitude of species including humans [3-15]. In rodents, Kp is expressed in the arcuate nucleus (ARC) of the hypothalamus and also in the rostral periventricular area of the 3rd ventricle (RP3V) which includes the anteroventral periventricular nucleus (AVPV). Rising estrogen levels have a positive feedback effect on Kp expression in the AVPV to initiate the LH surge required for ovulation in females [16-19]. Conversely, estrogen and testosterone have a negative feedback effect on Kp expression in the ARC that is regulated through estrogen receptor alpha (ERα) and also through the androgen receptor (AR) in males [16, 18-21].

What remains to be determined are the mechanisms controlling this sex steroid regulation of *Kiss1* and the consequences of loss of Kp signalling on downstream expression in the hypothalamus. We have generated *Gpr54* and *Kiss1* knockout mice that model the hypogonadotropic hypogonadism (HH) found in humans with *GPR54* mutations [2, 10, 22]. In order to assess what other genes may be affected in the hypothalamus, we compared gene expression between the two knockout genotypes as compared to wildtype mice of the same parental strain.

Affymetrix Exon 1.0 ST Array analysis of *Gpr54* and *Kiss1* KO mice hypothalami identified a novel gene list of possible regulators of *Gpr54* and *Kiss1* expression as well as genes potentially regulated by *Gpr54* and *Kiss1*. QPCR validation confirmed the novel gene list determined by the Affymetrix analysis and supported the current literature that GPR54/Kp are partially regulated through an endocrine feedback loop.

6.2 Material and Methods

6.2.1 Experimental procedure

i) To discover novel gene candidates that may be involved in the Kp-GPR54 signalling pathway, we assessed gene expression differences in the hypothalamus of *Kiss1* and *Gpr54* knockout mice (KKO, GKO) compared to wild-type mice (WT). An Affymetrix Exon 1.0 ST Array was used to assess gene expression initially, and these results were validated using real-time PCR and the Applied Biosystems Low Density Array (LDA).

ii) To account for hormonally regulated genes that may be differentially regulated as a consequence of sexual immaturity yet not directly affected by Kp and/or GPR54, a hormonally controlled group of mice were assessed. To ensure equal hormone exposure in all genotypes (GKO, KKO, WT), all mice were castrated prior to treatment. Treatment consisted of either a testosterone implant or an empty sialistic control. The hypothalamus was again isolated for assessment of differential gene expression, this time using a smaller LDA. The genes chosen for the smaller 48 gene LDA were based on the results of the previous larger 95

gene LDA that had been originally designed from the Affymetrix results. The experimental procedure is summarized in Figure 6.1.

6.2.2 Animals

Gpr54 and *Kiss1* knockout mice have been previously described [2, 10, 22]. Male 129S6/Sv/Ev wild-type, 129S6/Sv/Ev *Gpr54*- or 129S6/Sv/Ev *Kiss1*- knockout mice were housed under conditions of 12 hours of light with *ad libitum* access to food and water. The average age of the mice from the first analysis was 60-70 days and 90 days for the second hormonally controlled group. All experimental protocols were performed under the authority of a United Kingdom Home Office Project License and were approved by the Cambridge Animal Ethics Committee.

6.2.3 Castration and testosterone implants

Adult males were bilaterally castrated under general anaesthesia using Ketamin/Xyline. Castrated mice were divided into two groups: bilateral castration plus empty implant or bilateral castration plus testosterone implant. Testosterone implants were manually and aseptically prepared in the laboratory using silicone tubing (0.058 inch ID / 0.077 inch OD; Dow Corning) filled with crystalline testosterone (T-1500; Sigma Aldrich, UK), and sealed with adhesive silicone type A glue [23]. Implants were inserted subcutaneously at the time of castration. Mice were allowed approximately 3 - 4 weeks for recovery (the first week with paracetamol in the water supply), and killed by CO_2 exposure. Blood was collected in a heparinized syringe from the inferior vena cava and centrifuged at

1,000x g for 10 min at 4°C. The plasma supernatant samples were collected and stored at -20°C.

6.2.4 Testosterone assay

Free testosterone was measured by using an ELISA kit (DB52181; IBL, Hamburg, Germany) with a sensitivity of 0.17 pg/ml and intra- and inter-assay variation coefficients respectively 8.9% and 8.8% [22].

6.2.5 Hypothalamus extraction

After removal of the brain, the meninges and optic chiasm were discarded and the hypothalamus was isolated. The external limits for this dissection are: lateral, the external border of the medial preoptic area and more caudally, the lateral borders of the mammillary nucleus; dorsal, 1.5 mm depth; anterior, the anterior limit of the nucleus of the vertical limb of the diagonal band (bregma + 1.34 mm); and posterior, the posterior limit of the mammillary nucleus (bregma – 3.40 mm). The whole hypothalamus contained at least the following main hypothalamic nuclei: anteroventral periventricular nucleus, anterodorsal preoptic nucleus, anterior-hypothalamic nucleus, magnocellular lateral hypothalamic nucleus, supraoptic and paraventricular nuclei, medial preoptic area and the nucleus of the vertical limb of the diagonal band. Immediately after dissection, hypothalami samples were collected in 600 µl of RNAlater (Applied Biosystems, UK) and kept at 4°C for 24 h and then stored at -20°C until RNA extraction.

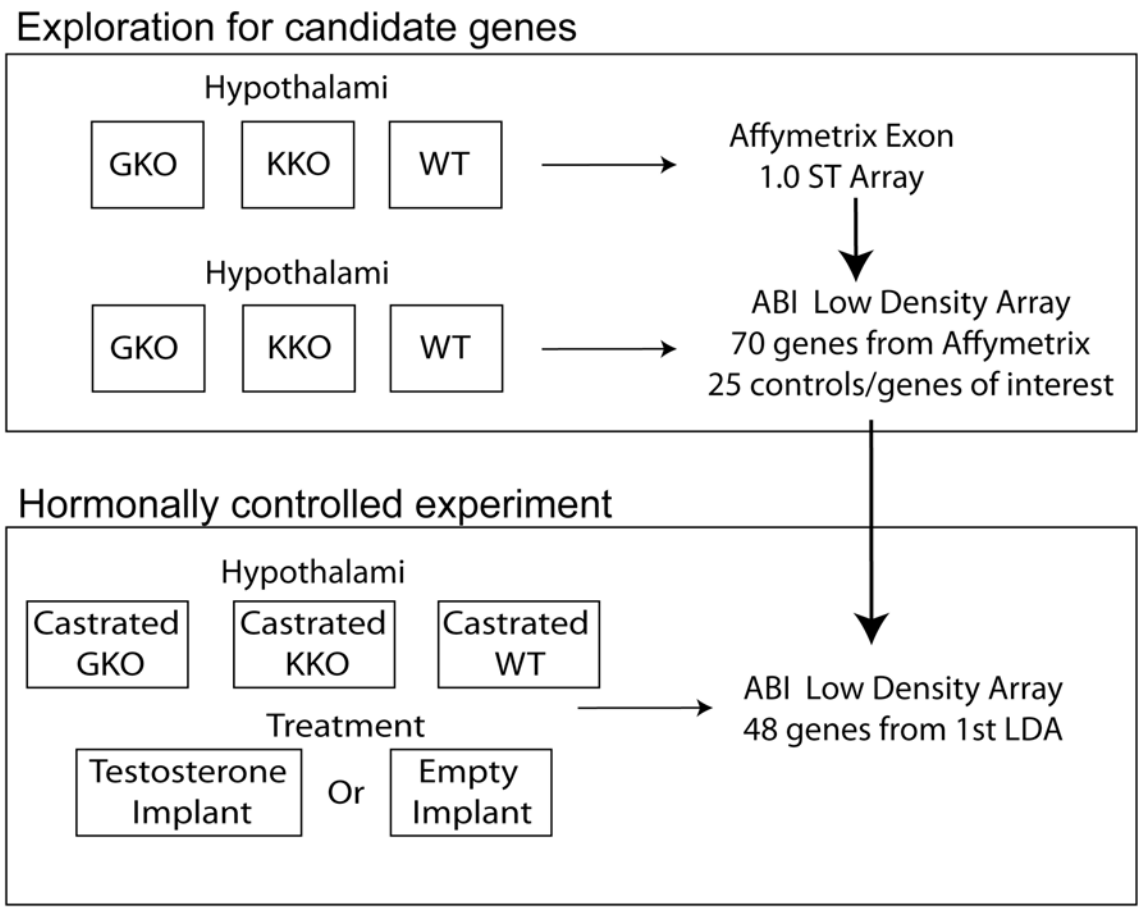

Figure 6.1 Summary of the experimental procedure

Initially, gene expression was analyzed using the Affymetrix Exon 1.0 ST Array and the results from this analysis were validated using the ABI Low Density Array (LDA) covering 70 genes from the Affymetrix analysis. Both platforms used RNA from mouse hypothalami from the three different genotypes: *Kiss1* knockout mice (KKO), *Gpr54* knockout mice (GKO), and wildtype mice (WT). Based on the results from the larger LDA array, a second smaller LDA was used to assess hormonally controlled genes. The mice for this experiment were all castrated and either treated with testosterone implant or an empty control prior to hypothalamus extraction. There were at least three biological replicates for each group.

6.2.6 RNA extraction

RNA was extracted using the Qiazol method (Invitrogen, Carlsbad, CA) that is recommended for fatty tissues. Whole hypothalami were removed from RNAlater, with excess reagent blotted off of the tissue with a kimwipe before being placed in 100-200 μl of Qiazol. The hypothalami were then homogenized using a Kontes Pellet Pestle (Fischer Scientific, Ottawa, ON) hand-held homogenizer until a uniform mixture was achieved. The remaining Qiazol up to 1 ml was added to the homogenized mixture and the total homogenate was placed in a phase lock tube to separate out the aqueous phase through centrifugation. RNA was precipitated out of the aqueous phase with an equal volume of isopropanol and pelletted by centrifugation. Seventy percent ethanol was used to wash the pellet that was then air-dried, resuspended into 5.5-11.5 μl of RNase/DNase free water, and heated to 65°C for 10 min. One and a half microliters of RNA was set aside for Agilent analysis and RNA was stored at -80°C.

6.2.7 Agilent analysis

RNA concentration and integrity (RIN) were determined by the Agilent 2100 bioanalyzer as per manufacturer's recommendations. Briefly, 1 μl of heat denatured RNA mixed with 5 μl of Nano Marker was run on a RNA 6000 NanoChip (Agilent Technologies, Mississauga, ON) already prepped with pre-filtered Nano gel matrix mixed with Nano dye concentrate. RNA samples were compared to 1 μl of NanoChip RNA 6000 ladder.

6.2.8 Affymetrix procedure

Hypothalamic RNA was prepared using the GeneChip Whole Transcript (WT) Sense Target (ST) Labeling Assay (Affymetrix, Santa Clara, CA) as per manufacturer's recommendations before being hybridized to a GeneChip Mouse Exon 1.0 ST Array (Affymetrix, Santa Clara, CA). One microgram of RNA was used as starting material with all samples having a RIN ranging between 8.2-9.0. Starting RNA was mixed with Control Poly-A-RNA before using the RiboMinus Transcriptome Isolation Kit (Invitrogen, Carlsbad, CA) as per Affymetrix recommendations to reduce the rRNA contamination within the sample. Each batch of RNA run through the RiboMinus kit was run in parallel with mouse liver RNA that had either been rRNA reduced or left unreduced as a comparison for effectiveness of the RiboMinus assay. The rRNA reduced samples and liver controls were then purified and concentrated using the GeneChip In Vitro Transcription (IVT) cRNA Cleanup Kit and assessed by Agilent analysis. Successfully rRNA reduced samples continued forward to first strand cDNA synthesis using T7-(N)6 primers and SuperScript II enzyme before second strand cDNA synthesis using DNA Polymerase I. Within 10 min of second strand cDNA synthesis, samples were run through the GeneChip WT cDNA Amplification Kit before IVT cRNA Cleanup and then assessed for cRNA concentration by NanoDrop analysis (NanoDrop Technologies, Wilmington, DE). Samples with cRNA concentrations equal to or greater than 10 μg in a total volume of 6.5 μl were carried forward to the second cycle of first-strand cDNA synthesis using the

GeneChip WT cDNA synthesis Kit. Random primers with a deoxyuridine triphosphate (dUTP) component mixed within the deoxynucleotide triphosphates (dNTPs) were used. The single stranded cDNA was then treated with RNase H to hydrolyze any remaining cRNA and run through the cDNA Cleanup Spin Columns as per the GeneChip Sample Cleanup Module. Eluted cDNA was assessed by NanoDrop for sample concentration. Finally, 5.5 μg of single stranded cDNA was fragmented with Uracil DNA Glycosylase (UDG) and APE 1 then labeled with biotin allonamide triphosphate (TdT) using the GeneChip WT Terminal Labeling Kit before being hybridized for 17 h to the Mouse 1.0 ST Array chip in the GeneChip Hybridization Oven 640 using the GeneChip Hybridization, Wash and Stain Kit. Before scanning the chip on the GeneChip Scanner 3000 7G, each chip was run through the GeneChip Fluidics Station 450 for washing and staining with Streptavidin Phycoerythrin (SAPE).

When the sample yielded a concentration less than required for subsequent steps in the protocol, the process was started over again and resulting volumes amalgamated (and in some instances vacuum centrifuged to increase the concentration) before moving forward to the next step. The protocol was also attempted with 2 μg of RNA as starting material as advised by technical support but an increase in yield was not achieved.

6.2.9 Quantitative real-time polymerase chain reaction (QPCR)

The Applied Biosystems (ABI) 7900HT Fast Real-Time System was used to amplify and detect mouse cDNA transcripts (Applied Biosystems, Foster City,

CA). For high throughput detection a Taqman Low Density Array (LDA) Card from ABI consisting of 95 genes including two endogenous control genes (GAPDH and 18S rRNA) was custom ordered. To assess the second group of mice independent of hormonal feedback, a second smaller LDA set was ordered consisting of 48 genes. The gene list was similar to that of the first larger LDA and had the same two endogenous controls. The complete list of genes used for both LDAs are in Tables 6.1 and 6.2.

6.2.10 Statistical analysis

Stratagene ArrayAssist version 5.1.0 was used to analyzed the Affymetrix data and create volcano plots. Affymetrix chip data was quantile normalized and corrected using the GC-RMA algorithm [24]. Experimental groups were compared using unpaired t-tests. QPCR data was analyzed using linear mixed effects models, with experimental groups compared via the likelihood ratio chi-square statistic [25, 26]. Adjustment for multiple comparisons was performed using the Benjamini-Hochberg method [27].

For the testosterone challenge experiment, a linear mixed effects model was used to relate raw Ct values from loading control genes (*Gapdh*, 18S RNA) and test genes. Although all factors could have been put in one 2x2x3 model, for simplicity the data from *Kiss1* and *Gpr54* knockouts was modeled separately, in two 2x2x2 factorial designs (genotype: wt or ko; treatment: T- or T+; gene: test or loading control). The relationship between the model-estimated means for each of the conditions allowed inference of four main effects (Figure 6.2).

Table 6.1 Gene list for the 96 Low Density Array

96 Low Density Array*					
Gene	**Assay ID**	**Gene**	**Assay ID**	**Gene**	**Assay ID**
18S	Hs99999901_s1	*Htr2c*	Mm00434127_m1	*Pdyn*	Mm00457572_m1
Abca8a	Mm00462440_m1	*Hyal2*	Mm00477731_m1	*Pgm1*	Mm00804141_m1
Acsm3	Mm00489774_m1	*Il15ra*	Mm00500457_m1	*Pgr*	Mm00435625_m1
Adamtsl4	Mm00523242_m1	*Il8ra*	Mm00731329_s1	*Phtf2*	Mm00557065_m1
Ar	Mm00442688_m1	*Irak3*	Mm00518541_m1	*Ppef2*	Mm00448366_m1
Boc	Mm00552900_m1	*Itgax*	Mm00498698_m1	*Prickle3*	Mm00558283_m1
Centd2	Mm00546699_g1	*Itgbl1*	Mm00520942_m1	*Pus7*	Mm00617871_m1
Cln3	Mm00487021_m1	*Itpr3*	Mm00446540_m1	*Rab34*	Mm00446343_g1
Col7a1	Mm00483818_m1	*Kiss1*	Mm00617576_m1	*Rragd*	Mm00546741_m1
Ddx3y	Mm00465349_m1	*Kiss1r*	Mm00475046_m1	*Sh3tc2*	Mm00553970_m1
Dmn	Mm00809202_s1	*Krit1*	Mm00459502_m1	*Six2*	Mm00807058_m1
Dmrt3	Mm00616649_m1	*Lep*	Mm00434759_m1	*Slc25a25*	Mm00525104_m1
Eda	Mm00438653_m1	*Lepr*	Mm00440174_m1	*Slc26a8*	Mm00524836_m1
Edc4	Mm00725090_m1	*Lhb*	Mm00656868_g1	*Sp1*	Mm00489039_m1
Eif2s3y	Mm00468995_g1	*Lhcgr*	Mm00442931_m1	*Speer4b*	Mm00835620_m1
Elk3	Mm00469054_m1	*Lrdd*	Mm00502614_m1	*Sphk1*	Mm01252544_m1
Elmo3	Mm00555221_g1	*Mapk1*	Mm00442479_m1	*Srpk3*	Mm00444746_m1
Ermap	Mm00469273_m1	*Med23*	Mm00518410_m1	*Srrm1*	Mm00489728_m1
Ero1l	Mm00469296_m1	*Metapl1*	Mm00491809_m1	*Sult1c2*	Mm00471845_m1
Esr1	Mm00433149_m1	*Mmp2*	Mm00439506_m1	*Svep1*	Mm00465696_m1
Esr2	Mm00599819_m1	*Mmp28*	Mm00712992_m1	*Tac2*	Mm00436885_m1
Fsd2	Mm00556634_m1	*Mmp9*	Mm00442991_m1	*Tacr3*	Mm00445346_m1
Fzd10	Mm00558396_s1	*Mobkl2c*	Mm00774347_m1	*Taf12*	Mm00499416_m1
Gapdh	Mm99999915_g1	*Myh9*	Mm00502575_m1	*Tcf7l2*	Mm00501505_m1
Glis3	Mm00615386_m1	*Ngef*	Mm00451232_m1	*Tec*	Mm00443230_m1
Gnrhr	Mm00439143_m1	*Npas4*	Mm00463644_m1	*Tmem144*	Mm00510477_m1
Golt1a	Mm00503319_m1	*Npy*	Mm00445771_m1	*Txnip*	Mm00452393_m1
Gpr143	Mm00440553_m1	*Nr2f2*	Mm00772789_m1	*Ucn*	Mm00445261_m1
Gpr146	Mm01700739_m1	*Oas3*	Mm00460944_m1	*Wnt5a*	Mm00437347_m1
Gtf3a	Mm00550608_m1	*Olig2*	Mm01210556_m1	*Zfp472*	Mm00461969_m1
Hapln2	Mm00480745_m1	*Opn4*	Mm00443523_m1	*Zmym6*	Mm00624222_m1
Hhip	Mm00469580_m1	*Pbx2*	Mm00479560_m1		

*One position is empty on the part of the manufacturer

Table 6.2 Gene list for the 48 Low Density Array

48 Low Density Array			
Gene	**Assay ID**	**Gene**	**Assay ID**
18S	Hs99999901_s1	*Klk1b22*	Mm02343755_g1
Abca8a	Mm00462440_m1	*Krit1*	Mm00459502_m1
Acsm3	Mm00489774_m1	*Lhcgr*	Mm00442931_m1
Ar	Mm00442688_m1	*Lrdd*	Mm00502614_m1
Arap1	Mm00546699_g1	*Mmp2*	Mm00439508_m1
Boc	Mm00552900_m1	*Mmp28*	Mm00712992_m1
Ddx3y	Mm00465349_m1	*Mmp9*	Mm00442991_m1
Eif2s3y	Mm00468995_g1	*Mobkl2c*	Mm00774347_m1
Elk3	Mm00469054_m1	*Ngef*	Mm00451232_m1
Ero1l	Mm00469296_m1	*Npas4*	Mm00463644_m1
Esr1	Mm00433149_m1	*Nr2f2*	Mm00772789_m1
Esr2	Mm00599819_m1	*Olig2*	Mm01210556_m1
Fzd10	Mm00558396_s1	*Pgm1*	Mm00804141_m1
Gapdh	Mm99999915_g1	*Pgr*	Mm00435625_m1
Glis3	Mm00615386_m1	*Phtf2*	Mm00557065_m1
Gnrhr	Mm00439143_m1	*Six2*	Mm00807058_m1
Hapln2	Mm00480745_m1	*Synm*	Mm00809202_s1
Hhip	Mm00469580_m1	*Tac2*	Mm00436885_m1
Htr2c	Mm00434127_m1	*Taf12*	Mm00499416_m1
Hyal2	Mm00477731_m1	*Tcf7l2*	Mm00501505_m1
Il15ra	Mm00500457_m1	*Tec*	Mm00443230_m1
Itgax	Mm00498698_m1	*Tmem144*	Mm00510477_m1
Kiss1	Mm00617576_m1	*Txnip*	Mm00452393_m1
Kiss1r	Mm00475046_m1	*Wnt5a*	Mm00437347_m1

Dmn=Synm, Centd2=Arap1

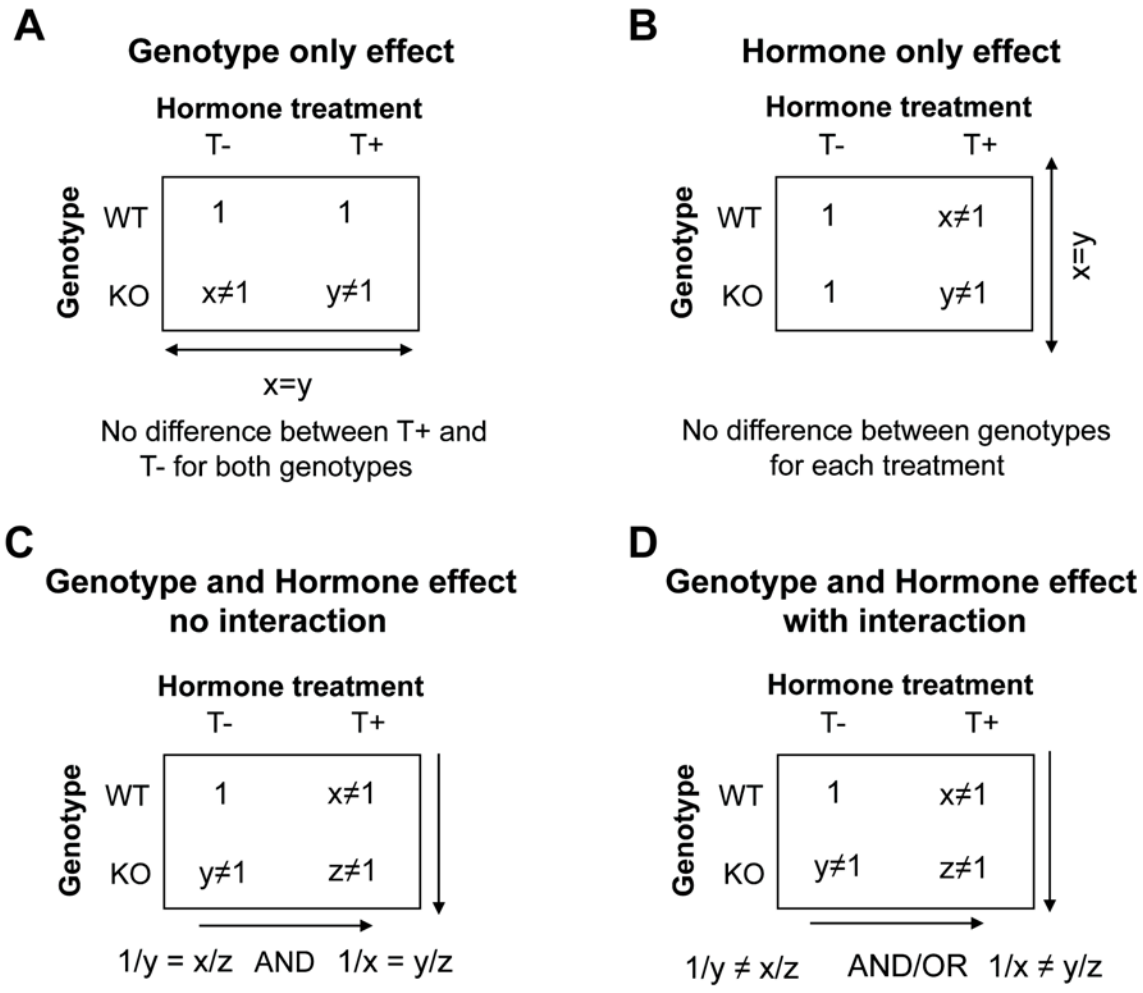

Figure 6.2 Summary of statistical grouping of the hormonally controlled LDA assay

A) Results were deemed to be a genotype only effect if the measured fold change between the wildtype (WT) and knockout (KO) mice was the same in both of the hormone T+ and T- conditions. B) Similarly, result is a hormone only effect if the measured fold change between the T+ and T- conditions was the same for both of the genotype WT and KO conditions. C) Genes were classified as having both a genotype and hormone effect when there was a proportional difference between the two comparisons. D) Genes were classified as having a genotype and hormone effects with interaction when there was a disproportionate difference between the two comparisons that was not equal to 1.

These were: genotype only transcription differences, hormone treatment only transcription differences, hormone and genotype differences where the rate of difference is symmetrical (no interaction between effects), and hormone and genotype differences where the differences between groups show an effect interaction (eg the difference for hormone treatment is disproportionately greater in one genotype). The linear model was fitted using the lmer function from the lme4 package implemented in R-2.7.0 [28, 29]. The model output reports the estimated mean, 95% confidence intervals, relative effect and direction, and p-value (adjusted for multiple corrections by the Benjamini Hochberg method).

6.3 Results

6.3.1 Microarray identification of candidate hypothalamic deregulated transcripts from GPR54 and kisspeptin knockout mice.

We compared quantile normalized probe intensity values from Affymetrix whole mouse exon array chips where hybridization was performed with wildtype, *Gpr54* knockout (GKO) or *Kiss1* knockout (KKO) hypothalamic RNA. Affymetrix results were compared between genotypes, specifically gene expression of all wildtype mice were grouped together and compared with all knockout mice grouped together (WT vs KO) or with *Kiss1* knockout mice alone (WT vs KKO) or *Gpr54* knockout mice alone (WT vs GKO). Additionally, *Gpr54* knockout mice were compared with *Kiss1* knockout mice (GKO vs KKO) to give a total of four comparative groups. Gene level and probe level summarization was used in the

comparisons and from each of these we selected gene loci showing $p<0.05$ and expression fold differences of 1.5 or greater, as candidates for further analysis (yellow highlighted regions in the volcano plots shown in Figure 6.2).

This analysis yielded 213 gene loci, putatively exhibiting differential transcription (Figure 6.3). To validate the initial array based measurements of transcription, we selected 70 candidate loci for further validation by QPCR. The selections were prioritized on the strength of expression, consistency of differences between knockouts, known or suspected roles in hormonal regulation, and the availability of quenching QPCR probes for low density 384 well plate QPCR plate analysis. Within these criteria, all transcription factors, transmembrane receptors, and signalling molecules that were available were included. QPCR sets for 18S RNA and *Gapdh* were included in each array as loading controls. Finally, we also selected a number of genes of interest based on known role in gonadotropic signalling, that were not differentially expressed by microarray analysis. This resulted in 95 gene loci, of which 25 were controls or genes of interest and 70 were genes selected on the basis of differential expression by array analysis (Table 6.1).

We used the LDA QPCR array to assay each gene locus at least 3 times per sample (technical replicates) and over 3 biological replicates of RNA prepared from microdissected hypothalamic tissue from wildtype, GKO and KKO mice. After analysis of the CT values by a linear mixed effects model, 18 loci showed statistically significant differential expression in one of the groups (Table 6.3) and were carried forward for further analysis.

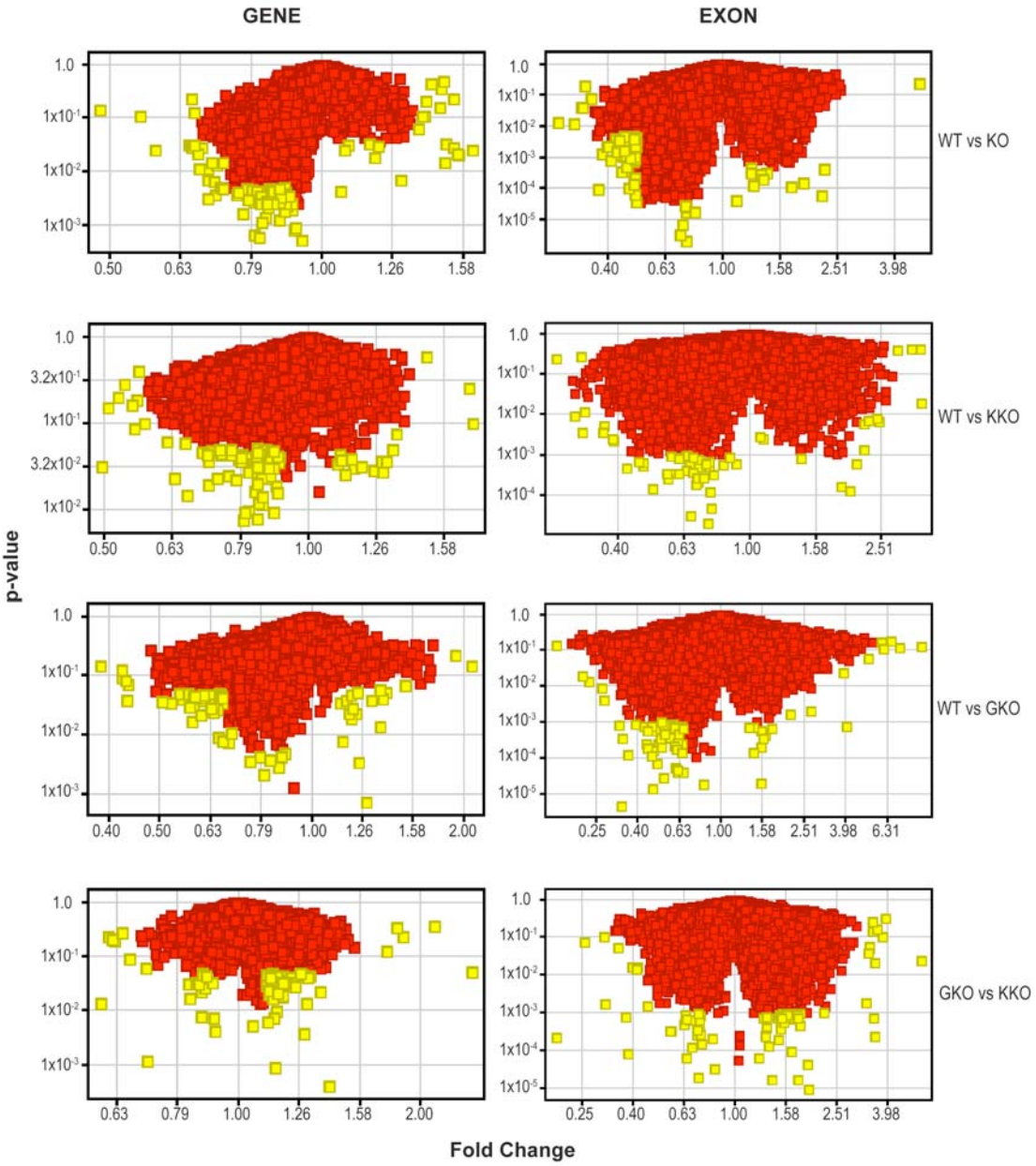

Figure 6.3 Volcano plots from the Affymetrix Exon 1.0 ST Array

Genes that were considered for further analysis are represented by yellow squares. The x-axis is the fold change for comparison groups that are indicated on the left side of the figures. The y-axis is the p-value among biological replicates.

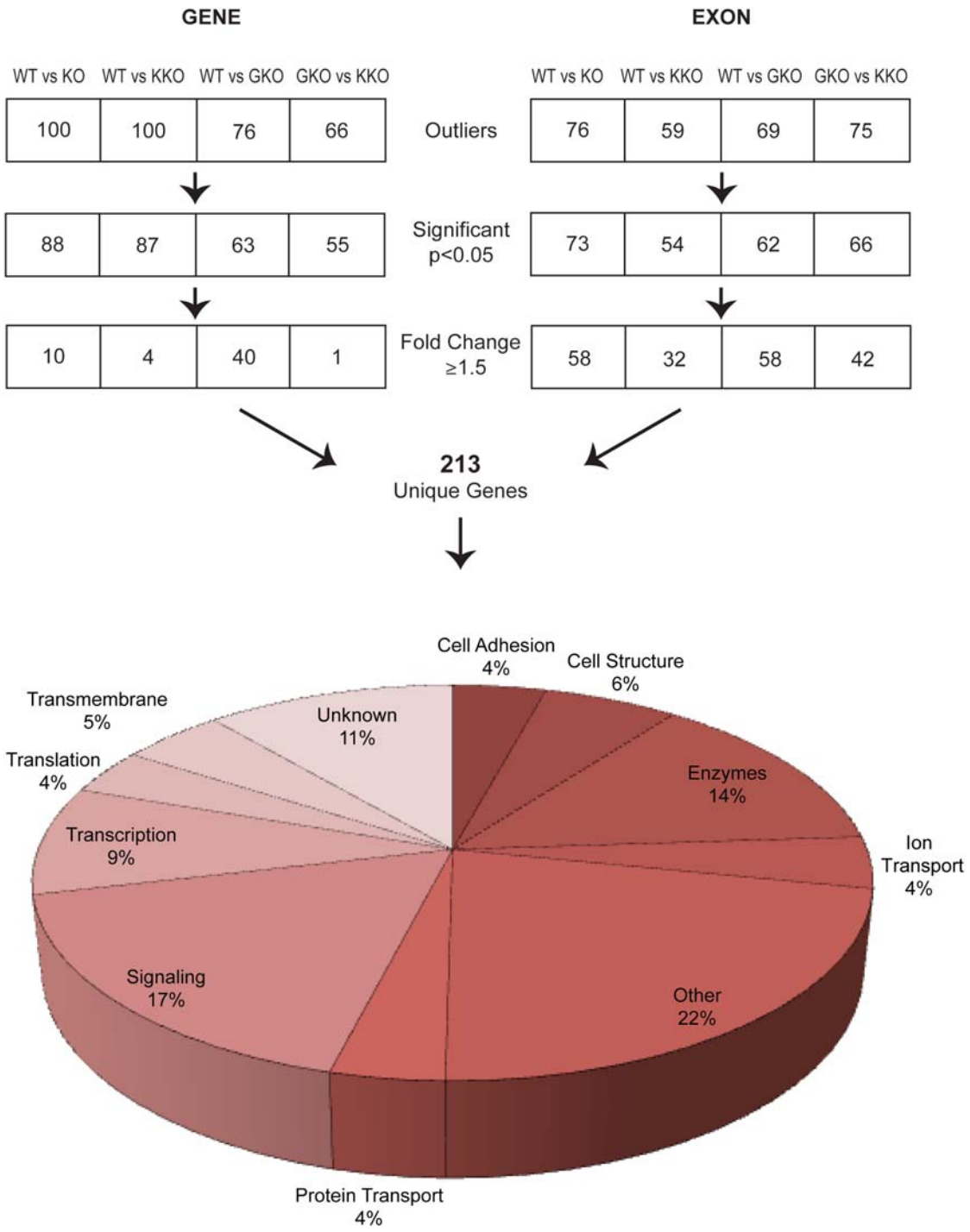

Figure 6.4 Summary of Affymetrix results

The pie chart represents the breakdown of the 213 unique genes by function based on the ExPASy proteomics server (http://www.expasy.ch).

Table 6.3 Genes with significant fold change from the 95 LDA assay

	GKO vs WT		KKO vs WT	
GeneTarget	**FC (95%LCI-UCI)**	**Direction**	**FC (95%LCI-UCI)**	**Direction**
Abca8a	1.85 (1.33-2.59)	Up	1.97 (1.33-2.90)	Up
Esr1	1.62 (1.37-1.92)	Up	1.54 (1.32-1.78)	Up
Htr2c	1.34 (1.14-1.58)	Up	1.41 (1.21-1.64)	Up
Kiss1	14.03 (10.86-18.11)	Up	NA	NA
Mmp2	1.51 (1.18-1.94)	Up	n.s.	n.s.
Npas4	2.60 (2.09-3.25)	Up	n.s.	n.s.
Boc	n.s.	n.s.	1.38 (1.14-1.67)	Up
Eif2s3y	n.s.	n.s.	1.26 (1.11-1.43)	Up
Ero1l	n.s.	n.s.	1.23 (1.07-1.41)	Up
Hapln2	n.s.	n.s.	1.20 (1.05-1.37)	Up
Hhip	n.s.	n.s.	1.34 (1.11-1.63)	Up
Krit1	n.s.	n.s.	1.21 (1.05-1.39)	Up
Lrdd	n.s.	n.s.	1.80 (1.29-2.51)	Up
Ngef	n.s.	n.s.	1.22 (1.07-1.40)	Up
Pgm1	n.s.	n.s.	1.26 (1.07-1.48)	Up
Taf12	n.s.	n.s.	1.31 (1.14-1.50)	Up
Tmem144	n.s.	n.s.	2.61 (2.21-3.08)	Up
Txnip	n.s.	n.s.	1.36 (1.13-1.65)	Up

6.3.2 Dissection of hormone dependent and independent hypothalamic gene transcription in *Gpr54* and *Kiss1* knockout mice.

Expression analysis of intact GKO and KKO hypothalamus RNA revealed significant differential transcription. However, since GKO and KKO mice fail to undergo puberty an unknown number of these transcriptional differences could be explained by the hormonal milieu. To address this, we asked which of the gene loci exhibited hormonally responsive transcription, by measuring transcriptional differences in the presence or absence of testosterone (T). WT, GKO and KKO male mice were castrated and implanted with either a sham silastic implant, or a T containing implant. After 4 weeks exposure, the mice were sacrificed, the hypothalamus microdissected, and the RNA extracted. To confirm exposure to T, we measured serum testosterone (Table 6.4). Each gene locus was assayed with at least 4 technical replicates and 3 biological replicates. To reduce the chance genotype or hormonal variance of loading controls, we used two independent loading controls, 18S RNA and *Gapdh*. The data were analysed separately using a linear mixed effects model (methods). We analyzed GKO and KKO mice separately in a 2x2x2 design, considering gene (test, loading control), treatment (T+, T-) and genotype (WT, GKO, KKO) as categorical variables. Tables 6.5-6.12 summarize the statistically significant ($p<0.05$) model results for hormone effect, genotype effect, hormone+genotype no interaction and hormone+genotype with interaction, for the GKO and KKO mice, separately.

Table 6.4 Characteristics of hormonally treated mice

Mouse ID	Genotype	Sex	Weight	Testosterone	Implant	Date of Birth	Date of Castration	Date of Sacrifice
KJ74	K-/-	Male	20 g	0 pg/mL	Empty	29/08/08	15/10/08	12/11/08
KJ59	K-/-	Male	21 g	0 pg/mL	Empty	17/08/08	15/10/08	12/11/08
KJ10	K-/-	Male	20 g	0 pg/mL	Empty	04/08/08	30/09/08	21/10/08
KJ25	K-/-	Male	20 g	5.7 pg/mL	Testosterone	25/07/08	30/09/08	21/10/08
KJ55	K-/-	Male	21 g	2.8 pg/mL	Testosterone	15/08/08	15/10/08	12/11/08
KJ47	K-/-	Male	22 g	3.4 pg/mL	Testosterone	14/08/08	15/10/08	12/11/08
HL73	G-/-	Male	21 g	0 pg/mL	Empty	18/08/08	15/10/08	12/11/08
HL74	G-/-	Male	21 g	0 pg/mL	Empty	18/08/08	15/10/08	12/11/08
HL27	G-/-	Male	20 g	0 pg/mL	Empty	19/07/08	30/09/08	21/10/08
HL61	G-/-	Male	17 g	5.1 pg/mL	Testosterone	24/08/08	15/10/08	12/11/08
HK97	G-/-	Male	23 g	8.8 pg/mL	Testosterone	07/07/08	30/09/08	21/10/08
HL49	G-/-	Male	22 g	3.6 pg/mL	Testosterone	25/07/08	30/09/08	21/10/08
KJ52	WT+/+	Male	21 g	0 pg/mL	Empty	14/08/08	15/10/08	12/11/08
KJ64	WT+/+	Male	29 g	0 pg/mL	Empty	19/08/08	15/10/08	12/11/08
HL31	WT+/+	Male	28 g	0 pg/mL	Empty	19/07/08	30/09/08	21/10/08
HL30	WT+/+	Male	26 g	4.2 pg/mL	Testosterone	19/07/08	30/09/08	21/10/08
HK99	WT+/+	Male	27 g	4.8 pg/mL	Testosterone	07/07/08	30/09/08	21/10/08
KJ46	WT+/+	Male	24 g	7 pg/mL	Testosterone	14/08/08	15/10/08	12/11/08

Table 6.5 KKO hormone only effect genes compared with testosterone treatment

KKO	**Hormonal Effect**		
Gene Target	FC (95%CI)	Direction	p-value
Esr1	1.23(1.14-1.32)	Down	0.0003
Gnrhr	2.33(1.63-3.33)	Down	0.0009

Table 6.6 GKO hormone only effect genes compared with testosterone treatment

GKO	**Hormonal Effect**		
Gene Target	FC (95%CI)	Direction	p-value
Abca8a	1.57(1.33-1.87)	Down	2.83E-05
Hyal2	1.13(1.05-1.22)	Down	0.0422
Klk1b22	2.16(1.43-3.25)	Down	0.0042
Mmp2	1.23(1.10-1.37)	Down	0.0113
Pgr	1.28(1.18-1.37)	Up	5.66E-07

Table 6.7 KKO genotype effect only genes compared with WT

KKO	Genotype Effect		
Gene Target	FC (95%CI)	Direction	p-value
Ar	1.16(1.08-1.25)	Down	0.0272
Eif2s3y	1.27(1.09-1.48)	Down	0.0225
Tmem144	1.50(1.30-1.73)	Up	6.12E-06

Table 6.8 GKO genotype effect only genes compared with WT

GKO	Genotype Effect		
Gene Target	FC (95%CI)	Direction	p-value
Arap1	1.21(1.10-1.34)	Down	0.0042
Boc	1.24(1.11-1.39)	Down	0.0136
Eif2s3y	1.48(1.25-1.74)	Down	0.0003
Ero1l	1.27(1.18-1.38)	Down	2.61E-05
Esr2	1.63(1.46-1.82)	Down	4.10E-10
Glis3	1.32(1.18-1.48)	Down	0.0004
Hapln2	1.43(1.28-1.60)	Down	2.61E-05
Hhip	1.63(1.46-1.82)	Down	4.68E-10
Htr2c	1.24(1.13-1.36)	Down	0.0003
Hyal2	1.25(1.13-1.39)	Down	0.0004
Lhcgr	1.61(1.30-1.99)	Down	0.0042
Lrdd	1.47(1.28-1.68)	Down	3.88E-05
Mmp28	1.48(1.25-1.73)	Down	0.0023
Mobkl2c	1.36(1.22-1.53)	Down	0.0004
Npas4	1.65(1.45-1.87)	Down	2.06E-08
Nr2f2	1.28(1.18-1.39)	Down	2.46E-05
Olig2	1.48(1.32-1.65)	Down	8.34E-07
Pgm1	1.26(1.15-1.37)	Down	0.0003
Phtf2	1.25(1.15-1.36)	Down	0.0001
Taf12	1.28(1.17-1.39)	Down	1.57E-05
Wnt5a	1.36(1.24-1.50)	Down	1.39E-06

Table 6.9 KKO genes with independent genotype and hormone effect

KKO	Genotype Effect		Hormonal Effect		
Gene Target	FC (95%CI)	Direction	FC (95%CI)	Direction	p-value
Abca8a	1.21(1.06-1.37)	Up	1.64(1.45-1.86)	Down	1.75E-09
Ddx3y	1.18(1.06-1.32)	Down	1.25(1.12-1.39)	Down	0.0013
Fzd10	1.16(1.03-1.31)	Up	1.18(1.05-1.33)	Down	0.0272

Table 6.10 GKO genes with independent genotype and hormone effect

GKO	Genotype Effect		Hormonal Effect		
Gene Target	FC (95%CI)	Direction	FC (95%CI)	Direction	p-value
Abca8a	1.35(1.15-1.58)	Down	1.58(1.35-1.85)	Down	1.39E-07
Ddx3y	1.52(1.31-1.77)	Down	1.21(1.04-1.40)	Down	1.52E-05
Esr1	1.13(1.04-1.22)	Up	1.40(1.30-1.52)	Down	5.45E-10
Fzd10	1.36(1.20-1.53)	Down	1.16(1.03-1.31)	Down	6.00E-05
Kiss1	1.96(1.64-2.35)	Up	12.15(10.15-14.55)	Down	4.29E-46
Mmp2	1.41(1.27-1.56)	Down	1.23(1.12-1.36)	Down	2.06E-08
Pgr	1.28(1.18-1.38)	Down	1.27(1.18-1.37)	Down	1.51E-10
Tmem144	1.86(1.60-2.16)	Down	1.28(1.10-1.48)	Down	1.51E-10

Table 6.11 KKO hormone and genotype effect interacting genes

	Genotype Effect within Hormonal Group				Hormonal Effect within Genotype				
KKO	No testosterone		Testosterone		Wildtype		Knockout		
Gene Target	FC (95%CI)	Dir	FC (95%CI)	Dir	FC (95%CI)	Dir	FC (95%CI)	Dir	p-value
Acsm3	1.23(1.03-1.48)	Up	1.27(1.03-1.56)	Down	1.86(1.53-2.26)	Up	1.19(0.98-1.45)	Up	9.80E-06
Klk1b22	1.30(0.92-1.84)	Down	4.30(3.01-6.15)	Down	1.34(0.97-1.84)	Down	4.43(3.01-6.51)	Down	1.75E-09
Mmp9	1.03(0.68-1.56)	Down	3.75(2.44-5.75)	Down	1.08(0.74-1.59)	Down	3.93(2.48-6.22)	Down	0.0127
Six2	1.08(0.84-1.40)	Up	1.99(1.54-2.57)	Up	1.60(1.24-2.07)	Down	1.15(0.89-1.49)	Up	1.14E-05
Tec	1.07(0.86-1.32)	Down	1.72(1.39-2.12)	Up	1.63(1.32-2.02)	Down	1.12(0.91-1.38)	Up	1.82E-05
Txnip	1.16(1.00-1.34)	Down	1.22(1.06-1.41)	Up	1.66(1.44-1.92)	Down	1.18(1.02-1.36)	Down	3.01E-08

Table 6.12 GKO hormone and genotype effect interacting genes

	Genotype Effect within Hormonal Group				Hormonal Effect within Genotype				
GKO	No testosterone		Testosterone		Wildtype		Knockout		
Gene Target	FC (95%CI)	Dir	FC (95%CI)	Dir	FC (95%CI)	Dir	FC (95%CI)	Dir	p-value
Acsm3	1.26(1.01-1.57)	Up	2.28(1.84-2.83)	Down	1.93(1.54-2.42)	Up	1.49(1.21-1.84)	Down	2.02E-08
Ar	1.23(1.09-1.38)	Down	1.56(1.39-1.75)	Down	1.13(1.01-1.27)	Up	1.12(1.00-1.25)	Down	1.02E-08
Ddx3y	1.30(1.13-1.50)	Down	1.01(0.88-1.16)	Up	1.38(1.20-1.59)	Down	1.05(0.91-1.20)	Down	0.0004
Eif2s3y	1.33(1.10-1.61)	Down	1.10(0.91-1.33)	Up	1.40(1.16-1.70)	Down	1.05(0.87-1.27)	Up	0.0196
Elk3	1.47(1.29-1.66)	Down	1.15(1.02-1.31)	Down	1.09(0.96-1.23)	Down	1.17(1.03-1.32)	Up	2.88E-06
Esr1	1.08(0.96-1.21)	Down	1.31(1.17-1.47)	Down	1.28(1.14-1.43)	Down	1.55(1.39-1.74)	Down	1.36E-11
Fzd10	1.20(1.01-1.42)	Down	1.17(0.99-1.40)	Up	1.37(1.15-1.63)	Down	1.02(0.86-1.22)	Up	0.0315
Gnrhr	1.28(0.78-2.12)	Down	3.20(1.70-6.03)	Up	2.29(1.37-3.82)	Down	1.80(0.96-3.35)	Up	1.80E-03
Kiss1	1.95(1.58-2.42)	Up	1.09(0.88-1.35)	Up	9.12(7.36-11.29)	Down	16.34(13.19-20.23)	Down	5.71E-52
Klk1b22	1.25(0.71-2.20)	Up	2.70(1.61-4.52)	Down	1.08(0.61-1.91)	Down	3.65(2.17-6.12)	Down	0.0004
Krit1	1.10(0.94-1.28)	Down	1.48(1.27-1.73)	Down	1.06(0.91-1.24)	Up	1.27(1.09-1.48)	Down	0.0005
Mmp9	1.11(0.91-1.35)	Up	1.70(1.40-2.07)	Up	1.11(0.92-1.35)	Up	1.71(1.41-2.08)	Up	2.69E-07
Ngef	1.09(0.97-1.23)	Down	1.44(1.27-1.62)	Down	1.15(1.01-1.29)	Up	1.15(1.02-1.30)	Down	5.16E-05
Six2	1.05(0.73-1.50)	Down	2.22(1.55-3.18)	Up	1.78(1.24-2.55)	Down	1.30(0.91-1.87)	Up	0.0022
Tec	2.48(1.95-3.14)	Down	1.75(1.38-2.22)	Down	1.63(1.29-2.07)	Down	1.16(0.91-1.46)	Down	2.03E-13
Txnip	1.04(0.80-1.34)	Down	1.81(1.40-2.35)	Up	1.85(1.43-2.39)	Down	1.02(0.79-1.32)	Up	8.79E-05

Genotype Only Effect		Hormone Only Effect	
GKO Esr2 Hhip Lhcgr Npas4	**KKO** Tmem144	**GKO** Abca8a Klk1b22	**KKO** Gnrhr
GKO Abca8a Ddx3y Kiss1 Tmem144	**KKO** Abca8a	**GKO** Acsm3 Ar Esr1 Gnrhr Kiss1 Klk1b22 Mmp9 Six2 Tec Txnip	**KKO** Acsm3 Klk1b22 Mmp9 Six2 Tec Txnip
Genotype and Hormone Effect no Interaction		Genotype and Hormone Effect with Interaction	

Figure 6.5 Summary of gene expression differences with a fold change ≥1.5
Each quadrant contains the gene list for both the *Gpr54* knockout mice (GKO) and the *Kiss1* knockout mice (KKO) that had a fold change difference of ≥ 1.5 in at least one of the comparisons. The quadrants are labeled by gene expression effect as determined by the hormonally controlled experiment.

The strongest effect is in the *Kiss1* locus, measured in GKO mice which showed strong hormonal regulation (approximately 12 fold downregulated with T) and moderate genotype dependence (approximately 2 fold upregulated). With the 18S loading control, the regulation shows a weak interaction whereas with the *Gapdh* loading control, hormonal regulation and genotype regulation vary symmetrically and thus no interaction is reported. The next largest effects are for the locus *Klk1b22* (also known as NGF peptidase) and the GnRH receptor (*Gnrhr*) both of which show a strong hormonal effect and a weak genotype effect but with some interaction (the hormone effect is relatively stronger in the KO than the WT mice), however with large variances.

Fewer statistically significant effects were seen in the KKO mice, although some loci (eg *Klk1b22*, *Gnrhr*, *Tmem144*) were regulated in common with GKO mice, albeit with effects of different magnitude. Many of the statistically significant results are of small absolute magnitude and considering the variances of the loading controls between genotypes and treatments, interpretation of differences of less than 1.5 fold may not be meaningful.

Thus considering effects of 1.5 fold or greater we observed the following: For *Kiss1* knockout mice, only *Gnrhr* showed a pure hormonal effect, only *Tmem144* showed a purely genotype driven change. Among the genotype and hormone variant changes, *Klk1b22* showed the greatest differences, more than 2 fold, with testosterone exposure and this effect was greater in the knockout mice than the wildtypes (ie there is a statistically significant interaction).

For *Gpr54* knockout mice, *Klk1b22* showed a hormone only effect (downregulation) with the *Gapdh* control, but a hormone effect with genotype interaction using the 18S loading control. The direction and magnitude is similar to that observed in the KKO mice. *Gnrhr* shows a strong hormonal effect with a genotype interaction – downregulation in the wildtype mice and upregulation in the knockout mice. *Tmem144* shows a strong genotype effect in the GKO mice (downregulation), as with KKO mice, but with an additional marginal hormone effect without genotype interaction. Genotype only effects were seen at > 1.5 fold but less than 2 fold for ERβ (*Esr2*), *Hhip*, *Lhcgr*, and *Npas4*. We also noticed several matrix metalloproteinase (MMP) family members (*Mmp2*, *Mmp9, Mmp28*) showing mixed hormonal and genotype effects, but in aggregate less than 1.5 fold in any direction.

6.4 Conclusions

We have assessed gene expression variability amongst *Kiss1* and *Gpr54* knockout mice to determine potential gene candidates involved in the regulation of this ligand-receptor pair. Initially an Affymetrix Exon 1.0 ST Array consisting of more than 1 million probe sets (exons) was used to assess gene expression differences between genotype. We re-validated the array estimated differences, using QPCR. This yielded a set of 70 gene loci with confirmed transcriptional differences between genotypes, which was supplemented with additional genes of interest and controls. However the differences observed could also be explained by secondary hormonal differences between wildtype and knockout

mice. To account for this hormonal feedback, all mice were castrated and treated with or without hormonal implant prior to gene expression comparisons between genotypes.

Considering the results of the hormone/genotype experiment, the observations of strong regulation for *Gnrhr* and *Kiss1* show that the relevant region of the hypothalamus was sampled and that the methods used were capable of picking up transcriptional differences in two genes known to be strongly hormonally regulated. We have shown for the first time, counter regulation of *Kiss1* in *Gpr54* knockout mice. This is consistent with previous reports showing that upon interruption of the hypothalamic-pituitary-gonadal axis, *Kiss1* is elevated in the hypothalamus of mice [21] and rats [30].

Relatively few large differences were seen in the initial Affymetrix array experiment, and fewer changes were seen in the *Kiss1* knockout mice, overall, than in the *Gpr54* knockout mice. Although both genotypes fail to undergo puberty, it has been reported that the kisspeptin loss of function alleles may be less penetrant than the *Gpr54* knockout mice [31]. Endogenous basal activity of the GPR54 receptor could explain such a difference, as could the existence of an as yet unknown weaker binding peptide ligand for GPR54.

Some of the gene expression differences could be cell type specific. The hypothalamus is a relatively small region in the brain and is divided into discrete nuclei. These nuclei consist of a cluster of cells responsible for mediating various physiological functions. It has been established that the anteroventral periventricular nucleus (AVPV) and the arcuate nucleus (ARC) are the key

regions in the rodent hypothalamus involved in *Kiss1* expression [16, 18-21, 32]. It is possible that the gene expression differences observed are neuron, specific reflecting only a few cells. Nevertheless, we were able to observe robust regulation of *Gnrhr*, which is predominantly restricted to the 500 GnRH expressing neurons in the hypothalamus, which suggests that the experiment was powered to detect transcription changes in at least this number of cell bodies. Small differences, although statistically significant, should be cautiously interpreted and considering the degree of variation seen with the load control estimates for the samples, we did not consider absolute effects of less than 1.5 fold to be interpretable, even if statistically significant. It should also be noted that especially where interaction effects are seen, differences in secondary sexual maturation of the hypothalamus at the time of castration could account for the effect. For example, regulation of GnRH receptor levels may show a “priming” effect in wildtype castrate mice from prior exposure to testosterone, compared with knockouts. The reversed direction of hormonal effect for *Gnrhr* between GKO and WT is consistent with such an explanation [33].

We did not compare directly the controlled group of castrate T+, T- mice with the intact knockout mice. Although the intact knockout mice are also sexually immature, the intact immature testis will still have active feedback to the hypothalamus and transcription differences would be expected. Indeed, a greater difference in *Kiss1* expression was seen in the intact GKO mice than the castrate GKO mice. This is consistent with reports showing that *Kiss1* expression is upregulated in the hypothalamus of castrated rodents [12, 21].

It has been shown that Kp can downregulate MMP9 expression [34]. We observe that *Mmp9* is upregulated when there is *Kiss1* loss in the KKO mice and the expected downregulation is observed when there is gain of *Kiss1* expression, as we see in the GKO mice, however these observations are specific to the hormonally treated group for both mutants. Similarly MMP2 has been shown to be downregulated by Kp [35] and in our study *Mmp2* is downregulated in the highly expressing *Kiss1* GKO mice, although at a fold change less than 1.5. Additionally, reciprocal up-regulation is absent in the KKO mice. Although it has been demonstrated that MMP9/2 can cleave and inactivate Kp, the precise relationship between the MMPs and Kp remains to be elucidated. As *Mmp28* showed the greatest fold change difference in the GKO genotype only effect, with a fold change of 1.48, there may be a novel MMP involved in Kp-GPR54 regulation. Although all the MMPs continually appeared as significant transcription changes in our analysis, the fold change values were less than 1.5 for *Mmp2* and *Mmp28* and hence these results should be interpreted with caution.

Another potentially Kp modifying gene is *Klk1b22*, which is downregulated greater than 2 fold in the hormone only effect in the GKO mice yet has one of the largest fold change in the GKO mice and the largest fold change in the KKO mice for the genotype and hormone interaction comparison. From the family of kallikreins, this translated peptide may have a role in processing the 145 aa KiSS1 protein to the active kisspeptins. The *Klk1b22* sequence is one that would recognize and cleave an Arginine-Serine bond. This arrangement of amino acids

is found in the mouse sequence for the Kiss1 peptide; that requires cleavage to created the N-terminus of the 52 aa mouse equivalent of the human Kp-54. The precise *Klk1b22* gene is not found in humans, however neither is the R-S bond in the KiSS1 peptide as a putative cleavage site. Rather, the bond that is cleaved in the human sequence is between R-G to create the N-terminus of Kp-54 [36, 37]. To address the possibility of Klk1b22 as a Kp modifying enzyme, one could incubate active Klk1b22 with full-length mouse Kiss1 peptide and perform mass spectrometry on the resulting products.

Tmem144 was the only gene that was consistently upregulated in the KKO mice as compared with WT mice regardless of treatment. *Tmem144* has the sequence of a 10-transmembrane receptor that can form ion transport and peptide channels. Non-selective cation channels and potassium channels have been shown to be necessary for Kp to depolarize GnRH neurons [38]. As *Tmem144* is consistently upregulated in mice lacking *Kiss1* expression, and GPR54 is co-localized to GnRH neurons [10], it may be that Tmem144 is the cation channel involved in GnRH neuronal activation through GPR54 stimulation.

The transcription factor gene *Npas4* was upregulated in the GKO mice with one of the largest fold changes after *Kiss1*, yet was downregulated by 1.65 fold in castrated mice independent of hormonal feedback. Npas4 has been credited with regulating the development of inhibitory synapses in activating neurons in the hippocampus [39]. What is most striking is that *Npas4* is activated by an influx of calcium [39] and is independent of the MAP kinase pathway [40]. It has been recently reported that Kp-GPR54 signalling in the hypothalamus of rats

is independent of the MAP kinase pathway as well [38]. These results suggest that Npas4 may also have a role in Kp-GPR54 signalling, specifically as a transcription factor downstream of GPR54 activation that may induce GnRH release. However, as Npas4 is involved in neuronal maturation, the gene expression variability we are seeing between GKO and WT may be a result of the sexually immature phenotype of the knockout mice.

Expression of the estrogen receptor beta (*Esr2*) was downregulated by 1.63 fold in the genotype only effect GKO mice. Similarly, the androgen receptor (*Ar*) was also hormonally independent in KKO mice, however with a fold change less than 1.2. *Esr2* function in the hypothalamic-pituitary-gonadal axis is currently unknown as hormonal feedback occurs almost entirely through estrogen receptor alpha (*Esr1)*, [16, 18, 21] as supported in our study. This feedback is also partially regulated through the Ar in male mice [21]. This is the first report that *Esr2* may be involved in the hypothalamic-pituitary-gonadal axis, as regulated by Kp and GPR54.

We compared *Kiss1* and *Gpr54* knockout mice hypothalamic gene expression to determine novel genes involved in the endocrine signalling pathway, however we were anticipating that novel genes also involved in the anti-metastatic pathway of Kp-GPR54 epithelial signalling may also arise. One such candidate is Wnt5a. In the GKO mice *Wnt5a* expression was decreased as compared with wildtype, although at levels lower than 1.5 fold, and this was independent of hormonal influence. Wnt5a signals in the non-canonical pathway and has been suggested to decrease Kp expression in melanoma cells thus

inducing the metastatic potential of these cells [41]. Furthermore, Wnt5a is an independent marker for poor prognosis in melanoma [42], the disease that first characterized Kp as a metastasis supressor [43]. Most recently, Wnt5a has been implicated in embryo implantation yet is not affected by hormones [44]. We propose that Wnt5a and the Kp-GPR54 ligand-receptor pair may play a role in trophoblast invasion in the placenta that is a fine balance between the regulation of these genes.

In our study we have determined a panel of genes that are involved in the regulation of the Kp-GPR54 ligand-receptor pair independent of, or in addition to, hormonal effects. However, further analysis is required to definitively include these candidates as part of either the endocrine or autocrine pathways regulated by *Kiss1* and *Gpr54*.

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7 SUMMARY, OVERALL CONCLUSIONS, AND FUTURE DIRECTIONS

7.1 Summary

Kp and GPR54 have been studied as anti-metastatic agents [1-6] and have been determined to initiate sex hormone release through activation of the hypothalamic-pituitary-gonadal axis [7-15]. This dissertation hypothesized that expression of Kp and GPR54 would be predictive of disease outcome and anti-metastatic behaviour would be altered with the loss of GPR54 in a malignant epithelial cell line. An existing tumour that has Kp expression may inhibit metastasis through autocrine signalling assuming concurrent expression of GPR54 within the tumour. An overarching hypothesis would be that the secreted Kp may be released back into the system from this same tumour and stimulate sex hormone production through an endocrine pathway.

To address our hypothesis that Kp and GPR54 expression predict clinical outcome we used IHC on tissue microarrays. Prior to my research, Kp and GPR54 expression had been assessed in a number of cancers, usually using RNA *in situ* hybridization or QPCR technology, and that loss of either Kp and/or GPR54 was equated to a poorer outcome [16-21]. However, assessment of Kp and GPR54 expression by immunohistochemical methods had not been accomplished successfully. Although a few cases of breast and ovarian cancers have been studied, large cohorts with outcome data have not been used.

Furthermore, this ligand-receptor pair had not been studied in prostate or testicular cancers prior to this dissertation.

We have established, using the antibodies and protocols optimized as describe in the thesis, the pattern of immunoreactivity for Kp and GPR54 in breast, ovarian, testicular, and prostate cancers. Specifically we have determined that: (1) loss of GPR54 is associated with poor prognosis in node positive breast cancer patients, (2) that seminomas express higher levels of GPR54 and Kp as compared with the more malignant testicular cancers, (3) that GPR54 is highly expressed in benign prostatic hyperplasia as compared to cancer, and (4) that both GPR54 and Kp are independent markers for favourable prognosis in ovarian cancer. Additionally we have correlated Kp and GPR54 expression with the clear cell subtype of ovarian cancer, further describing the least characterized ovarian cancer subtype. These favourable outcome associations are consistent with the literature and consistent with the notion that Kp and GPR54 may have a role in tumour migration, but perhaps not tumour initiation. Furthermore, we have shown for the first time, the range of tumours that may co-express GPR54 and Kp. An important caveat to these studies is the specificity of the antibodies used.

Although clinical outcome data for the patients from the testicular germ cell array and the prostate array was unavailable, we can infer that GPR54 expression may correlate with a more favourable prognosis based on GPR54 expression specific to subtype. Seminomas have a more favourable outcome compared to non-seminomas and the seminomas in our array highly express GPR54 and Kp as compared to the more malignant testicular cancers. For Kp,

specificity was determined by examining the ability of blocking peptides to abrogate binding in the IHC pattern and also by examining pattern specific expression in placental tissue, where the expression of GPR54 and kisspeptin has been localized [2, 6, 22]. GPR54 specificity was determined by western blot, and revealed one clear band at 42 kD, the established protein weight of GPR54. These controls show that the antibodies and protocol used recognize GPR54 and Kp, but they do not completely exclude other proteins that may also be bound (at unknown affinity) by these antibodies.

As Kp is a secreted peptide, we set out to determine whether Kp expression in tumours is then secreted into the bloodstream. Using plasma samples from a variety of gynaecological cancer patients, we were able to determine that Kp is indeed secreted into the bloodstream of these patients. Previously it had been determined that Kp is barely measurable in human plasma under normal physiological conditions except in pregnancy where it is dramatically elevated [23-27]. Two other groups also established plasma Kp elevation in cancer patients throughout the course of my research; in gestational trophoblastic neoplasia (GTN) [25] and in pancreatic cancer [28]. In GTN, elevated Kp returns to normal physiological levels after surgical removal of the tumour and chemotherapy. In pancreatic cancer patients, plasma Kp is elevated as compared to normal healthy volunteers, yet there is no correlation with any prognostic markers such as survival, grade or stage. Similarly, we also determined that Kp was elevated in plasma of gynaecological cancer patients but did not have the benefit of post-treatment blood samples to determine disease

specificity. However, there were significantly higher Kp levels in patients with a metastasis to the ovary than those that had a history of cancer, suggesting that plasma Kp does return to near physiological levels after treatment.

Following that Kp is expressed in tumours and can be released into the bloodstream of cancer patients, we could hypothesize further whether Kp could then act upon the hypothalamus in an endocrine manner. Our group did not specifically assess whether plasma Kp could affect the hypothalamic-pituitary-gonadal axis through neuronal GPR54. Throughout the course of my research, other groups had determined that when peripheral Kp is injected into mammals, there is desensitization of the Kp-GPR54 endocrine axis *in vivo* [29, 30]. Furthermore, direct stimulation of GnRH neurons with Kp *in vitro* produced desensitization of these neurons for at least 20-30 min [31] thus we could hypothesize that plasma Kp when released from cancer would not be able to have a long term effect on hypothalamic signalling. However, if there is pulsatile Kp released from the tumour cell then it may be possible that there is still stimulation of the hypothalamic-pituitary-axis, as the desensitization of the GnRH neurons is temporary [29, 31].

Our second hypothesis was that anti-metastatic behaviour would be altered with the loss of GPR54 in a malignant epithelial cell line. Whether signalling desensitization occurs in malignant cells expressing GPR54 was/is unknown, however chronic expression of Kp in animal xenograft models of melanoma and breast cancer metastasis has been shown capable of reducing cell migration [3, 4]. My objective was to manipulate GPR54 signalling in breast

and/or ovarian cancer cell lines, to assess possible anti-metastatic effects and the role of autocrine signalling, if any. Several studies [1, 2, 5, 6, 32, 33] have examined the consequences of gain of function (forced overexpression) of GPR54 in metastatic cell lines and shown anti-migratory effects. What was not known, certainly for breast and ovarian cancers, is whether manipulation of endogenous GPR54 expression would result in altered migratory behaviour. To address this we were first required to survey breast and ovarian cancer cell lines for GPR54 expression and secondly, to show that downregulation of GPR54 could alter signalling. We were able to show expression of GPR54 in several breast and ovarian cancer cell lines, including exceptionally high expression in MCF-7 cells. The first important discovery was that none of these cell lines signal through the canonical calcium mobilization pathway, although some showed pERK1/2 responses. However when we attempted to manipulate GPR54 protein levels with shRNA constructs, it was discovered that in cell lines where strong knockdown of the protein could be observed, there was no significant alteration of MAPK signalling.

While examining the effect of knockdown by immunofluorescence, it became apparent that GPR54 was not detectable on the plasma membrane. Rather, GPR54 was cytoplasmic in four of the endogenously expressing cell lines. GPR54 has been described as a G-protein coupled receptor that signals through the $G\alpha_q$ calcium-releasing pathway [2, 5, 6]. However, upon Kp stimulation of the GPR54 endogenously expressing cells, there was no intracellular calcium release, consistent with the absence of FACS or IHC

detectable membrane expression. It remains possible that low levels of membranous GPR54 are present and the lack of calcium mobilization would be due to absence of the relevant intracellular G-proteins. We were able to activate the MAP kinase pathway of the GPR54 internalized cells upon Kp stimulation, but for most of the cell lines, only by using higher than physiological levels of Kp. Whether there was true activation of the MAP kinase pathway via cytoplasmic GPR54 or whether very low levels of surface receptor are present, is unknown.

We explored the possibility that the cytoplasmic receptor was the result of chronic endogenous activation by Kp, by knocking down *Kiss1* transcripts by siRNA. Although GPR54 could be co-localized with β-arrestin, the *Kiss1* transcriptional knockdown did not result in re-localization of GPR54 to the membrane. It is important to consider that in Chapter 2 we describe that Kp IHC and *Kiss1* RNA transcripts do not match. Therefore we acknowledge that the QPCR knockdown results of *Kiss1* may inaccurately reflect Kp protein concentrations for this experiment. A full exploration of these observations would require the availability of a potent and specific small molecular inhibitor, however none have been described to date. Finally, it remains possible that the signalling seen through the MAPK pathway may represent activation (possible low affinity activation) of other peptide receptors, or the existence of an alternative receptor for kisspeptins, as has been speculated in the literature [34, 35].

Lastly, we set out to discover novel genes that may be involved in Kp and GPR54 signalling with the ultimate aim of comparing physiologically activated/repressed hypothalamic genes with those activated by GPR54

signalling in malignant cells. Since we could not show GPR54 *specific* signalling in the malignant cells, this comparison could not be made. Nevertheless, using genome wide transcript expression profiling methods we were able to show for the first time which gene loci have transcription that is dependent on intact *Gpr54* and *Kiss1* and which of these are likely hormonally regulated.

Using Affymetrix Exon 1.0 Mouse ST Array hybridization of microdissected hypothalamic RNA, we first estimated differential gene expression between knockout and wildtype mice. Microdissection was used to limit the analysis to the anatomical region of the hypothalamus where GnRH and GPR54 expressing neurons exist. The initial analysis found 213 unique transcripts, to be differentially expressed between knockout and wildtype animals at greater than 1.5 fold. 70 of these genes were re-evaluated with an additional 25 controls and genes of interest that were not apparently differentially expressed on the Affymetrix arrays. This was achieved by using Low Density arrays; a 384 well probe-quenching QPCR platform. After this first pass analysis, 18 array derived gene loci survived re-evaluation by showing reproducible changes.

These were carried forward with 25 additional control/interest genes to a final experiment where the effects of genotype and hormonal influence were separated. To achieve this, RNA from castrated male wildtype, *Gpr54* knockout, and *Kiss1* knockout mice that were implanted either with testosterone containing implants or vehicle only implants, was analyzed by QPCR. The results were analyzed using a 2x2x2 (genotype, hormone treatment, and test vs control groups) linear mixed effects model for the *Gpr54* and *Kiss1* knockouts.

From this analysis and addressing gene loci with greater than 1.5 fold change, we determined that from the *Kiss1* knockout mice, only *Gnrhr* showed a pure hormonal effect and only *Tmem144* showed a genotype effect. Among the genotype and hormone variant changes, *Klk1b22* showed the greatest differences. For *Gpr54* knockout mice, *Klk1b22* showed a hormone only effect and a hormone effect with genotype interaction depending on the loading control. *Gnrhr* shows a strong hormonal effect with a genotype interaction while *Tmem144* shows a strong genotype effect and a slight hormone effect without genotype interaction. Genotype only effects were seen with ERβ (*Esr2*), *Hhip*, *Lhcgr*, and *Npas4*.

This is the first study to assess gene expression differences in *Kiss1* and *Gpr54* knockout mice and has created a list of gene candidates that should be further explored in their capacity to affect Kp-GPR54 signalling and regulation. We set out to identify novel gene candidates involved in Kp-GPR54 epithelial signalling using a neuronal system since the physiological role of Kp and GPR54 has been more definitively defined in the hypothalamus. Had we successfully created a GPR54 knockdown cell line model, we would have explored the gene candidates discovered in the neuronal system using the cell line model to determine if they are relevant in the Kp-GPR54 epithelial signalling.

7.2 Significance and Potential Applications

The importance of considering both the neuroendocrine and epithelial characteristics of Kp and GPR54 arise when assessing Kp as a potential

therapeutic or diagnostic marker. Firstly, Kp could be considered as a potential therapeutic due to its anti-metastatic properties. It is known from previous studies that peripheral injections of Kp can stimulate the hypothalamic axis due to the increased levels of LH and FSH in humans after injection [23, 24], and furthermore there were no adverse affects in humans. As it has also been reported that GPR54 becomes desensitized to Kp after prolonged exposure [29-31], there is little risk of increasing sex steroid levels by activating the hypothalamic-pituitary-gonadal axis through continuous administration of Kp. It is important to mention that there was testicular degeneration in rats with a single bolus of Kp administered subcutaneously [36] but this could be avoided while maintaining maximal gondaotropin release by adjusting the dose. Further studies need to be conducted before confirming that Kp has no adverse affects in humans.

The anti-metastatic behaviour of Kp and GPR54 overexpressing cell lines has been well established [1-6, 37, 38]. However, whether there are anti-proliferative effects of Kp activation of GPR54 are still in question. That the MAP kinase pathway is activated upon Kp stimulation of GPR54 suggests that proliferation is not inhibited. Regardless of the contradictory results for anti-proliferative behaviour using *in vitro* studies, Kp expression in clinical samples is higher than in normal tissues yet Kp expression maintains a favourable prognostic marker phenotype in all cancers except hepatocellular cancer. These characteristics of Kp follow that of a tumourigenic agent that has anti-metastatic properties and should be taken into consideration when assessing Kp as a

potential therapeutic. Therefore, although Kp may not be tumourigenic through stimulation of sex steroid release, it may still be tumourigenic via an unknown mechanism. It may not be advantageous to use Kp as an anti-metastatic agent if there is the risk of Kp inducing cell proliferation. However a rigorous study conducted by Nash et al. [38] suggests that Kp induces dormancy in *in vivo* studies. By overexpressing Kp in a highly malignant melanoma cell line, it was determined that these cells could migrate to the lungs of injected mice, yet these cells failed to colonize. Additionally it was determined that this failure to colonize was specific to the cell's ability to secrete Kp, indicating the possibility of GPR54 paracrine signalling mediating the anti-metastatic effects rather than autocrine signalling. Due to the slight possibility of Kp and GPR54 having a proliferative effect, and as yet unknown potential degenerative effects, it is not likely that Kp will soon be used as a therapeutic.

However the use of Kp as a diagnostic marker may be plausible. Further studies attempting to measure Kp in plasma samples of cancer patients would be required to achieve this aim. Assuming Kp plasma levels were representative of Kp expression within the tumour, the plasma levels could then be correlated with outcome. Kp would be an ideal candidate as a diagnostic marker in neoplastic disease where early stage is difficult to detect, such as ovarian cancer. Ovarian cancer is problematic in that it is an internal organ and patients are usually unaware of their disease in the early stages since there are no obvious symptoms [39]. We have determined that plasma Kp is elevated in patients with ovarian cancer and other gyneacological diseases. Unfortunately, we did not

have a proper control group of healthy volunteers and could not confirm within our study that normal physiological levels of Kp are <2 pmol/l as has been previously reported with this assay [23-25]. A Kp plasma screen would give a significant advantage to the patient by early detection and facilitate treatment of this disease, however further analysis is required to determine if plasma Kp can be distinguished between malignancy and benign disease.

The major issue encountered throughout this thesis has been reproducible immunohistochemical analysis with respect to the Kp antibody. The KiSS1 antibody from Santa Cruz that was used in these IHC experiments yielded robust results on the human placenta that was used as a positive control. The placenta was the only tissue that had intense and reproducible staining; regardless of whether the assay was performed manually or using the automated Ventana system.

The IHC variability between cancer cohorts cannot be simply attributed to tissue type, although it is most likely that in the case of prostate cancer Kp is expressed at extremely low levels. Kp consistently correlated with the ovarian clear cell subtype in two independent cohorts, however these results were more striking in the original ovarian array. In the gynaecological array used as a validation cohort, the statistical analysis was also performed by grouping Kp IHC scores +1 and +2 together for the positive group. This grouping produced more positive clear cell cancer cases than when the +2 cases were considered as positive alone. However, the IHC score of +1 consisted of any Kp staining and included cases that had very weak signal. It is not realistic that an IHC biomarker

would be useful when the pathologist is expected to consider even weak staining as positive.

Furthermore, that Kp can be a favourable prognostic marker in one cohort of clear cell cancer patients yet not in a separate cohort of clear cell cancer patients from a different institution is worrying. Details of the treatment of these cohorts are unavailable to assess as a possible reason for the inconsistent results. One would not realistically expect that difference of tissue treatment within the same country could account for the IHC results, and most likely reflects lack of reproducibility due to the antibody.

7.3 Overall Conclusions

In summary, we have determined that Kp and GPR54 expression does predict clinical outcome; this expression correlates with favourable prognosis in ovarian cancer and GPR54 loss correlates with poor prognosis in node positive breast cancer patients. Furthermore, Kp can be secreted into the plasma of patients with gynealogical disease. Although we were unable to directly address our second hypothesis that GPR54 loss would alter Kp-GPR54 signalling, we have demonstrated that endogenous GPR54 is internalized in cancer cell lines. Moreover we have demonstrated that this cytoplasmic GPR54 does not signal through the canonical $G\alpha_q$ signalling pathway upon Kp stimulation. Finally, we have discovered novel genes involved in the regulation of the Kp-GPR54 system.

7.4 Future Directions

Due to the internalized GPR54 encountered in our cancer cell lines, we did not successfully establish a GPR54 knockdown model from a cancer cell line expressing a functioning plasma membrane receptor. A cell line that endogenously expresses GPR54 on the plasma membrane would be required in order for this to be accomplished, and as yet none have been reported. However, few studies have attempted to localize endogenous GPR54 in cancer cells, yet the literature supports that there are several lines with endogenous GPR54 that could be explored [37, 40-43]. Upon discovery of such a cell line, stably transfected knockdowns could be created using short hairpin RNA.

With the creation of this model, the effects of endogenous GPR54 signalling could be studied and compared directly to the absence of GPR54 signalling. In the literature it has been established that upon Kp stimulation, cells expressing GPR54 have less invasive ability. Similar studies could be conducted such as: matrigel invasion assays, wound healing assay, and migration chamber assays. Cell proliferation assays of this system would be informative and would help support whether or not the arrest of proliferation in GPR54 overexpressing cell lines is due to the presence of GPR54.

Although the above model system would help determine the mechanism involved in the anti-metastatic autocrine signalling effect of GPR54 by Kp, it would also be useful to explore cytoplasmic GPR54 further. As this is the first description of cytoplasmic GPR54 expressed endogenously, it would be helpful to know how frequently GPR54 is located in the cytoplasm. Identifying a cell line

with endogenously expressing GPR54 located on the plasma membrane as we have described above would help to elucidate this. This may be a common state for GPR54 in cancer cell lines, as we have determined that this is the case in all four of the cell lines studied. Similarly, it would be informative to discover the mechanism behind GPR54 endogenous internalization. We have co-localized GPR54 with β-arrestin, but GPR54 may also be encapsulated in an endocytic vesicle. Further co-localization immunofluorescent studies would help determine the exact nature of GPR54 internalization using antibodies specific for proteins involved in endosomic vesicles such as clatherin [44]. Further verification of β-arrestin's role in GPR54 internalization would also be valuable. Using short interfering RNAs to reduce β-arrestin expression may be sufficient to bring GPR54 back to the plasma membrane. If this were true, then it would be advisable to determine if returning GPR54 to the cell membrane is sufficient to activate calcium release upon Kp stimulation.

The Affymetrix analysis described in Chapter 6 comparing gene expression differences between knockout and wildtype mice was performed in order to discover new genes involved in Kp-GPR54 signalling. We successfully validated several genes through real-time PCR after controlling for sex hormone influence and have identified multiple gene candidates that deserve greater scrutiny. Of these candidates we are particularly interested in the 10-transmembrane protein, Tmem144, and the transcription factor Npas4. Npas4 was recently described to have a role in inducing inhibitory synapse development, and is activated by calcium ions [45]. This group also has an

antibody available for Npas4, so a starting point would be to determine if GPR54 and Npas4 are co-expressed within the same neurons in the hypothalamus. As the *Gpr54* promoter is paired with an IRESlacZ sequence in the *Gpr54* knockout mouse, there is β-galactosidase activity in cells and tissues that would ordinarily express GPR54 [15]. The Npas4 antibody could be treated on hypothalamic tissues from the GPR54 knockout mouse to determine if there is co-localization with *GPR54* expression.

There are currently no publications describing *Tmem144,* however a similar study could be conducted as suggested for Npas4, as the *Kiss1* knockout mouse was similarly created with a IRESlacZ sequence paired to the *Kiss1* promoter [46]. In this instance one could develop a RNA *in situ* probe for *Tmem144* that could be hybridized to hypothalamic tissues in the *Kiss1* knockout mouse. However, in this case we would expect that *Tmem144* would colocalize with *Gpr54* expressing neurons if we hypothesize that cation released through a Tmem144 channel would stimulate GnRH neurons.

Finally, a more comprehensive study assessing Kp in plasma of cancer patients would be extremely valuable. Such a study should be designed to include a true normal group of healthy volunteers as in our experiment all patients either had or were likely to have been affected by disease. Patients with benign disease should still be included, and all patients that are diagnosed with a malignancy. Furthermore, it would be extremely valuable to have a post-treatment sample from all patients regardless of their disease to establish whether Kp is disease specific. It has been established that plasma Kp returns to

normal after the delivery of the placenta in the case of pregnancy [25, 26] as well as after removal and treatment of the tumour in gestational trophoblast neoplasia [25]. Ours is the first report of plasma Kp elevation in benign disease, yet this may still be disease specific and once treated, plasma Kp may again return to normal physiological levels. Elevation of Kp in benign disease does not eliminate Kp as a potential plasma biomarker; rather it is still informative, as these benign diseases still require treatment.

The reason for the elevated Kp levels in BRCA mutation carriers and those free of disease yet having a history of cancer is unexpected and potentially a cause for concern. It is the one resounding argument against using Kp as a potential plasma biomarker. However, these women should not be immediately disregarded and assumed free of disease. As it has been repeatedly reported that Kp at normal physiological levels is negligible [23-27], it is important to consider something more may be occurring with these patients. As the stress and risks associated with exploratory surgery and further testing in these patients may outweigh the potential benefits of discovering an unknown disease, it would be unethical at this point to consider contacting these women. However, it would be advisable to link any future reports of disease in these patients with their current results if possible.

In summary, Kp and GPR54 are not currently diagnostic markers for malignancy, and though this ligand-receptor pair has the potential to become clinically useful, there is much more research required before this could be implemented.

7.5 References

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