

**Gonadotropin and kisspeptin receptor function in
granulosa cells from women with and without
Polycystic Ovary Syndrome**

Dr Lisa Owens

Institute of Reproductive and Developmental Biology

Department of Surgery and Cancer

Imperial College London

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Abstract

Polycystic ovary syndrome (PCOS) is a common, complex and heterogenous condition which affects 8-13% of women. The underlying aetiology is poorly understood and evidence suggests that it is a multigenic disorder with strong epigenetic and environmental influences. Recent genome wide association studies have found hits close to loci encoding FSHR and LHCGR, implicating gonadotropin receptor action in the pathogenesis of the condition. I therefore studied the actions of the gonadotropin receptors in granulosa lutein cells (GLCs) from women with and without PCOS. I also studied the actions of receptors which have been shown to affect ovarian gonadotropin action, the androgen and kisspeptin receptors. Lastly, I examined gene expression of gonadotropin receptors, steroid enzymes and growth factors in granulosa cells (GCs) from small antral follicles from women with and without polycystic ovaries (PCO).

Gonadotropin receptors are dependent on internalisation for the majority of gonadotropin receptor G α s signal generation and LHR G α s-cAMP signalling is enhanced in PCOS GLCs. This may be related to enhanced internalisation and trafficking of LHR to the very early endosome, as β -arrestin-1 and GIPC expression are augmented. In PCOS LHR displays signal bias of G α s over G α q/11 but no difference in ERK signalling. Downstream there is augmented activation of cellular phosphoproteins and upregulation of LHR, but no difference in progesterone output into culture media. FSHR signalling is similar in control and PCOS GLCs but downstream phosphoprotein activation differs. *In vitro* androgen treatment enhances LH induced cAMP and ERK signalling. *In vitro* kisspeptin treatment activates kisspeptin receptor G α q/11 and ERK signalling but does not contribute to steroidogenesis. *In vivo* kisspeptin given as an IVF maturation trigger in women with PCOS augments gonadotropin receptor and steroid enzyme gene expression. GCs from women with PCO display higher LHCGR in certain follicles, with higher steroid enzyme expression and lower FSHR and androgen receptor expression. In conclusion, GCs and GLCs from women with PCOS display distinct gonadotropin receptor signalling and regulation.

Statement of Originality

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List of abbreviations

3 β HSD	3 β -hydroxy steroid dehydrogenase
AC	Adenylyl cyclase
ACAT2	Acetyl-CoA Acetyltransferase 2
ACTB	Beta actin
AMH	Anti-Müllerian hormone
AMHR2	AMH receptor 2
ANOVA	Analysis of variance
Anov PCOS	anovulatory PCOS
APPL1	Adaptor protein, phosphotyrosine interacting with PH domain and leucine zipper 1
AR	Androgen receptor
ARKO	Androgen receptor knock out
AREG	Amphiregulin
ARRB1/ARRB2	Beta arrestin 1/2
ATP	Adenosine triphosphate
B2M	Beta-2-Microglobulin
BCA	Bicinchoninic acid
BMI	Body Mass Index
BSA	Bovine Serum Albumin
Ca ²⁺	Calcium
cAMP	Cyclic adenosine monophosphate

CCP	Clathrin coated pits
CHO	Chinese hamster ovary
CL	Corpus luteum
cOCP	Combined oral contraceptive pill
CPM	Counts per Million
CREB	cAMP response element binding protein
CRISPR	Clustered regularly interspaced short palindromic repeats
DHT	Dihydrotestosterone
DMEM	Dulbecco's modified eagle medium
DMSO	Dimethyl sulfoxide
DNA	Deoxyribonucleic acid
DPBS	Dulbecco's phosphate-buffered saline
DTT	Dithiothreitol
eCAVIAR	eQTL and GWAS Causal Variant Identification in Associated Regions
ECL	Enhanced chemiluminescence
EE	Early endosome
EEA1	Early Endosome Antigen 1
EGF	Epidermal growth factor
EGFR	Epidermal growth factor receptor
eQTL	expression quantitative trait loci
EREG	Epiregulin

ERK	Extracellular signal-regulated protein kinase
ER α	Oestrogen receptor α
ER β	Oestrogen receptor β
FBS	Fetal bovine serum
FDR	False Detection Rate
FSH	Follicle Stimulating hormone
FSHR	Follicle stimulating hormone receptor
FST	Follistatin
GAPDH	Glyceraldehyde 3-phosphate dehydrogenase
GC	Granulosa cell
GFP	Green fluorescent protein
GIPC	PDZ domain containing family member 1
GLC	Granulosa lutein cell
GnRHa	Gonadotropin releasing hormone agonist
GPCR	G protein coupled receptors
GWAS	Genome wide association studies
hCG	Human chorionic gonadotropin
HEK	Human embryonic kidney
HMGCS2	3-Hydroxy-3-Methylglutaryl-CoA Synthase 2
HPG	Hypothalamus-pituitary-gonadal
HRP	Horseradish peroxidase

hSAFs	Human small antral follicles
HTRF	Homogeneous Time Resolved Fluorescence
IBMX-	3-isobutyl-1-methylxanthine
ICSI	Intra-cytoplasmic sperm injection
IGF1	Insulin-like growth factor 1
IGF1R	Insulin-like growth factor 1 receptor
IgG	Immunoglobulin G
INH A	Inhibin
INHBA	Inhibin A
INHBB	Inhibin B
IP-1	Inositol monophosphate
IP-3	Inositol triphosphate
ITSX	Insulin transferrin sodium selenite
IVF	In vitro fertilization
JNK-	c-Jun N-terminal kinases
KNDy	Kisspeptin, neurokinin B, dynorphin
Kp-54	Kisspeptin 54
Kp-10	Kisspeptin 10
LH	Luteinising hormone
LHCGR	Luteinising hormone/choriogonadotropin hormone receptor
MAPK	Mitogen-activated protein kinase

mRNA	Messenger ribonucleic acid
MVD	Mevalonate Diphosphate Decarboxylase
NGS	Normal goat serum
nM	Nanomolar
OHSS	Ovarian hyperstimulation syndrome
OvPCOS	Ovulatory PCOS
P234	Peptide 234
P4	Progesterone
pAMPK	Phospho adenosine monophosphate-activated protein kinase
PBS	Phosphate-buffered saline
PCO	Polycystic ovaries
PCOS	Polycystic ovarian syndrome
PCR	Polymerase chain reaction
pCREB	phosphorylated cAMP response element-binding protein
PEDF	Pigment derived endothelial factor
pERK	phosphorylated extracellular signal-regulated kinases
PI3K	Phosphatidylinositol 3-kinase
PIP2	Phosphatidylinositol-4,5-bisphosphate
PIP3	Phosphatidylinositol-3,4,5-triphosphate
PKA	Protein kinase A
PKC	Protein kinase C
PLC	Phospholipase C
RGB	Red-Green-Blue

RIPA	Radioimmunoprecipitation assay
RNA	ribonucleic acid
RPM	Revolutions per minute
RT-qPCR	Quantitative reverse transcription polymerase chain reaction
SEM	Standard error of the mean
SERPINF1	Serpin Family F Member 1
SiRNA	Short interfering RNA
STAR	Steroidogenic acute regulatory protein
TBS	Tris-buffered saline
TBS-T	Tris-buffered saline-Tween
TGF	Transforming growth factor
TM	Transmembrane
VEE	Very early endosome
VEGF	Vascular endothelial growth factor
WES	capillary automated western immunoassay
17-OHP	17 hydroxyprogesterone

Chapter 1

Introduction

1.1 Ovarian follicle development

The ovary supports reproduction through the production of oocytes capable of fertilisation and the secretion of steroid hormones essential for reproduction. The ovary consists of follicles which are surrounded by stroma and enclosed within ovarian surface epithelium. Wallace and Kelsey estimated that at birth, girls have an average of 295,000 follicles per ovary, with a wide predicted range of 34,800 to 2,508,000 follicles per ovary (Wallace and Kelsey, 2010).

Throughout the reproductive lifespan a small number of follicles are consistently being activated, and the primordial follicle pool progressively declines. At any time, the ovary contains follicles at most stages of development. Activation of these primordial follicles occurs with 3 morphological transformations; oocyte growth, granulosa cell (Longcope, 1986) proliferation and transformation from flattened to cuboidal, however the order these occur in is unclear (Lintern-Moore and Moore, 1979). The development of follicles from primordial through to large ovulatory follicles occurs in two phases; the pre-antral, gonadotropin independent phase and the antral, gonadotropin dependent phase (Figure 1.1). Most follicles will die without ever progressing to the large ovulatory stage.

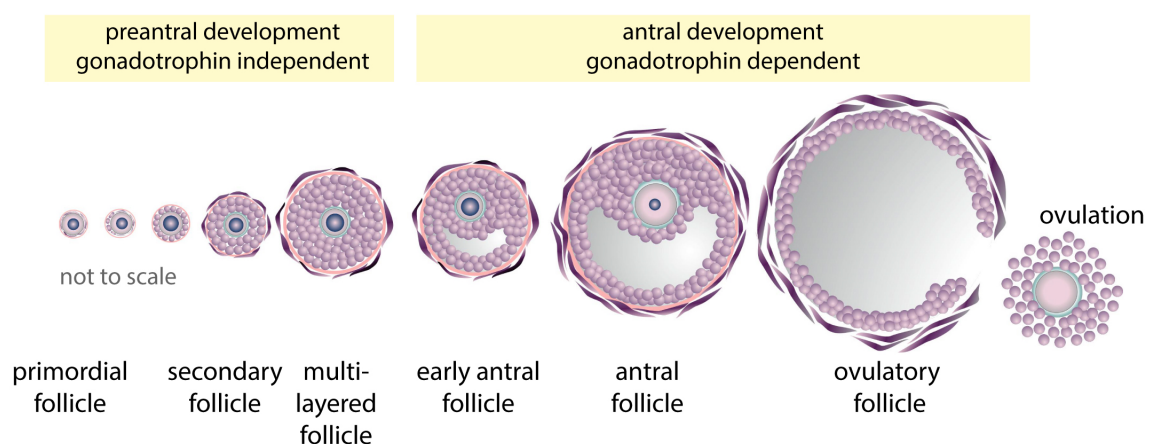


Figure 1.1 Follicle development in the mammalian ovary. After activation, dormant primordial follicles progress through the pre-antral phase, from primary, to secondary, to multi-layered follicles. These stages are largely independent of gonadotropins. Primordial follicles contain an immature oocyte surrounded by flat granulosa cells. After activation, granulosa cells become cuboidal, marking the primary follicle. Secondary follicles have theca and a basal lamina. Formation of the antrum, or central cavity, marks the transition to dependence and responsiveness to gonadotropins. The granulosa cells of the tertiary follicle undergo differentiation into four subtypes: corona radiata, membrana, periantral, and cumulus oophorous. Certain follicles ultimately progress to reach the pre-ovulatory stage, after which ovulation occurs, and oocytes are released for fertilisation. (Figure courtesy of Kate Hardy).

1.2 Ovarian steroid production

The ovary produces three main sex steroid hormones; androgens, oestrogens and progesterone. These hormones are critical for reproduction; they regulate puberty and development of secondary sexual characteristics, they support follicle development, they regulate the menstrual cycle, and they support pregnancy and lactation. Androgens, such as androstenedione and testosterone, are produced from cholesterol in theca cells in response to luteinising hormone (LH). Testosterone is reduced to Dihydrotestosterone (DHT) by 5 alpha reductase. Testosterone and androstenedione then diffuse into GCs, where they are aromatised into oestrogens by the enzyme aromatase (CYP19A1), stimulated by follicle stimulating hormone (FSH) (Dorrington and Armstrong, 1979, McNatty et al., 1979). This theca/GC interaction comprises the so-called two-cell two-gonadotropin hypothesis (Berger and Taymor, 1971). Following ovulation, GCs, now termed granulosa lutein cells (GLCs) within the corpus luteum, predominantly make progesterone, stimulated by LH (McNatty et al., 1979). Progesterone production from cholesterol is supported by the enzymes STAR (Steroidogenic acute regulatory protein), CYP11A1 (P450scc) and HSD3B2 (3 β Hydroxysteroid dehydrogenase 2) (Figure 1.2).

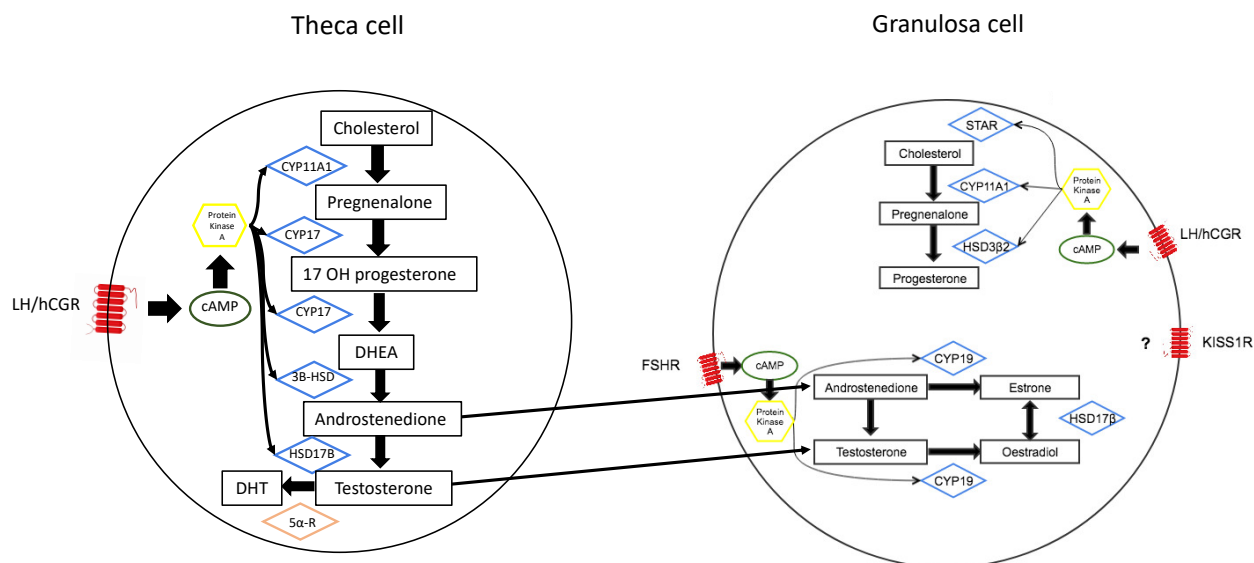


Figure 1.2 Ovarian steroidogenesis. LH stimulates theca cells to produce androgens from cholesterol. The androgens testosterone and androstenedione diffuse into granulosa cells and are converted to oestrogens via the enzyme aromatase (CYP19A1). This is mediated by FSHR. Progesterone production predominantly occurs in GLCs, again mediated by LHR. Progesterone is synthesised by pregnenolone, which is derived from cholesterol. The rate limiting step in progesterone production is the delivery of cholesterol to the mitochondrial cholesterol side chain cleavage system and this is mediated by the enzyme STAR. The conversion of cholesterol to pregnenolone is catalysed by the enzyme CYP11A1 and the conversion of pregnenolone to progesterone is catalysed by the enzyme HSD3B2.. The possible role of kisspeptin receptor in GLC steroidogenesis will be discussed further in 1.7 below.

1.3 Human Hypothalamic Pituitary Ovarian (HPO) axis

Kisspeptin neurons are in close proximity to gonadotropin releasing hormone (GnRH) neurons in the hypothalamus (Rance et al., 1994). They are co-located with Dynorphin and Neurokinin B, the so called 'KNDy neurons', which regulate kisspeptin release. GnRH neurons express the kisspeptin receptor (KISS1R) (d'Anglemont de Tassigny et al., 2008) and kisspeptin release

stimulates GnRH neurons to release GnRH (Liu et al., 2008). GnRH then travels to the anterior pituitary via the hypophyseal portal system and binds to receptors in the anterior pituitary, stimulating LH and FSH release into the bloodstream. FSH and LH primarily stimulate the ovaries to make androgens, oestradiol and progesterone. Oestradiol and progesterone then provide negative feedback to the hypothalamus, reducing GnRH release. Inhibin, which is also made by the ovary in response to FSH, downregulates FSH production and inhibits activin. Activin, made by multiple tissues, increases FSH binding and aromatisation and enhances LH-induced androgen synthesis. Activin is inhibited by both inhibin and follistatin (Figure 1.3).

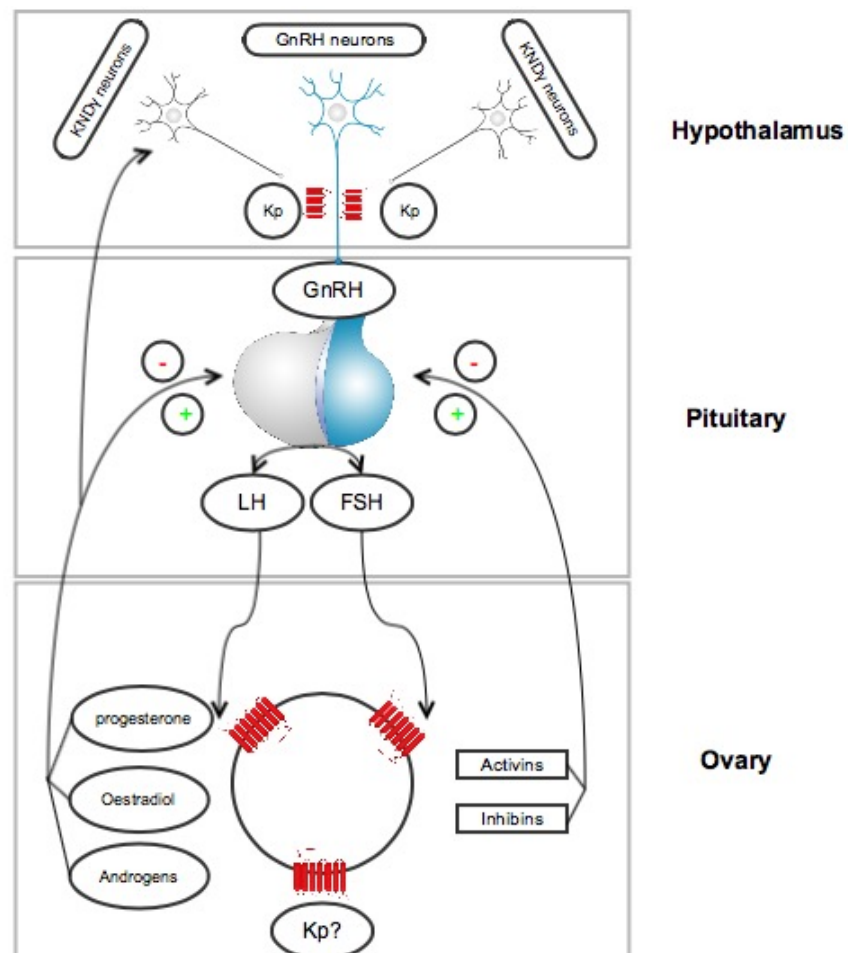


Figure 1.3 The human hypothalamic ovarian axis. Kisspeptin neurons are located within the hypothalamus with neurokinin B and Dynorphin neurons (KNDy neurons), which control its release. GnRH neurons in the hypothalamus express Kisspeptin receptor and kisspeptin stimulates GnRH release. GnRH then stimulates the anterior pituitary to release LH and FSH, which act predominantly on the ovary. LH stimulates androgen production and androgens are converted to oestradiol by the action of FSH. LH also stimulates progesterone production post ovulation. Oestradiol and progesterone negatively feedback to the hypothalamus to inhibit GnRH release. Feedback also occurs at the level of the pituitary. Inhibin, which is also made by the ovary in response to FSH, downregulates FSH production and inhibits activin. Activin, made by multiple tissues, increases FSH binding and aromatisation and enhances LH induced androgen synthesis. Kisspeptin receptor is also expressed by the human adult ovary, however its direct role in the HPG axis is unknown.

1.4 The menstrual cycle

The ovarian (menstrual) cycle is divided into the follicular phase (days 1-14), ovulation (approximately day 14), and the luteal phase (days 15-28). For the uterus this cycle is divided into menstruation (days 1-5/7), the proliferative phase (days 7-14) and the secretory phase (days 15-28). Oestrogen progressively increases in the follicular phase, ending menstrual bleeding and stimulating proliferation of the endometrial lining. Several ovarian follicles begin enlarging, however only one or two will become dominant, the rest involute and die. Mid-way through the cycle, 24–36 hours after the LH surge, ovulation occurs and the dominant follicle releases an ovum. After ovulation, the ovum will survive for approximately 24 hours unless it is fertilised. The remnants of the dominant follicle in the ovary become the corpus luteum, which predominantly produces progesterone to support implantation. Progesterone stimulates increased blood flow, endometrial secretions and reduced smooth muscle contractility of the endometrium, preparing it for implantation. If implantation does not occur, the corpus luteum will involute, causing a sharp drop in levels of both progesterone and oestrogen. This drop stimulates shedding of the uterine lining and menstruation begins.

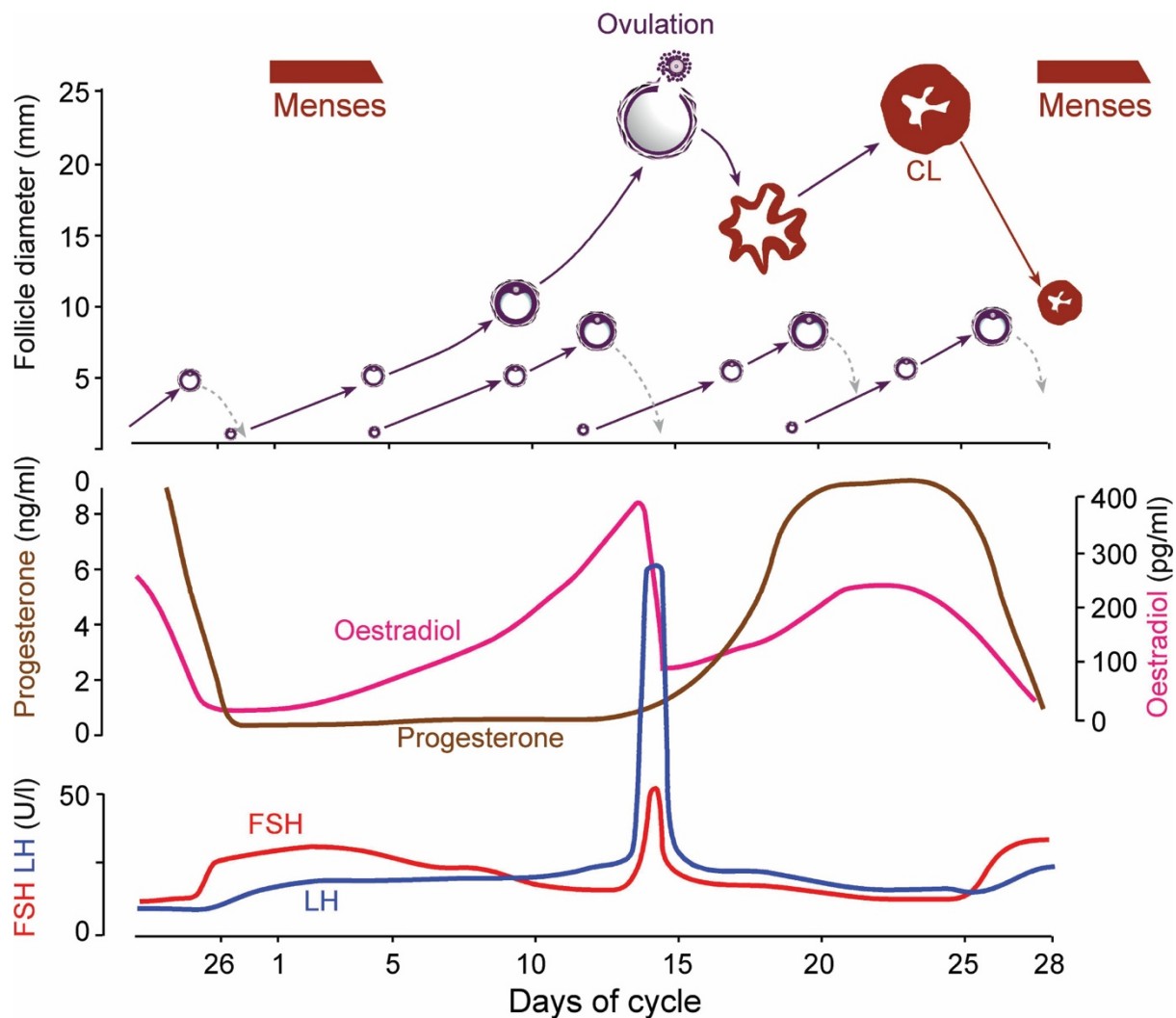


Figure 1.4 The menstrual cycle. Oestrogen progressively increases in the follicular phase (days 1-14), ending menstrual bleeding (menses) and stimulating proliferation of the endometrial lining. Several ovarian follicles begin enlarging. Mid way through the cycle LH, and to a lesser extent FSH, surge. 24–36 hours after this LH surge, ovulation occurs and the dominant follicle releases an ovum. This ovum will survive for approximately 24 hours unless fertilisation occurs. The other follicles that do not become dominant die. The remnants of the dominant follicle in the ovary become the corpus luteum (CL), which predominantly produces progesterone to support implantation. If implantation does not occur, the CL will involute, causing a sharp drop in levels of both progesterone and oestrogen. This drop stimulates shedding of the uterine lining and menstruation/menses begins (figure courtesy of Kate Hardy).

1.5 Androgen actions in the ovary

Androgens play a critical role in the normal development and functioning of the female reproductive system. They act as a precursor for oestradiol, but they also have direct roles, via the androgen receptor (AR). Androgens are synthesised by theca cells, and subsequently diffuse into GCs where they are converted to oestrone or oestradiol by aromatase, as discussed above in 1.2, termed the two-cell two-gonadotropin hypothesis (Hillier, 1994, Burger, 2002, Ghayee and Auchus, 2007). The production of androgens within the ovarian follicle is under the control of luteinising hormone (LH), with LH acting via LH receptors on theca cells to stimulate conversion of cholesterol to pregnenolone (Longcope et al., 1986, Erickson et al., 1985).

The AR functions by binding to genomic androgen response elements (ARE), which influence transcription of AR target genes, and via membrane-initiated non-genomic signalling. Rapid, non-genomic AR signalling likely involves G-protein-coupled receptors (GPCRs) acting as steroid extranuclear receptors, such as the androgen regulated GPCR Family C group 6 Member A (GPRC6A), which stimulates signalling in response to androgen in cells lacking the AR (Pi et al., 2010). Non-genomic signalling also relies on nuclear receptors localising to the plasma membrane, from where they can alter signal transduction (Wilkenfeld et al., 2018). The AR associates with the plasma membrane at caveolin lipid rafts through interactions with proteins Caveolin-1 and Src, from where it can activate G proteins and alter signalling pathways (Deng et al., 2017, Yu et al., 2012). However the non-genomic actions of AR in the ovary have not been extensively studied. It is known that androgens can stimulate extracellular signal-regulated protein kinase (ERK) signalling and steroidogenesis through transactivation of the epidermal growth factor receptor (EGFR), a process which is mediated by matrix metalloproteinases (Carbajal et al., 2011, Evaul and Hammes, 2008).

The AR is expressed by theca cells, GCs and the oocyte throughout follicular development (Suzuki et al., 1994). Expression is highest in preantral and antral stages and declines as follicles mature (Suzuki et al., 1994) (Figure 1.5). It is unclear what regulates expression of the AR. Androgen effects on follicle development have mostly been demonstrated in animal models, however it can be difficult to separate androgen effects from that of oestrogen through

aromatisation. The critical role of androgens in the ovary has been highlighted through mouse knockout models. The global and granulosa cell-specific AR knockout (ARKO) (Funes et al., 2003) mice have been shown to be sub-fertile with fewer ovulations and reduced litter size (Sen and Hammes, 2010, Hu et al., 2004b, Walters et al., 2007) . The ovaries of mice from the GC ARKO model have more pre-antral and atretic follicles and less antral follicles, suggesting that AR in GCs is necessary for follicle progression. The oocyte-specific ARKO model has normal reproductive function, suggesting that androgen activity in the oocyte is not necessary for reproduction (Sen and Hammes, 2010).

Androgens also interact synergistically with gonadotropins and this relationship appears to be key to regulating ovarian function treatment with testosterone increases FSH receptor expression in primate follicles and mouse granulosa cells (Laird et al., 2017, Weil et al., 1999, Sen and Hammes, 2010). In addition, in mouse ovaries, *in-vitro* androgen treatment has been shown to augment FSH-induced follicle growth, increase antrum formation, increase expression of Star and reduce expression of Cyp11a1 (Laird et al., 2017). These androgen effects were reversed by the androgen receptor antagonist flutamide. Incubation with androstenedione *in vitro* also augments FSH-dependent oestradiol secretion in bovine granulosa cells (Hamel et al., 2005). In rat granulosa cells, DHT caused a reduction in ERK phosphorylation in response to FSH, leading to reduced cyclin D2 mRNA expression that culminated in cell cycle arrest.

The role of androgens around the time of ovulation is less clear. *In vitro* treatment with androgens in mature rat follicles was shown to inhibit GC proliferation and increase apoptosis (Billig et al., 1993, Pradeep et al., 2002). Androgens produced by the LH surge may be involved in ovulation in mice, as *in vitro* treatment of whole mouse ovaries with DHT increased the expression and actions of Cox2 and Areg, both of which are necessary for ovulation and which contain AREs (Yazawa et al., 2013).

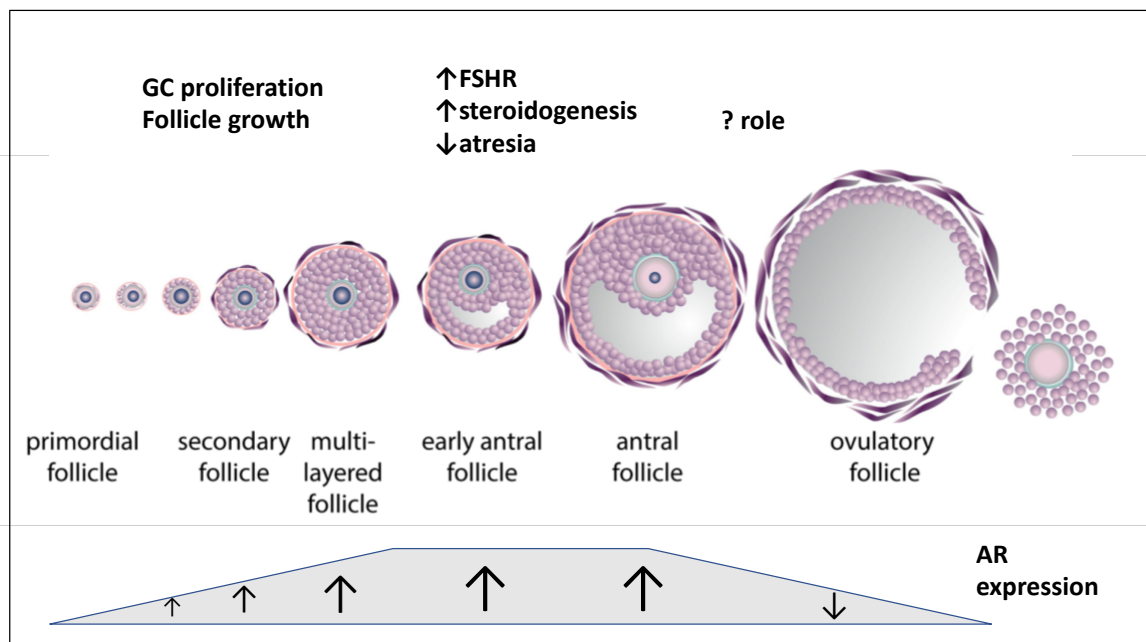


Figure 1.5 Androgen actions and AR expression in the human ovarian follicle. AR increases in expression until the follicles reach the antral stage, then decrease as the follicle reaches ovulation. Androgen stimulates follicle growth and transition from primary to secondary follicle, promoting antrum formation and proliferation of GCs. In animal models it increases expression of FSHR and expression of AR is positively correlated with FSHR and also augments steroid enzyme expression and augments FSH induced oestradiol synthesis. The role of androgens in the peri-ovulatory follicle is unknown (Figure adapted from Kate Hardy).

The importance of androgens in the ovary is clear, however there appears to be a fine balance, with excess androgen resulting in ovulatory dysfunction and is a key defining feature of PCOS. Androgen excess in animal models replicates human PCOS characteristics and there is strong evidence to support a direct role for AR-mediated signalling in the pathogenesis of PCOS. Androgen excess in congenital adrenal hyperplasia also creates a PCOS type phenotype. Hyperandrogenism in PCOS will be discussed in more detail in 1.6.4 below.

1.6 Polycystic Ovary Syndrome (PCOS)

PCOS is the most common endocrinopathy in women of childbearing age, affecting between 8 and 13% of women, depending on what populations are studied and what criteria are used for diagnosis (Azziz et al., 2016, Teede et al., 2010). It is a complex condition that is associated with

reproductive, metabolic and psychological sequelae (Azziz et al., 2016, Teede et al., 2010). It was first described in 1935 by Stein and Leventhal as the combination of hirsutism, amenorrhoea, chronic anovulation and infertility, obesity and enlarged polycystic ovaries (Stein, 1935).

Irregular cycles and ovulatory dysfunction are common in PCOS and can lead to subfertility. Hyperandrogenism is a key feature of PCOS and occurs in between 60 and 100% of women with the condition (Teede et al., 2018). The most common androgen-related symptoms in PCOS are hirsutism, acne and androgenic alopecia. The long term health of women can also be affected by PCOS, as it is associated with insulin resistance and type 2 diabetes mellitus (T2DM), obesity, psychological morbidity, endometrial cancer and possibly cardiovascular disease (CVD).

Women with PCOS have increased prevalence of impaired glucose tolerance (Odds ratio (OR) 2.48) and T2DM (OR 4.43) (Moran et al., 2010). This increased prevalence is independent of, but compounded by, obesity. A Danish registry study found that T2DM was four times higher and was diagnosed four years earlier in women with PCOS (Rubin et al., 2017). It is recommended that all women with PCOS should have glycaemic status measured at baseline, and ongoing assessment should be every one to three years, influenced by the presence of other risk factors for T2DM (Teede et al., 2018).

Women with PCOS may be at risk of cardiovascular disease (CVD), and certainly often have risk factors for CVD, including obesity, insulin resistance and dyslipidaemia. However longitudinal studies in this area are lacking and existing studies focus on younger women, or on risk factors for CVD as opposed to actual clinical events (Calderon-Margalit et al., 2014). The Nurses' Health study found that women with irregular menstrual cycles were at increased risk of CVD (53% increase) and had a 2 fold increased risk of T2DM (Solomon et al., 2001, Solomon et al., 2002).

Health related quality of life is affected by PCOS. There is a high prevalence of anxiety and depressive symptoms in women with PCOS and an increased rate of diagnosis of a psychological

disorder, independent of obesity (Cooney et al., 2017, Dokras et al., 2018). There is also a higher prevalence of eating disorders and disordered eating (Cesta et al., 2016, Månsson et al., 2008).

1.6.1 Diagnosis of PCOS

The two main criteria used for diagnosing PCOS are the Rotterdam criteria and the National Institutes of Health Criteria (NIH) criteria. The Rotterdam Criteria (2003) require two of three of the following; oligo- or anovulation, clinical and/or biochemical hyperandrogenism, or polycystic ovaries on ultrasound, after exclusion of related disorders (Group, 2004). The Rotterdam criteria are endorsed by the International PCOS guidelines 2018 (Teede et al., 2018). The NIH criteria (1990) require the presence of clinical and/or biochemical hyperandrogenism and oligo/amenorrhea anovulation (Zawadski and Dunaif, 1992).

In clinical practice however diagnosis of PCOS can be difficult and diagnosis can often be delayed. Measuring serum androgens in women, while it is part of the diagnostic criteria, can be difficult as most high-throughput inexpensive methods of measuring testosterone are insensitive and inaccurate in women. Endocrine Society Guidelines recommend using high-quality assays such as liquid chromatography-mass spectrometry (LCMS) and extraction/chromatography immunoassays to measure testosterone, (Teede et al., 2018), however these are expensive and not readily available in many centres.

Serum anti-mullerian hormone (AMH) is elevated in women with PCOS (Cook et al., 2002, Seifer and Maclaughlin, 2007) and has been suggested as a diagnostic marker of the condition, however there is a lack of standardisation between assays and further studies validating thresholds and cut off levels in populations of different ages and ethnicities are required prior to AMH being adopted as part of the diagnosis of PCOS.

1.6.2 The aetiology of PCOS

PCOS is a heterogenous condition, the aetiology of which is complex and incompletely understood. Evidence suggests it is multigenic disorder with strong epigenetic and environmental influences, including ethnicity, diet and lifestyle factors and prenatal exposure to insults such as excess androgen.

The heritability of PCOS was estimated to be 70% in a monozygotic twin study (Vink et al., 2006). Studies in sisters of women with PCOS showed that 22% of them fulfilled diagnostic criteria for PCOS and an additional 24% had hyperandrogenaemia with regular menstrual cycles (Legro et al., 1998). This led to an interest in using genome wide association studies to study PCOS. The first GWAS and subsequent follow up in Han Chinese populations identified the following 11 PCOS candidate loci: *FSHR*, *LHCGR*, *DENND1A*, *INSR*, *YAP1*, *C9orf3*, *RAB5B*, *HMG2A2*, *TOX3*, *SUMO1P1/ZNF217* and *THADA* (Shi et al., 2012, Chen et al., 2011). Replication studies in European populations have also found that variants at these loci also confer risk for PCOS. The strongest PCOS loci in European populations are; *DENND1A*, *THADA*, *LHCGR*, *FSHR*, *YAP1* and *RAB5* (Welt and Duran, 2014). A further study in European populations with PCOS as diagnosed by NIH criteria also implicated gonadotropin and gonadotropin receptor action in the aetiology of PCOS, replicating the *FSHB*, *FSHR* and *LHCGR* loci found by other studies (Hayes et al., 2015). They also found that *FSHB* subunit of FSH was significantly associated with serum LH levels. A recent meta-analysis from 10,074 PCOS cases and 103,164 controls of European ancestry identified 3 novel loci (near *PLGRKT*, *ZBTB16* and *MAPRE1*), and replicated the 11 previously reported loci (Day et al., 2018). These again included *FSHR* and *FSHB*, and *FSHB* was shown to be associated with ovulatory dysfunction, serum LH and FSH levels. Interestingly the observed susceptibility loci in PCOS are similar for those diagnosed using NIH criteria and those who self-report having PCOS, suggesting a common genetic background in the condition despite varying severity.

However, GWAS do not identify a gene associated with a disorder, rather highlight a genetic locus of interest, a variant which may affect the nearest gene or a gene that is farther away. Further studies must examine if these variants are causal by demonstrating a functional effect

that shows how a variant predisposes a person to develop the disorder. Thus far, studies have looked at the functional role of one of the variants identified as a PCOS locus, *DENND1A*. It encodes a protein associated with clathrin coated pits and is thought to be involved in endocytosis and receptor-mediated turnover (Allaire et al., 2010). It is expressed by the cytoplasm and nuclei of theca cells. A splice variant of *DENND1A* (*DENND1A.V2*) was found to be over-expressed in theca cells and in urine from women with PCOS (McAllister et al., 2014). Overexpression of *DENND1A.V2* in theca cells from women without PCOS resulted in a PCOS phenotype, with increased *CYP17A1* and *CYP11A1* gene expression and androgen biosynthesis. Knocking down *DENND1A.V2* in PCOS theca cells reduced androgen biosynthesis and *CYP17A1* and *CYP11A1* gene transcription. An IgG specific to *DENND1A.V2* also reduced androgen biosynthesis and *CYP17* and *CYP11A1* mRNA when incubated with PCOS theca cells in vitro (McAllister et al., 2014).

The limitations of GWAS studies have led to the development of further analysis tools. The vast majority of GWAS risk loci fall in non-coding regions of the genome and do not account for tissue-specific effects. Also GWAS risk loci may alter the individual's disease risk via their effect on gene expression in different tissues. In addition, multiple testing correction can miss the causal variants that confer genetic effects (Wang et al., 2007). Genes can also function together rather than working alone (Cordell, 2009). The genes identified by GWAS are often functionally unrelated, providing limited information on the pathogenesis of complex diseases. The proportion of heritability accounted for by the PCOS loci identified so far by GWAS is <10%, which is vastly different from the 70% suggested by twin studies.

Expression quantitative trait loci (eQTL) analysis (Nica and Dermitzakis, 2013) involves a direct association test between markers of genetic variation with gene expression levels. This has previously been done comparing GWAS data in PCOS with serum, fat and skin biopsies from women with and without PCOS (Pau et al., 2017). They found associations between certain genotypes and inflammation and variations in gonadotropins and gene expression in skin and fat. Analyses such as eQTL help to determine which genes are affected in which tissues. Another related tool is the eCAVIAR analysis (eQTL and GWAS Causal Variant Identification in Associated

Regions). This model allows for allelic heterogeneity, where multiple variants can be causal in a single locus, a phenomenon that is widespread in eQTL data (Hormozdiari et al., 2016).

It is likely that PCOS is multigenic, where predisposing genetic variants interact with environmental or epigenetic influences (Escobar-Morreale, 2018). These environmental influences may include diet and lifestyle factors that are strongly influenced by race and ethnicity. Insults during pregnancy, for example maternal diabetes or smoking, can result in a low birth weight infant (Ibáñez et al., 2006). These children are then predisposed to obesity, insulin resistance and PCOS (Ibáñez et al., 2001). This could then be compounded by a poor diet and sedentary lifestyle, factors which can cluster in families (Figure 1.6).

However PCOS is not just seen in women who were born at low birth weight, it can be seen in those who were large for gestational age, whose mothers have obesity or hyperandrogenism (van Hooff and Lambalk, 1998, Cresswell et al., 1997). This theory of prenatal exposure to androgens as a causative factor for PCOS has been explored in animal models. Pregnant monkeys were injected with testosterone at two stages during their pregnancy. When their progeny were followed up beyond puberty, many developed polycystic ovaries, high serum concentrations of LH and insulin resistance (Abbott et al., 2005). Studies of the ovaries of prenatally androgenised female sheep indicate that exposure to androgens *in utero* influences early follicular development, with their ovaries demonstrating a lower percentage of primordial follicles and a higher percentage of primary follicles (Forsdike et al., 2007). Thus hyperandrogenism may explain the disordered pattern of folliculogenesis characteristic of the polycystic ovary.

Placental function may also play a role in the aetiology of PCOS. Placental tissue from women with PCOS were found to have high 3 β -HSD-1 and lower aromatase activity, which could increase placental steroidogenesis and androgen production during pregnancy (Maliqueo et al., 2013). Prenatal testosterone exposure in rodents reduces placental and foetal growth and amino acid transfer (Sun et al., 2012, Sathishkumar et al., 2011).

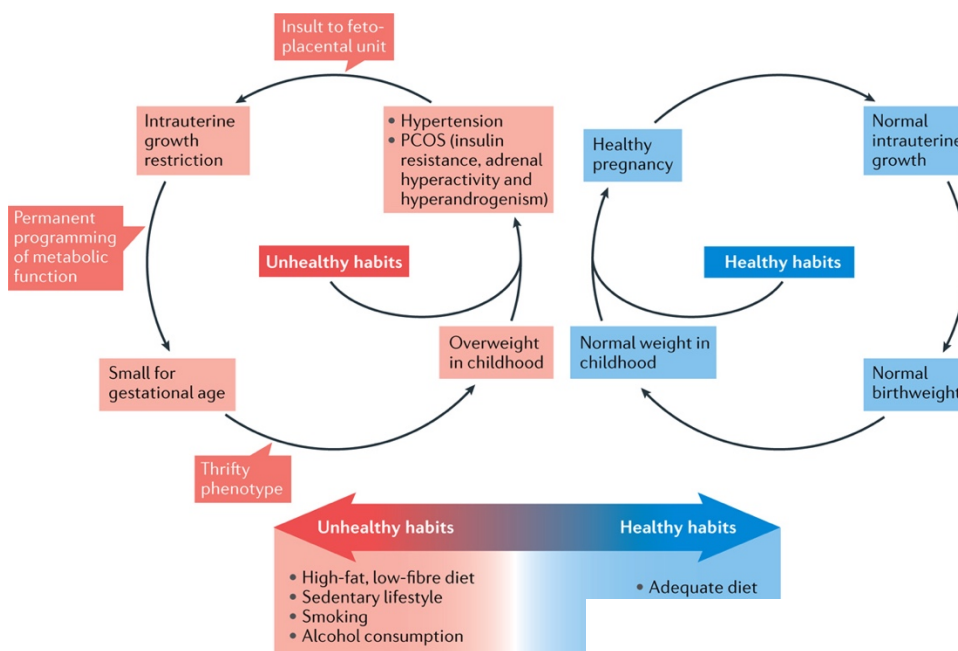


Figure 1.6 Environmental factors influencing PCOS. Adapted with permission from (Escobar-Morreale, 2018) An insult in utero may result in intrauterine growth restriction and low birth weight, which then predisposes to overweight in childhood. That in combination with unhealthy lifestyle habits, such as poor diet and lack of exercise, increase the risk of hypertension and PCOS in later life. A healthy pregnancy and healthy lifestyle habits on the other hand, lead to a healthy baby, child and therefore adult.

1.6.3 The HPG axis in PCOS

GnRH pulsatility in the hypothalamus of women with PCOS is persistently rapid, favouring LH synthesis over FSH in the pituitary, which results in a high circulating serum LH and high LH:FSH ratio (Taylor et al., 1997), without cyclic variation (Waldstreicher et al., 1988). LH then stimulates androgen synthesis by theca cells, perpetuating the hyperandrogenaemia seen in PCOS. Insulin resistance and hyperinsulinaemia also contribute to androgen synthesis (discussed in detail in 1.6.4 below). Reduced FSH production by the pituitary results in decreased aromatisation of androgens to oestrogens and impaired follicle development. Androgens reduce sex steroid-

mediated negative feedback to the GnRH pulse generator through reduced sensitivity. Lack of luteal phase progesterone production in PCOS also affects GnRH pulsatility, as progesterone is the primary mediator of GnRH pulse frequency (Soules et al., 1984), with and to a lesser extent, oestradiol (Nippoldt et al., 1989).

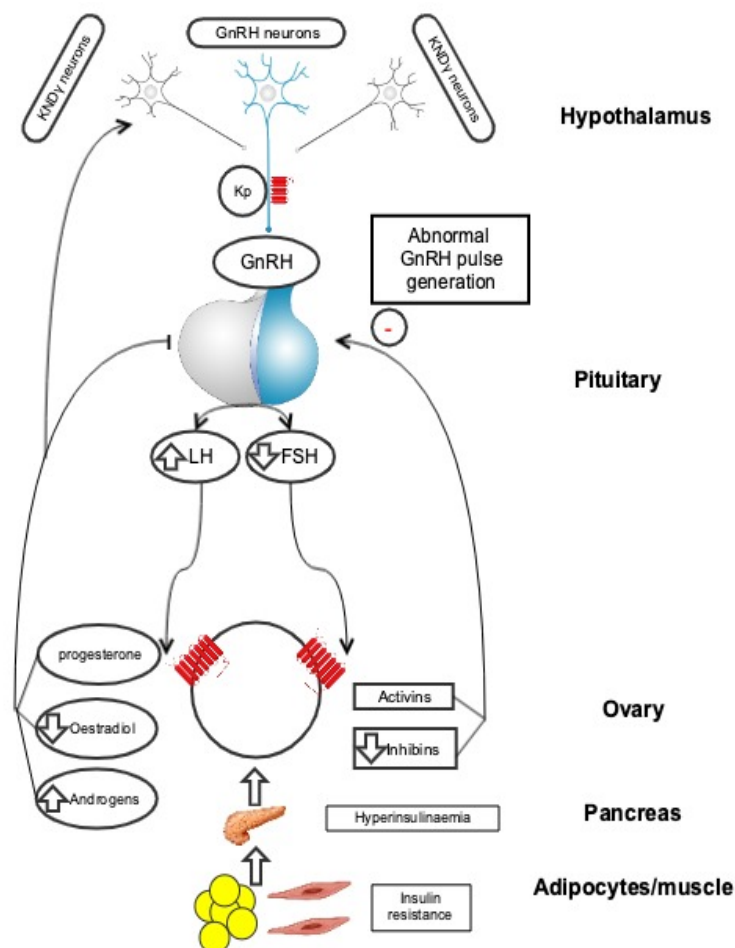


Figure 1.7 The HPG axis in PCOS. Rapid pulsatile GnRH release from the hypothalamus favours LH release over FSH. This LH then acts on the ovary to stimulate androgen synthesis. There is a lack of sex steroid mediated negative feedback, so further GnRH pulses are generated and LH release occurs. Insulin resistance and consequent hyperinsulinaemia also contribute to hyperandrogenism and therefore lack of negative feedback to the hypothalamus/pituitary.

1.6.4 The pathophysiology of PCOS

While the pathophysiology underlying the development of PCOS in those who are predisposed to developing the condition has not been clearly elucidated, several key metabolic features of PCOS have been described. Insulin resistance is a common feature in PCOS. Women with PCOS have exaggerated insulin responses to glucose tolerance testing, beyond that of obesity alone (Burghen et al., 1980) and can have acanthosis nigricans, the severity of which is correlated with the degree of insulin resistance (Dunaif et al., 1991). Women with hyperinsulinaemia of any cause have an increased prevalence of PCOS, such as those with T2DM (Peppard et al., 2001) (Tosi et al., 2011), obesity (Alvarez-Blasco et al., 2006), or rare insulin receptor mutations (Musso et al., 2004).

Insulin acts as a co-gonadotropin in the ovary, augmenting LH-induced androgen production in theca cells, augmenting CYP17 activity (Munir et al., 2004, Franks et al., 1999, Nestler and Strauss, 1991). PCOS theca cells have a higher expression of insulin receptor substrate (IRS) 1 and 2, which transmit signals from insulin receptor (INSR) and insulin-like growth factor-1 (IGF-1) to the AKT and ERK pathways (Yen et al., 2004). Insulin augments FSH-induced oestradiol and LH-induced luteinisation in GCs (Franks et al., 1999). Insulin enhances adrenal androgen production (Carmina et al., 1999). As discussed above, LH pulsatility is affected in PCOS, and this too may be related to hyperinsulinaemia, as insulin enhances gonadotropin secretion from the pituitary (Adashi et al., 1981). Insulin may also contribute to the follicle arrest seen in PCOS, acting with LH to promote premature LHR responsiveness in GCs from small antral follicles (Willis et al., 1998, Willis et al., 1996, Rice et al., 2005). Follicle arrest will be discussed in more detail in 1.6.6 below.

However not all women with PCOS have insulin resistance, and not all those with insulin resistance have PCOS. Therefore hyperandrogenism may be the primary defect, which may result from a complex interplay between varying triggers, including insulin resistance. PCOS may result from a vicious circle of hyperandrogenaemia, which favours abdominal adipose tissue deposition and visceral adiposity by inducing insulin resistance and hyperinsulinism, which further facilitates androgen secretion by the ovaries and adrenal glands in women with PCOS

(see Figure 1.8 below) (Escobar-Morreale, 2018). Profiles of adipose tissue from women with PCOS are different from healthy women and are closer to that of men, suggesting androgens directly cause adipose tissue abnormalities (Cortón et al., 2008, Cortón et al., 2007, Martínez-García et al., 2013, Montes-Nieto et al., 2013).

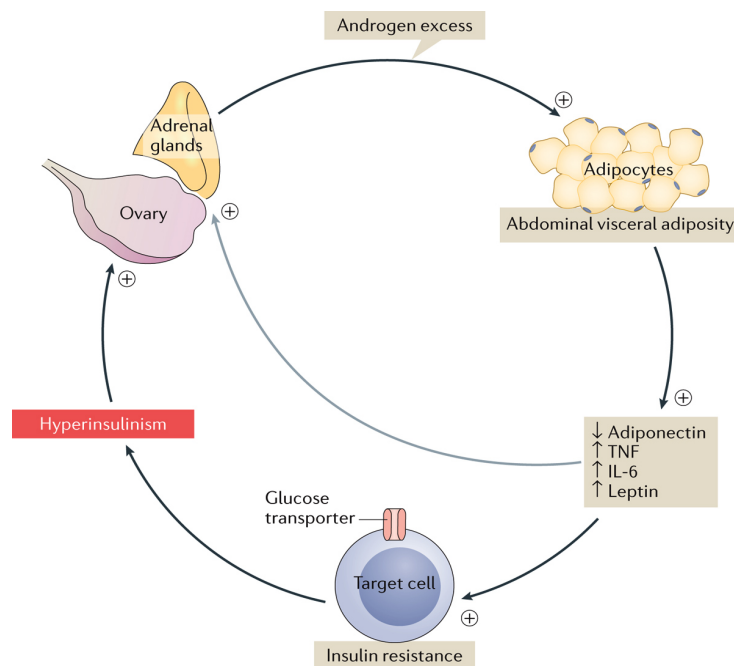


Figure 1.8 The interplay between PCOS and abdominal adiposity. PCOS may involve a vicious circle of androgen excess, which results in increased abdominal adiposity, which then causes further excess androgen production from the ovary through mediators such as adiponectin (which is downregulated), tumour necrosis factor (TNF), interleukin-6 (IL-6) and leptin, which are upregulated, and also induces insulin resistance and hyperinsulinaemia. Reproduced with permission from (Escobar-Morreale, 2018).

1.6.5 Disordered follicle development in PCOS

Hyperandrogenaemia in PCOS is in part related to excess ovarian androgen production, termed 'functional ovarian hyperandrogenism'. Women with PCOS have been shown to have exaggerated 17-hydroxyprogesterone (17-OHP) responses to GnRH agonist or human chorionic gonadotropin (hCG) administration, suggestive of enhanced theca cell CYP17 activity (Ehrmann et al., 1995), (Gilling-Smith et al., 1997). This theca cell hyperresponsiveness has also been demonstrated in vitro, as PCOS theca demonstrates increased expression of CYP11A1 and CYP17, and increased activity of CYP17, HSD3B, 17 β hydroxysteroid dehydrogenase and increased production of progesterone, 17-OHP and testosterone (Nelson et al., 2001, Nelson et al., 1999). Hyperandrogenaemia in PCOS may also be related to adrenal production of androgens, as when women with PCOS are given GnRH agonists (GnRHa) to suppress ovarian androgen production, they still have excess androgen compared to control women, suggesting the adrenals are also contributing to hyperandrogenism in PCOS (Hoffman et al., 1984, Barnes et al., 1989, Carmina et al., 1992). This may be explained by dysregulation of 11 β -hydroxysteroid dehydrogenase which causes increased oxidation of cortisol to cortisone (Rodin et al., 1994).

AMH has also been implicated in the pathophysiology of PCOS. AMH is a key member of the TGF- β superfamily of growth factors that are important for normal follicle development. It's role in follicle arrest will be discussed in 1.6.6 below. It has recently been reported that treatment of pregnant mice with AMH results in a PCOS like phenotype in the offspring, via a mechanism that implies a neuroendocrine action of AMH (Tata et al., 2018). GnRH antagonist treatment of these offspring normalised their PCOS neuroendocrine phenotype.

1.6.6 The aetiology of follicle arrest and ovulatory dysfunction in PCOS

The 'polycystic' appearance of the ovaries in PCOS is caused by the accumulation of follicles of varying stages of maturation and atresia, with a higher number of pre-antral and small antral follicles (Dewailly et al., 2014, Franks et al., 2006). Disordered ovarian follicular development in PCOS can be observed even at the early preantral stages (Webber et al., 2003). The later, gonadotropin-dependent stages of follicle development are characterised by arrest at the small-

medium sized antral follicle stage and failure to progress to ovulation (Hughesdon, 1982, Webber et al., 2003, Maciel et al., 2004) (Figure 1.9). The exact cause of aberrant follicle development is unknown, but follicle arrest is associated with elevated serum concentrations of LH, AMH, insulin and androgens and a relative deficiency of FSH (Franks et al., 2008). Inappropriate (premature) responsiveness of GCs to LH may also play a part in follicle arrest in PCOS. Willis and colleagues examined GCs from human small antral follicles (hSAFs) taken from women with normal ovaries and regular cycles, which, as expected, showed a response to FSH but no response to LH in terms of steroid production (Willis et al., 1998, Mason et al., 1994). GCs from women with anovulatory PCOS, however, showed significantly more variability of responsiveness to LH compared to ovulatory women (either with or without polycystic ovaries), with some follicles behaving as normal ovaries (i.e. no response to LH), but with a significant sub-cohort displaying inappropriate, or premature, responsiveness to LH. In addition to inappropriate responsiveness to LH, cultured GCs from PCOS women also show augmented oestradiol and progesterone production in response to FSH (Willis et al., 1998, Mason et al., 1994).

The aberrant LH responsiveness of GCs in PCOS is likely to be due to early acquisition and/or function of LHR in GCs, which may result in terminal differentiation in the GC layer and subsequent arrest of follicle growth. However, Jeppesen and colleagues (Jeppesen et al., 2012a) detected *LHCGR* gene expression in GCs from follicles as small as 3mm. Jakimiuk and colleagues also studied GCs from women with PCOS and found increased gene expression of *LHCGR* and *CYP11A1* in GCs from hSAFs compared to cells from regularly cycling women (Jakimiuk et al., 2001). However, that study did not include quantitative RT-PCR analysis and has not been replicated, nor has a comprehensive study of expression of gonadotropin, AR and steroidogenic enzymes been carried out.

Another factor that has been implicated in the mechanism of arrested follicle growth in PCOS is AMH. Most of the circulating AMH is produced by GCs of large pre-antral and small antral follicles (Weenen et al., 2004, Andersen et al., 2010). AMH has been shown to inhibit FSH-induced oestradiol production by GCs in vitro (Pellatt et al., 2011) and as AMH production is

higher in women with PCOS (Cook et al., 2002, Seifer and Maclaughlin, 2007), it has been postulated that the higher ovarian levels of AMH impair antral follicle function and contribute to follicle arrest (Dewailly et al., 2016). It has been concluded, on the basis of correlating serum AMH with antral follicle count on ultrasound, that the production of AMH per follicle is increased in PCOS (Bhide et al., 2015, Bhide et al., 2017). This view is supported by a study that found that concentrations of AMH (as measured by ELISA) were much higher in conditioned media of cultured GCs from women with PCOS compared with follicles from control subjects, although concentrations of AMH in follicular fluid between polycystic and normal ovaries was not measured (Pellatt et al., 2007). Although AMH gene expression has been measured in GLCs from gonadotropin-stimulated cycles (and reported to be higher than normal in women with PCOS) (Catteau-Jonard et al., 2008), it has not to my knowledge been measured in GCs from individual, matched, unstimulated follicles in women with and without PCO, the main cells involved in making AMH.

Other members of the TGF- β superfamily are plausible candidates for disrupted follicle function in PCOS. Follistatin, produced predominantly in GCs (Chang et al., 2006, Roberts et al., 1993), inhibits FSH secretion by binding and neutralising activin action (Nakamura et al., 1990, Ueno et al., 1987), and has been shown to be elevated in serum of women with PCOS (Teede et al., 2018). Conversely, inhibins A and B, which also suppress FSH secretion, were found, in one study, to be lower in follicular fluid in large size-matched follicles from women with PCOS (Welt et al., 2005).

Androgens and insulin may also be important in the mechanism of follicle arrest in PCOS, and have been discussed in detail above. Insulin can stimulate GC steroidogenesis and enhance GC responsiveness to LH (Willis et al., 1996) and increased androgen synthesis may augment FSH receptor expression, both of which may increase local production of cyclic AMP and encourage terminal differentiation of GCs (Franks et al., 2008). However it is unclear whether insulin and androgen receptors themselves are differentially expressed in PCOS GCs at the time of follicle arrest.

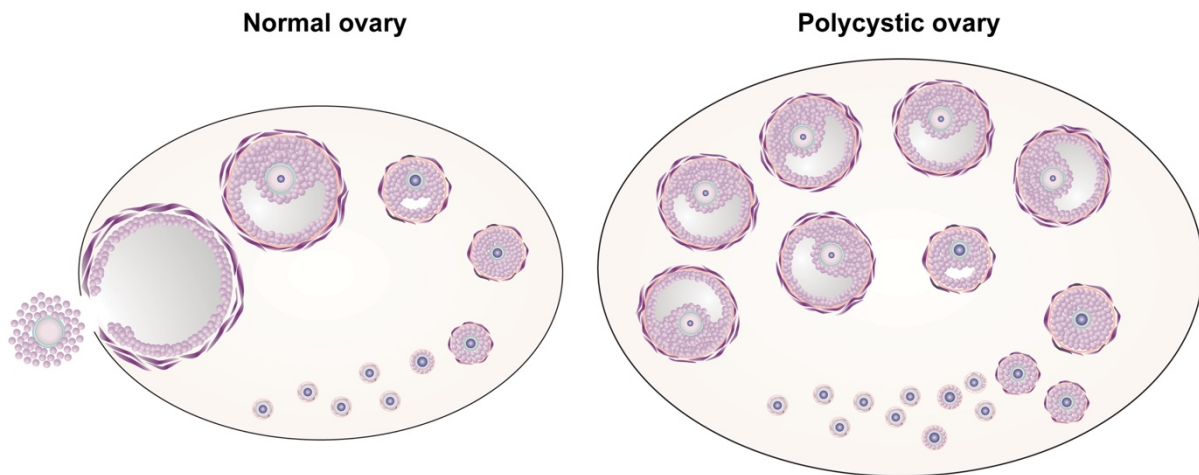


Figure 1.9 Disordered follicle development in the polycystic ovary. Polycystic ovaries display an increase in number of small growing pre antral follicles. Follicles in the polycystic ovary become arrested at the antral stage of development and accumulate under the surface of the ovary. The polycystic ovary is also larger than the normal ovary. (Figure courtesy of Kate hardy).

1.6.7 Pharmacological treatment of PCOS

Treatment of PCOS depends on the predominant symptom/symptoms which are affecting the individual. No individual treatment treats all features of PCOS, which is unsurprising given its heterogenous presentation. Lifestyle changes such as weight loss can improve cycle regularity, mood and hyperandrogenic symptoms (Moran et al., 2003, Clark et al., 1998, Thomson et al., 2010). The combined oral contraceptive pill (cOCP) improves symptoms of hyperandrogenism and irregular bleeding. Metformin does not improve hyperandrogenic symptoms but does increase ovulatory cycles, and modestly reduces weight, cholesterol and LDL in those with BMI > 25kg/m². Metformin is most useful for those at high risk of diabetes, as the Diabetes Prevention Program/Diabetes Prevention Program outcome study (DPP/DPPOS) showed that after an average follow-up of 2.8 years, metformin reduced the incidence of diabetes by 31% compared with placebo, and even after between 10 and 15 years, the risk reduction was 18% (Aroda et al., 2017).

There are few studies addressing anti-androgen use in PCOS, and the majority of these studies have used anti-androgens in combination with cOCP. They can be useful in treating androgenic symptoms and their use is recommended to treat hirsutism and androgen-related alopecia where COCPs are contraindicated or poorly tolerated, in the presence of other effective forms of contraception. The role of anti-obesity medications in improving health in general populations and those at high risk is becoming increasingly recognised. However, in PCOS and young women in general, the role of anti-obesity pharmacological agents remains unclear, due to a lack of evidence.

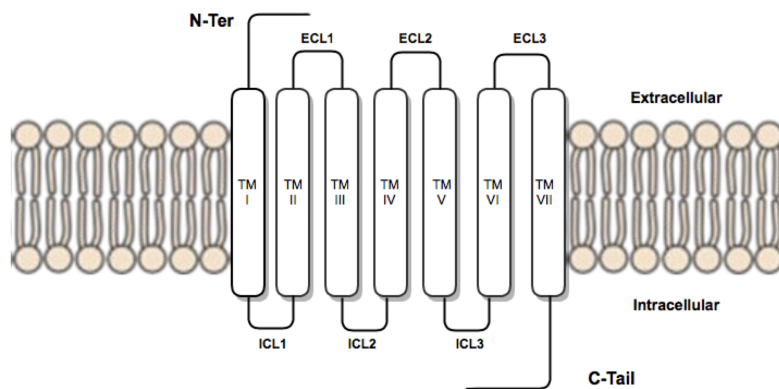
1.7 G-protein coupled receptors (GPCRs)

GPCRs are the largest family of membrane proteins and comprise over 4% of the total protein-coding human genome (Bjarnadottir et al., 2006). There are over 800 GPCR genes (Fredriksson et al., 2003). They mediate a vast array of biological processes, and most of our physiological responses, including to hormones, neurotransmitters, environmental stimuli, photons, nucleotides, ions, and amino acids (Stevens et al., 2013). Their diverse structure and function makes them therapeutic targets, and currently approximately 34% of all prescription medications target GPCRs (Rask-Andersen et al., 2011, Hauser et al., 2017). The classic role of GPCRs is to sense extracellular molecules, couple agonist binding to the activation of heterotrimeric G proteins, which then leads to activation and modulation of downstream effector proteins.

1.7.1 Structure and classification and of GPCRs

GPCRs all share a highly conserved structure composed of seven hydrophobic transmembrane (Christman et al., 1991) spanning alpha helices, linked by intracellular loops, an extracellular amino-terminus and an intracellular carboxyl-terminus. The extracellular domain is responsible for ligand binding and the intracellular domain interacts with cytosolic signalling proteins.

A



B

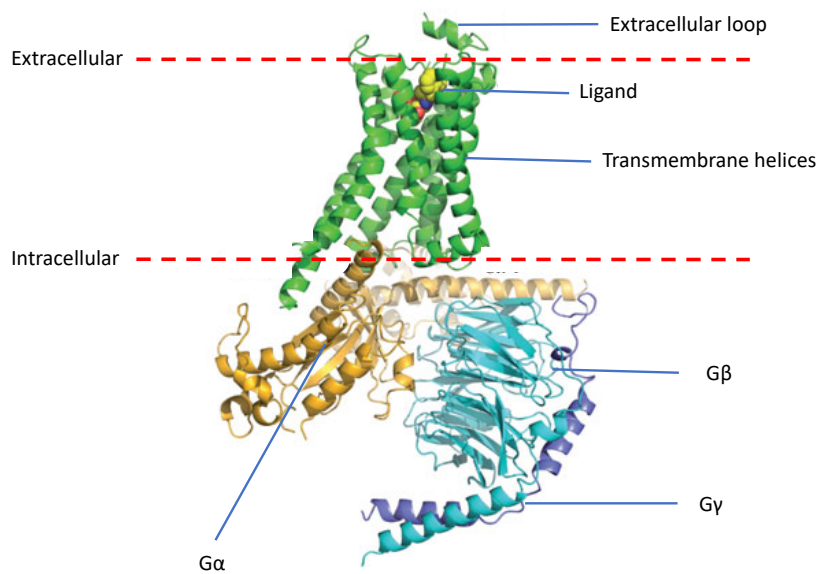


Figure 1.10 Schematic diagram and crystal structure of GPCRs. A GPCR structure, composed of seven transmembrane (Christman et al., 1991) helices, connected by three alternating extracellular (ECL) and three intracellular loops (ICL), with an extracellular amino-terminal domain (N-ter) and an intracellular carboxy-terminal tail (C-tail). **B** Crystal structure of the $\beta 2$

adrenergic receptor (β2AR) with ligand bound in complex with the G subunits. Adapted from (Rasmussen et al., 2011).

GPCRs are classified based on their sequence homology and functional similarity, and are divided into six classes, three large (A-C) and three subclasses (D-F). Class A, also termed the 'rhodopsin-like family' of receptors, account for 85% of all GPCR genes. The receptors in this class encompass a wide range of functions, such as neurotransmitters, olfactory receptors, light and hormone receptors (Attwood and Findlay, 1994). Class A receptors all share seven TM helices and have highly conserved amino acid sequences and a disulphide bridge that connects the first and second extracellular loops. Class B, or the 'secretin receptor family' are regulated by peptide hormones and glucagon (Harmar, 2001). They also have seven TM helices but have their own characteristic large extracellular domain for ligand binding and a network of three disulphide bonds defining a globular domain structure. Class C receptors are characterised by a long N-terminus with a large agonist-binding region and include receptors such as calcium sensing receptors and metabotropic glutamate receptors (Bonsi et al., 2005). Class D, the fungal pheromone mating factor receptors, STE2 and STE3, are structurally similar to Class A and B receptors and are used for chemical communication (Nakayama et al., 1985). Class E, the cAMP receptors, are structurally similar to Class A receptors and are involved in coordinating aggregation of individual cells in slime mould and regulating the expression of development regulated genes (Klein et al., 1988). Class F, 'frizzled/smoothed receptors' are involved for Wnt and hedgehog signalling pathways (Malbon, 2004).

1.7.2 Activation of GPCRs

The development of high-resolution crystal structures of GPCRs has facilitated significant advancement in understanding the processes involved in GPCR activation. The receptors exist in multiple active, inactive and intermediate states, determined by their interactions with ligands/binding proteins (Dror et al., 2011). Ligand binding disrupts the inter-helical stabilising interactions that maintain the receptor in the inactive state, thus the receptor undergoes a conformational change. GPCR ligands are divided into; agonists, which fully activate the receptor; partial agonists, which can activate the receptor to varying degrees but do not fully

activate the receptor even at saturating concentrations; antagonists, which bind to but do not activate the receptor and inhibit the function of agonists, and inverse agonists which do not activate the receptor and inhibit its basal activity (Kobilka, 2007). The efficacy and affinity of these ligands also influences receptor activation.

Despite a diverse array of ligands and ligand binding domains, GPCRs share a common mechanism of activation. GPCR structure is most similar in the regions that interact with the cytoplasmic G proteins (Mirzadegan et al., 2003). Heterotrimeric G proteins are made up of three subunits: $G\alpha$, $G\beta$ and $G\gamma$. In humans there are 21 different $G\alpha$ subunits, 6 $G\beta$ and 12 $G\gamma$. Binding of an agonist, and the subsequent conformational change in the receptor is transmitted to the bound $G\alpha$ subunit. In the resting state, $G\alpha$ is associated with GTP and the $G\beta\gamma$ dimer (Malbon, 2005). Upon activation, the $G\alpha$ subunit exchanges GTP for GDP, induced by GTPase-activating proteins (GAPs), which triggers dissociation of $G\alpha$ from the receptor and from the $G\beta\gamma$ dimer. The dissociated $G\alpha$ and $G\beta\gamma$ interact with other downstream effectors, while the dissociated receptor is free to form a complex with a new G protein complex, ready for signal transduction. $G\alpha$ has weak intrinsic GTPase activity, thus additional molecules called Regulators of G protein signalling (RGS) facilitate this process, triggering G protein complex re-association and the return to the basal state (McCudden et al., 2005, Oldham and Hamm, 2008) (Figure 1.11).

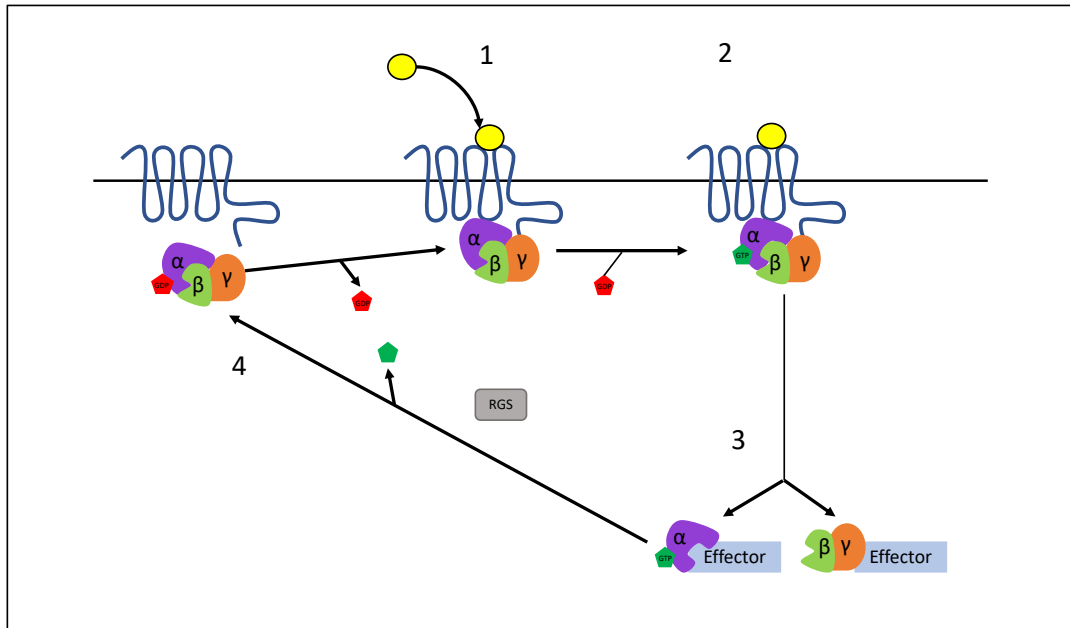


Figure 1.11 Model of GPCR activation of heterotrimeric G proteins. Ligand binds to the receptor (1), the activated receptor undergoes a conformational change and promotes the exchange of GDP for GTP for the $G\alpha$ subunit. Upon GTP binding, the $G\alpha$ subunit dissociates from the $G\beta\gamma$ and from the receptor and both commence signal transduction through effectors (3). Regulators of G protein signalling (RGS) stimulate the GTPase activity of $G\alpha$, thus $G\alpha$ re-associates with β/γ and the return to the basal state (4).

1.7.3 GPCR signalling pathways

$G\alpha$ proteins are divided into four main classes; $G\alpha_s$, $G\alpha_i$, $G\alpha_q/11$ and $G\alpha_{12/13}$ (Neves et al., 2002). $G\alpha_s$ and $G\alpha_i$ couple to membrane-anchoured enzymes called adenylyl cyclases (AC) and stimulate or inhibit these ACs, respectively. AC regulates levels of intracellular production of cyclic 3', 5-adenosine monophosphate (cAMP) by catalysing the conversion of ATP to cAMP. cAMP then activates cAMP-dependent kinases, specifically protein kinase A (PKA). The $G\alpha_q/11$ subunit act on phospholipases, activating phospholipase C (PLC) which hydrolyses phosphatidylinositol 4,5-bisphosphate (PIP₂) to inositol-1,4,5- trisphosphate (IP₃) and

diacylglycerol (DAG). IP₃ stimulates Ca²⁺ release from intracellular stores and DAG activates protein kinase C (PKC) (Strathmann and Simon, 1990) (Figure 1.12). G_{α12/13} activates Rho guanine nucleotide exchange factors (Rho-GEFs), which in turn activate small cytosolic Rho-GTPase, which activates numerous proteins such as cytoskeletal remodelling proteins (Strathmann and Simon, 1991) (Figure 1.12).

The Gβγ dimer acts as a guanine nucleotide dissociation inhibitor for G_α. It also couples to other effectors including PLC, PI3K, ACs and K⁺ and Ca²⁺ channels (Smrcka, 2008) and can activate the mitogen activated protein kinase (MAPK) pathway (Kiyono et al., 1999, Welch et al., 2002) (Figure 1.12). The Gβγ of one receptor can also mediate the signalling of another receptor, thus facilitating crosstalk between two receptors, without their physical interaction (Jean-Alphonse et al., 2017).

All of the G protein subunits contribute to the activation of the MAPK signalling pathway. There are four MAPKs: ERK, JNK (c-June N-terminal kinase), ERK5 and p38 (Li et al., 2016). The ERK pathway can be activated in both G-protein and non G-protein related manners and can involve β-arrestins (Lefkowitz and Shenoy, 2005). The ERK pathway, also known as the Ras-Raf-MEK-ERK cascade involves activation of Ras, recruitment of Raf kinase, which promotes activation of MEK kinase and ERK 1/2 phosphorylation, which regulates the activities of several transcription factors (Orton et al., 2005, Avruch et al., 2001).

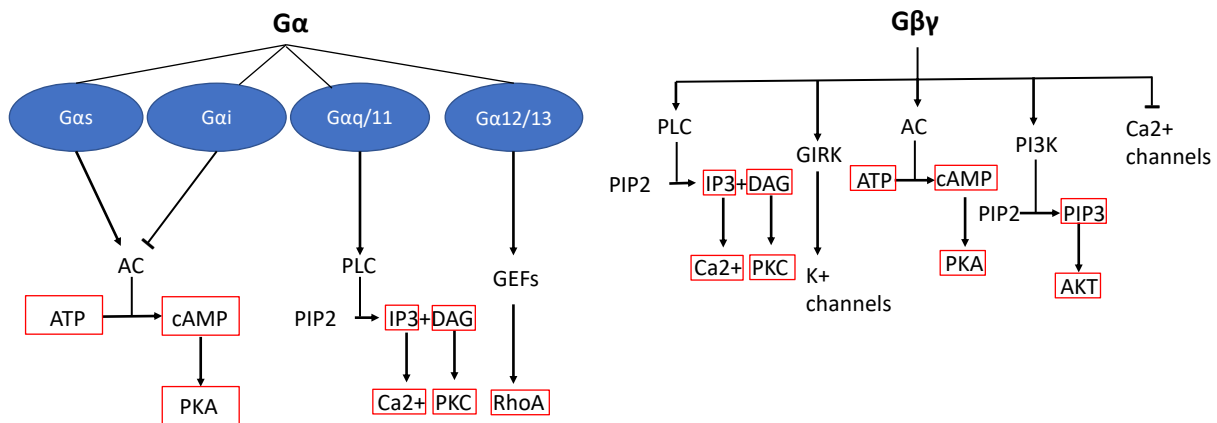


Figure 1.12 Signalling pathways activated by GPCR activation. There are 4 main classes of $G\alpha$; $G\alpha_s$, $G\alpha_i$, $G\alpha_q/11$ and $G\alpha_{12/13}$. $G\alpha_s$ activates AC which converts ATP into cAMP, $G\alpha_i$ inhibits cAMP production, $G\alpha_q/11$ activates PLC- β that converts PIP2 into DAG and IP3 which activate PKC and Ca^{2+} release, $G\alpha_{11/12}$ activates the Rho family of GTPases. Second messengers are in red boxes.

1.7.4 Diversification of GPCR signalling

GPCR signalling can be diversified in various ways and at different levels in the receptor-signalling cascade, and this facilitates receptors to mediate diverse and complex responses in different tissues. One way in which receptors alter their function is through alternate splicing, which facilitates a single gene to generate exponential protein coding capabilities. Alternatively spliced GPCRs can alter tissue distribution patterns, drug binding properties, and receptor signalling characteristics and can affect or cause disease. For example there are 4 known splice variants of the LHR. LHR-A is the full length receptor, LHR-B is lacking of exon 9, LHR-C is lacking part of exon 11 and LHR-D is lacking both exon 9 and part of exon 11 (Minegishi et al., 2007). LHR-B is known to be signalling deficient and reduces expression of both LHR-A and FSHR (Nakamura et al., 2004, Yamashita et al., 2005). LHR-D alters LHR-A signalling activity (Dickinson et al., 2009). Aberrant splice variants, which result in impaired ligand binding, have been

observed in individuals suffering from Leydig cell hypoplasia, hypospadias and micropenis (Han et al., 2012).

GPCRs associate with other GPCRs as dimers, and work in conjunction with each other. Homodimerisation, the association of two of the same GPCRs, amplifies signal generation and allows binding of more than one ligand simultaneously (Jonas et al., 2015, Capra et al., 2017). Heterodimerisation, the association of two different GPCRs, can change the function of both receptors, forming its own functional unit with unique properties. For example FSHR co-expression with LHR in granulosa cells activated $G\alpha_q/11$ and prolonged LH-induced calcium signalling (Jonas et al., 2018).

Membrane trafficking of the receptor can also regulate signal generation (Jean-Alphonse and Hanyaloglu, 2011). After a ligand binds, the receptor is rapidly internalised, which results in rapid signal desensitisation and occurs via clathrin-dependent endocytosis or clathrin-independent endocytosis. Clathrin-dependent endocytosis is generally mediated by β arrestins, driving receptor clustering to the clathrin coated pits (CCPs). Arrestin-bound GPCRs can also lead to activation of non-G-protein mediated signalling. Once receptors are localised to CCP, dynamin mediates endocytosis of CCPs from the plasma membrane and trafficking to endosomes. Clathrin-independent endocytosis uses a mechanism independent of dynamin but is poorly understood.

Once receptors are trafficked to endosomes, these endosomes act as receptor sorting stations, from which they can be targeted back to the plasma membrane for recycling or to lysosomes for degradation (Jean-Alphonse and Hanyaloglu, 2011) (Figure 1.13). Recent studies however, have demonstrated that G-protein signalling can continue or be reactivated following receptor internalisation (Irannejad et al., 2015). This will be discussed in more detail below.

1.7.5 Endosomal trafficking and signalling in GPCRs

Following endocytosis, receptors are trafficked to early endosomes (EE), the sorting compartment for internalised receptor (Goh and Sorkin, 2013, Hanyaloglu and von Zastrow, 2008). Receptors are then sorted from the EE for degradation, where they involute to form multi-vesicular bodies (MVBs), which fuse with lysosomes and degrade. Intracellular signal generation also occurs at the EE. Persistent cAMP signalling after internalisation has been demonstrated for the thyrotropin-stimulating hormone receptor (TSHR) and the parathyroid hormone receptor (PTHr) and this cAMP signalling is augmented by β -arrestin

(Ferrandon et al., 2009, Calebiro et al., 2009). EE signalling has also been demonstrated for other G protein pathways apart from $G_{\alpha s}$. $G_{\beta\gamma}$ coupled to B2AR can prolong the cAMP response of PTHr. $G_{\alpha q/11}$ -coupled receptors, such as kisspeptin receptors, display a sustained calcium response that is independent of receptor internalisation (Min et al., 2014).

Endosomal trafficking is diverse between GPCRs and involves endosomal compartments distinct from the EE. LHR, FSHR and $\beta 1AR$ (but not $\beta 2AR$) have been shown to be trafficked to a distinct compartment for sorting, the very early endosome (VEE) (Jean-Alphonse et al., 2014). The EE and VEE can be distinguished by unique markers, with the EE being marked by EE antigen 1 (EEA1), Rab 5 and phosphatidylinositol-3 phosphate (PI3P) (Jean-Alphonse et al., 2014) and the VEE containing and being distinguished by APPL1 (adaptor protein containing PH domain, PTB domain and leucine zipper motif) (Jean-Alphonse et al., 2014, Broussard et al., 2012). The receptor is sorted to the VEE via interactions with the C-tail of the LHR and the protein GIPC (Gai-interacting protein C terminus) (Jean-Alphonse et al., 2014, Hirakawa et al., 2003). Trafficking to the VEE is critical for recycling of the receptor back to the plasma membrane, as opposed to trafficking to lysosomes for degradation. APPL1 is essential for this recycling (Sposini et al., 2017). The VEE not only promotes recycling of the receptor, it can also mediate intracellular signal generation. This has been demonstrated for LHR, where trafficking to the VEE stimulates sustained MAPK (ERK 1/2) activation (Jean-Alphonse et al., 2014) and cAMP signalling (Sposini et al., 2017). Therefore endosomal trafficking to the VEE adds an extra layer to the compartmental biased signalling already seen between the plasma membrane and the EE (Tsvetanova and von Zastrow, 2014).

While it is clear that endosomal signalling plays a critical role in the function of GPCRs, what is not so clear is the downstream or biological significance of endosomal signalling in different endosomes and compared with classic plasma membrane signalling, and whether they play a role in disease. cAMP produced from endosomes is necessary for rapid dopaminergic signalling through dopamine-D1 receptor (Kotowski et al., 2011) and for insulin secretion by GLP1R (glucagon-like peptide 1 receptor) (Kuna et al., 2013), therefore it is possible that endosomal signalling may contribute to disease pathogenesis and targeting receptor trafficking may be used as novel treatment.

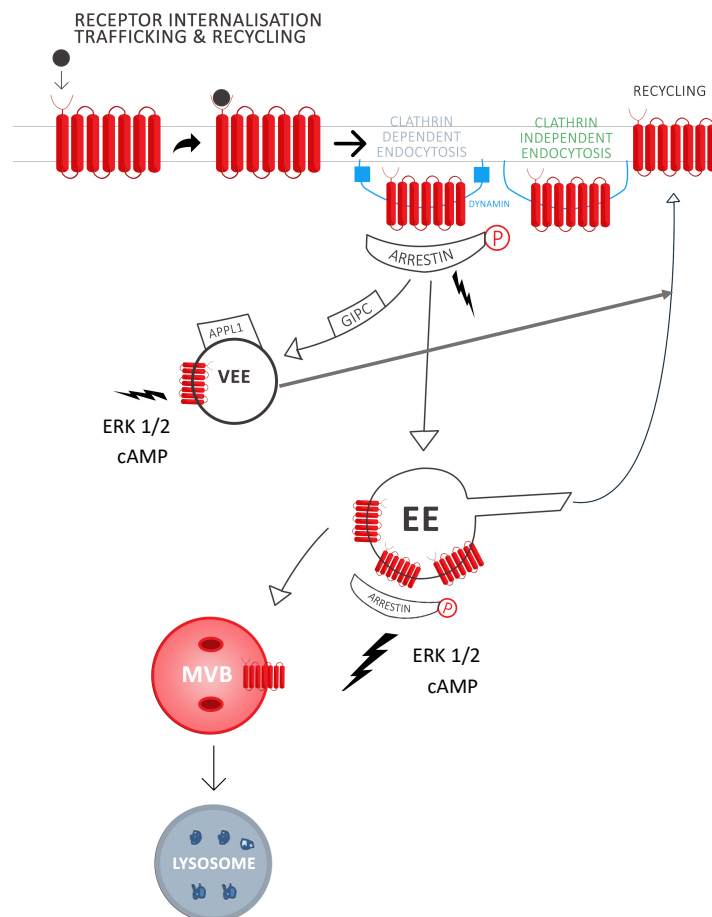


Figure 1.13 GPCR endosomal trafficking. Ligand binding to the receptor leads to activation of the receptor and signal transduction. After the G proteins dissociate, receptors are phosphorylated, which leads to arrestin recruitment. Arrestins then target the receptor-arrestin

complex to clathrin coated pits (CCPs). Dynamin mediates endocytosis or scission of the CCPs from the plasma membrane into vesicles which then fuse to endosomes. Endocytosis can also be independent of clathrin and dynamin. Receptors are targeted to the VEE via GIPC, marked by APPL1, from where signalling occurs and receptors are recycled to the plasma membrane. Receptors can also be trafficked to EE, from where signalling occurs and then they involute to form multi-vesicular bodies (MVBs), which fuse with lysosomes and degrade.

1.8 Gonadotropin receptors

LHR and FSHR are glycoprotein hormone receptors and members of the Class A 'rhodopsin-like' family of GPCRs. LHR or Lh/hCGR binds both LH and human chorionic gonadotropin (hCG) and has a critical role in ovarian steroidogenesis and ovulation. FSHR binds only FSH and regulates ovarian follicle development and oestrogen production. Functional receptors are also expressed by tissues outside of the gonads including bone, adipose tissue and uterus (Sacchi et al., 2018, Liu et al., 2017, Sun et al., 2006). These receptors are characterised by large, identical, horseshoe-shaped extracellular domains for ligand binding. The cognate G protein for glycoprotein receptors are $G\alpha_s$, therefore they activate the cAMP/PKA pathway (Puett et al., 2007). LHR also binds to $G\alpha_q/11$ in presence of high ligand and/or receptor concentrations (Breen et al., 2013), therefore mediating calcium release. $G\alpha_q/11$ signalling has been demonstrated for FSHR in cell lines with overexpressed FSHR, not in cells that endogenously express the receptor (Thomas et al., 2011). LHR couples to $G\alpha_i$ in leydig cells, a process which is also dependent on the receptor concentration (Moraga et al., 1997). Both receptors trigger sustained activation of the MAPK pathway via recruitment of β arrestins (Sposini and Hanyaloglu, 2018).

Ligand binding triggers a conformational change of the transmembrane region of the receptor facilitating activation of G proteins. The receptor is then internalised via clathrin dependent endocytosis, following the recruitment of arrestins, as described in Figure 1.13. Arrestins mediate receptor-G-protein uncoupling, internalisation and signalling (Shukla et al., 2014, Thomsen et al., 2016, Eichel et al., 2018). They can also enhance persistent signalling from endosomal compartments (Wehbi et al., 2013). After internalisation gonadotropin receptors are

trafficked to endosomes, either to the EE for degradation or to the VEE for recycling, as discussed in 1.7.5 above. Intracellular signalling occurs from these endosomal compartments. This trafficking to the VEE is steered by GIPC. This process appears similar for both LHR and FSHR, despite the FSHR C-tail recycling sequence lacking a PDZ binding sequence (Jean-Alphonse et al., 2014). APPL1 is essential for receptor recycling back to the plasma membrane from the VEE in gonadotropin receptors. APPL1 phosphorylation at serine 410 was activated by LHCGR induced G α s activation and this process appears to be necessary for APPL1 dependent receptor recycling (Sposini et al., 2017). Knockdown of APPL1 in HEK293 cells consistently augments cAMP accumulation in response to LH, which is not due to altered surface expression of LHR (Sposini et al., 2017). What is less clear are the downstream effects of gonadotropin receptor endosomal trafficking on ovarian function, and its potential role in disease pathogenesis and treatment. SiRNA knockdown of APPL1 in bovine theca cells was shown to augment LH-mediated androgen synthesis (Comim et al., 2013a), so APPL1 and the endosomal location of LHR may play a role in the regulation of ovarian steroidogenesis.

1.9 Assisted reproductive technology (ART)

ART is the application of technology to gametes and/or embryos for the purposes of reproduction. ART encompasses all medical procedures used primarily to treat infertility, including in vitro fertilisation (IVF), intracytoplasmic sperm injection (ICSI), ovulation induction and gamete cryopreservation. In 2016 in the UK there were 68,000 IVF cycles resulting in 20,028 live births. The success rates were 26% per fresh treatment cycle and 28% per frozen treatment cycle (HFEA, 2018). The most common reasons for IVF treatment cycles being carried out in the UK in 2016 were; male infertility (37%), unexplained (32%), ovulatory disorders (13%), tubal disease (12%) and endometriosis (6%) (HFEA, 2018).

IVF is the process of fertilisation of gametes outside of the body. An IVF cycle involves controlled ovarian hyperstimulation, during which gonadotropins stimulate multi-follicular development, treatment with GnRH agonist or antagonist suppresses the pituitary, preventing premature ovulation and final oocyte maturation is triggered with hCG or GnRH agonist. GnRH antagonists

bind competitively to GnRH receptors, thereby preventing GnRH from stimulating gonadotropin release from the pituitary. GnRH agonists result in prolonged activation of GnRH receptors by GnRH, which leads to desensitisation and suppression of gonadotropin treatment.

36 hours after trigger oocyte maturation transvaginal oocyte retrieval occurs under ultrasound guidance. Mature oocytes are placed in culture media and inseminated with sperm. In an ICSI cycle a single sperm is injected directly into a mature egg. Embryo transfer then occurs 2 to 6 days later.

The two main IVF cycle protocols used are long GnRH agonist (Long Day 21) or GnRH antagonist cycle. In a Long day -21 cycle in the Wolfson Fertility Centre, Hammersmith Hospital, gonadotropin-releasing hormone agonist is used to suppress the pituitary production of gonadotropins for two weeks starting from Day 21 of the menstrual cycle. Ovarian suppression is confirmed with an ultrasound scan and stimulation started using recombinant follicular stimulating hormone. FSH dose varies based on ovarian reserve and can range between 112.5 and 450 units. hCG is administered once at least three follicles reached ≥ 18 mm in diameter; oocyte retrieval follows 36 hours later. For antagonist cycles, ovarian stimulation starts on the second day of the menstrual cycle, a GnRH antagonist is used from Day 5 to prevent a premature LH surge. hCG or a GnRHa is administered when at least three follicles reached ≥ 18 mm in diameter, and transvaginal oocyte retrieval is carried out 36 hours thereafter.

The most dangerous complication of ART is ovarian hyperstimulation syndrome (OHSS), which can cause ovarian enlargement, ascites, hydrothorax, renal failure, acute respiratory distress syndrome, and can be life-threatening (Golan et al., 1989, Whelan and Vlahos, 2000). Severe OHSS occurs in between 2–9% of cycles and moderate OHSS in a further 10–16% (Toftager et al., 2016, HFEA, 2014). Moreover, the risk of severe OHSS is further increased in women with PCO to 12–15% (Lee et al., 2008, Swanton et al., 2010, Jacob et al., 2016) as well as those of young age (Delvigne and Rozenberg, 2002a, Delvigne and Rozenberg, 2002b). Guidelines for preventing OHSS for those at risk recommend a lower starting dose of FSH, using a GnRH antagonist cycle instead long agonist and using a GnRH agonist trigger instead of hCG (Society, 2014). Kisspeptin, discussed in detail in 1.9 below, has been studied as an alternative trigger of oocyte maturation and is associated with a low risk of OHSS.

Several studies have compared alterations in gene expression, gonadotropin levels, vascular endothelial markers and growth factor profiles in serum, follicular fluid and GLCs between hCG and GnRH agonist triggered IVF protocols (Doldi et al., 1997, Wang et al., 2002, Villasante et al., 2008, Cerrillo et al., 2011, Cerrillo et al., 2009, Haas et al., 2014, Miller et al., 2016). Levels of vascular endothelial growth factor (*VEGF*) (Doldi et al., 1997, Wang et al., 2002, Cerrillo et al., 2011, Cerrillo et al., 2009), Cadherin 5 (*CDH5*) (Villasante et al., 2008) and platelet endothelial-derived factor (*PEDF*, also known as *SERPINF1*) (Miller et al., 2016) are putative markers of OHSS and have been shown to be different between hCG and GnRHa triggered cycles (Cerrillo et al., 2009, Cerrillo et al., 2011, Haas et al., 2014). Microarray analysis of cumulus and mural GCs has shown differences in genes involved in lipid and steroid metabolism, and platelet degranulation (Borgbo et al., 2013). In addition, Haas and colleagues studied the differential effects on gene expression in granulosa cells from women who were triggered with hCG or GnRH agonists (Haas et al., 2014). They found that granulosa cells from women who had received GnRHa as a trigger had lower expression of genes related to steroidogenesis and gonadotropin regulation (*CYP19A1*, *CYP11A1*, *3BHSD*, *FSHR*, inhibin B and amphiregulin). These changes may reflect the different hormonal profiles and likelihood to induce OHSS between the two triggers.

Women with anovulatory PCOS often require ART to achieve pregnancy. First line medical treatment (after/with lifestyle change and weight loss) is ovulation induction with clomiphene citrate (cc) or letrozole (Kamath et al., 2010, Palomba et al., 2009). CC is a selective oestrogen receptor modulator that is predominantly anti-oestrogen. It binds to the oestrogen receptor and stimulates GnRH release, and may also have a direct oestrogenic effect on the gonadotrophs, enhancing sensitivity to GnRH. Letrozole is an aromatase inhibitor, which prevents oestrogen production and therefore stimulates gonadotropin release. Metformin can also induce ovulation but is less effective than cc or letrozole (Kjøtrød et al., 2011), but can be used in combination with these agents (Morley et al., 2017). Gonadotropins are used as second line agents in anovulatory women with PCOS who have failed first line oral ovulation induction with cc and letrozole. Gonadotropins are more effective with higher pregnancy rates but are associated with higher costs, increased risk of multiple pregnancy and the need for intensive monitoring (Homburg et al., 2012). IVF is used as third line therapy or where there is another indication for it, e.g. concomitant tubal issues or male factor infertility.

Women with PCOS are at high risk for developing OHSS, therefore the methods discussed above are often utilised to reduce the risk of OHSS. The differences in their ovarian stimulation protocols could affect the function of GLCs, especially gonadotropin receptor function, and the ability to study this accurately. hCG suppresses gonadotropin receptor function and has an elimination half-life of 30 hours. Serum LH levels only return close to baseline by day 6 after administration of hCG (embryo transfer day) and are completely back to normal if measured a week later (Fauser et al., 2002). GnRHa are used as maturation triggers in those at high risk of OHSS and have a short elimination half-life of approximately 80 minutes (Kuhl et al., 1984). Serum gonadotropins return to normal within 24 hours of administering the GnRHa buserelin (Fauser et al., 2002), therefore while it may have some impact on gonadotropin receptor function, it is likely to be relatively less than hCG.

Recombinant FSH has a relatively short half-life (elimination half-life is approximately 1 day (le Contonnec et al., 1994) and give only until the oocyte maturation trigger (36 hours before oocyte retrieval), therefore while FSH may have some impact on gonadotropin receptor function, it is likely to have less of an effect than other factors. All of these factors will be addressed and discussed in the GLC optimisation chapter (Chapter 3).

1.10 Female fertility cryopreservation

Women can cryopreserve their gametes either through oocyte vitrification (egg freezing) or ovarian cortex cryopreservation. These techniques are used for example by women with cancer commencing gonadotoxic medications, or by those who wish to postpone childbearing. In 2016 in the UK there were 1,173 egg freezing cycles, 519 thaw treatment cycles, with a birth rate of 19% per cycle (HFEA, 2018). The process of oocyte vitrification is similar to that of IVF, with controlled ovarian hyperstimulation followed by ultrasound guided retrieval.

Ovarian cortex cryopreservation is used primarily for young women with cancer. In 2004 the first live birth after ovarian tissue cryopreservation with auto-transplantation was described (Donnez et al., 2004). Since then transplantation of ovarian tissue has restored ovarian function in 95% of

women and resulted in the birth of over 130 children worldwide (Donnez and Dolmans, 2017, Gellert et al., 2018), half of whom have been born by natural conception.

Ovarian cortex cryopreservation also has significant research potential, and provides the opportunity to study oocytes, GCs, theca cells and follicular fluid from normal growing follicles from women who chose to donate excess or unneeded tissue. Studies have already begun giving an insight into the mechanisms underlying normal follicle growth and maturation (Kristensen et al., 2015, Kristensen et al., 2014, Jeppesen et al., 2012a). It also has the potential to study molecular mechanisms underlying polycystic ovaries, providing tissue that was previously very difficult to obtain. The Department of Fertility at Rigshospitalet, Copenhagen University Hospital, with whom we collaborate, have the most active ovarian cryopreservation programme worldwide.

1.11 Kisspeptin

Kisspeptins are a group of peptides encoded by the KISS1 gene. They act as ligands for a G-protein-coupled receptor, kisspeptin receptor (KISS1R), which was initially known as G-protein-coupled receptor 54 (GPR54). The KISS1 gene was first discovered in 1996 (Lee et al., 1996) and was initially called metastatin, as it was discovered in melanoma cell lines and thought to be relevant for recognising metastatic disease. The researchers however found diffuse distribution in hypothalamus and gonads and speculated that it may in fact play a role in reproduction (Lee et al., 1996, Ohtaki et al., 2001).

KISS1R and KISS1 are expressed by tissues throughout the body, including brain, gonads, placenta, and gastrointestinal tract (Lee et al., 1996, Ohtaki et al., 2001). Kisspeptin neurons are expressed in the hypothalamic infundibular and arcuate nuclei (Oakley et al., 2009, Rometo et al., 2007). The importance of the kisspeptin system in reproduction was recognised in 2003 when two groups discovered that mutations in KISS1R were associated with the idiopathic hypothalamic hypogonadism and impaired pubertal maturation (de Roux et al., 2003, Seminara

et al., 2003). Studies in mice with targeted deletions of Kiss1r also resulted in similar reproductive dysfunction (Seminara et al., 2003, Funes et al., 2003).

Kisspeptin-54 (Kp-54) is cleaved from the 145-amino acid protein encoded by KISS1 and is further cleaved to 14, 13, and 10 amino acid (kisspeptin-10/kp-10) sequences, all sharing the same C-terminal tail sequence which activates the receptor (Kotani et al., 2001, Clements et al., 2001) (Figure 1.14). All of the kisspeptin ligands bind to KISS1R with similar affinity in human placenta (Kotani et al., 2001), however this has not been tested in different tissues and it is known that different ligands can exert different actions in the same receptor in different tissues. *In vivo* Kp-54 is more potent than Kp-10, stimulating more gonadotropin release in men and rats (Jayasena et al., 2015). However all of the pharmacodynamics studies have involved *in vivo* or central administration of kisspeptins and have not involved *in vitro* studies of peripheral tissues or cells expressing the receptor.

Kisspeptins stimulate the hypothalamic-pituitary axis and stimulate gonadotropin release in both humans and animals as mentioned in 1.3 (Gottsch et al., 2004, Dhillo et al., 2005, Messenger et al., 2005). They predominantly stimulate LH secretion more than FSH secretion (Skorupskaite et al., 2014, Dhillo et al., 2005). Administration of a kisspeptin antagonist prevents GnRH secretion (Irwig et al., 2004, Roseweir et al., 2009). Kisspeptin neurons co-localise with dynorphin and neurokinin B (Goodman et al., 2007, Skorupskaite et al., 2014), forming the so called KNDy neurons, both of which influence LH secretion (Navarro et al., 2009). Inactivating mutations of the neurokinin 3 receptors have been found in people with hypogonadotropic hypogonadism (Guran et al., 2009, Topaloglu et al., 2009).

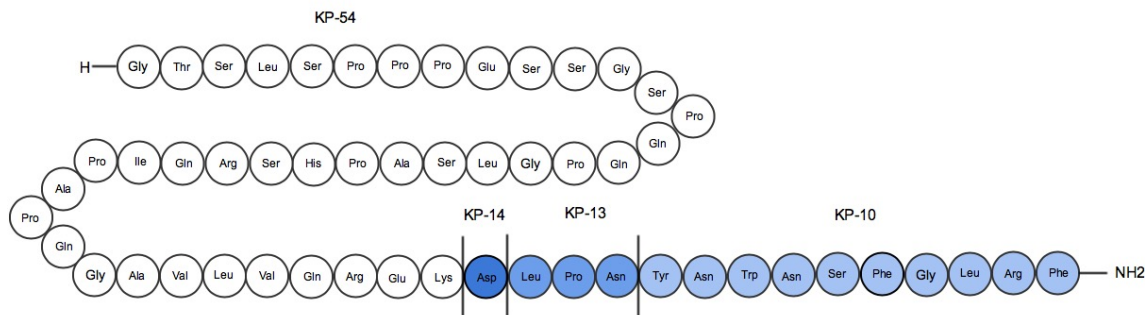


Figure 1.14 Kisspeptin amino acid sequences. All kisspeptin ligands share the same 10 same C-terminal decapeptide sequence tail (light blue). *KISS1* encodes a protein that is cleaved into a 54 amino acid sequence, and this can be further cleaved into *kp-10* (light blue), *kp13* (darker blue) or *kp14* (dark blue).

1.12 Kisspeptin receptor signalling

Kisspeptin signals via the kisspeptin receptor (KISS1R), which is a $G\alpha_q/11$ -coupled 7-transmembrane-spanning GPCR. Ligand binding to KISS1R activates multiple second messengers, some of which are tissue-specific, and some of which have not been entirely elucidated.

When kisspeptin binds to KISS1R, the phosphorylated $G\alpha_q/11$ protein activates phospholipase C (PLC)- β . This leads to activation of phosphatidylinositol 4,5-bisphosphate (PIP₂), accumulation of inositol-(1,4,5)-triphosphate (IP₃), protein kinase C (PKC) activation, and intracellular Ca^{2+} release (Stafford et al., 2002, Jiang et al., 2005, Kotani et al., 2001). PKC activation also activates the MEK-ERK (MAPK) pathway, which appears to be necessary for LH-induced progesterone synthesis (Woods and Johnson, 2007, Jamaluddin et al., 1994, Jiang et al., 2005, Yuan and Connor, 1997). Therefore, the PLC/PKC/ERK pathway mediates progesterone synthesis in granulosa-luteal cells. Reduced MAPK signalling is associated with granulosa cell survival, apoptosis and follicle atresia (Yang et al., 2017, Shiota et al., 2003). In addition kisspeptin or kisspeptin receptor mutant mice displayed increased follicular atresia (Colledge, 2009, Gaytan et al., 2014), suggesting the kisspeptin system may be involved in preventing cellular apoptosis.

Studies in Chinese hamster ovary (CHO) KISS1R cell lines have shown that kisspeptin stimulation results in a biphasic increase in intracellular Ca^{2+} , with a rapid acute increase followed by a more sustained second phase (Kuohung et al., 2010, Min et al., 2014). Activation of KISS1R by Kp-10 initiated calcium oscillations in GnRH neurons in the mouse nasal explants (Constantin et al., 2009). Calcium signalling in response to activation of KISS1R has not however been demonstrated in human ovarian tissue.

Activated KISS1R also recruits β -arrestin 1 & 2 to the cell membrane, which also modulate phosphorylated ERK 1/2 (Pampillo et al., 2009, Szereszewski et al., 2010, Goertzen et al., 2016). ERKs facilitate LH-induced secretion of progesterone. This arrestin signalling has been demonstrated in mouse models where ablation of $\text{G}\alpha_{q/11}$ in GnRH neurons only partially inactivates gonadotropin secretion (Babwah et al., 2015). In addition kisspeptin dependent LH secretion is diminished in mice lacking β -arrestin-1 or β -arrestin-2 (Ahow et al., 2014). This may explain why patients bearing some types of $\text{G}\alpha_{q/11}$ -uncoupled KISS1R mutants display only partial gonadotropic deficiency (Tenenbaum-Rakover et al., 2007, de Roux et al., 2003).

Kisspeptin has been shown to stimulate phosphorylation of PI3K/AKT in a tissue-specific manner. This signalling pathway plays an essential part in granulosa cell apoptosis as well as primordial follicular survival, growth and transition to primary follicles (Zheng et al., 2012, Cecconi et al., 2012). Kisspeptin administration has been shown to activate PI3K/AKT in pre-optic neurons and thyroid cancer cell lines stably overexpressing KISS1R (Hanchate et al., 2012, Stathatos et al., 2005), but not in rat luteal cells or papillary thyroid cancer cells (Peng et al., 2013, Ringel et al., 2002). In fact in colorectal cancer cell lines stably over-expressing KISS1R pAKT was inhibited by kp-10 administration (Chen et al., 2016). It is unclear however if intra-ovarian kisspeptin can modulate the AKT pathway in human cells.

1.13 Kisspeptin in the ovary

Kisspeptin and its receptor are expressed in the human and animal ovary (Cejudo Roman et al., 2012, Gaytan et al., 2009). Several studies, discussed in detail below, suggest that kisspeptin

plays a direct role in the control of ovarian function; including steroidogenesis, follicle development, oocyte maturation and ovulation. However this has not been clearly elucidated in humans.

Kisspeptin may have a very important role in ovarian progesterone synthesis. Firstly it is highly expressed in rat luteal cells (Gaytan et al., 2009, Peng et al., 2013). In addition two studies in chicken granulosa cells and rat luteal cells have shown a direct stimulatory effect of kp-10 on steroid enzymes and progesterone production (Peng et al., 2013, Xiao et al., 2011). In chicken GCs the gene expression of CYP11A1, 3 β -HSD and STAR were increased after *in vitro* treatment with kp-10. In rat luteal cells, treatment with kp-10 alone increased expression levels of StAR and CYP11A, and these effects were enhanced when cotreated with hCG and kp-10. Treatment with both kp-10 and hCG also significantly increased expression of 3 β -HSD. Also hCG-induced increase in progesterone production was suppressed by a kisspeptin antagonist P234. Another study (Laoharatchathanin et al., 2015) showed whole ovary expression of kisspeptin was increased after stimulation with hCG. This provides strong evidence for the direct role of kisspeptin in steroidogenesis, however this has not been well studied in humans.

1.14 Kisspeptin as an IVF maturation trigger

The most dangerous complication of ART is ovarian hyperstimulation syndrome (OHSS), which can be life-threatening (Golan et al., 1989, Whelan and Vlahos, 2000) and can occur in up to 25% of IVF cycles. (HFEA, 2014, Toftager et al., 2016). hCG has traditionally been used to trigger oocyte maturation but its use is complicated by OHSS (Delvigne and Rozenberg, 2002a, Delvigne and Rozenberg, 2002b, Elchalal and Schenker, 1997). GnRHa have a shorter duration of action than hCG and a lower risk of inducing OHSS, as discussed above in 1.9 (Babayof et al., 2006, Corbett et al., 2014, Melo et al., 2009). However, GnRHa are often used as second line agents due to concerns regarding implantation rates (Griesinger et al., 2006, Youssef et al., 2014). Kp-54 has been shown to trigger oocyte maturation with no clinically significant OHSS (Abbara et al., 2015).

Several studies have compared alterations in gene expression, gonadotropin levels, vascular endothelial markers and growth factor profiles in serum, follicular fluid and GLCs between hCG and GnRHa triggered IVF protocols as discussed above, however these have not been examined with kisspeptin use.

1.15 Kisspeptin in PCOS

Given that we know that kisspeptin predominantly stimulates LH release more than FSH, and that LH hypersecretion with a relative deficiency of FSH is a feature of PCOS, it has been postulated that kisspeptin may underly the neuroendocrine dysregulation seen in PCOS. *KISS1* expression in the hypothalamus is reduced in animal models of PCOS (Cernea et al., 2015). One study assessed pulsatile secretion of LH and kisspeptin in women with PCOS and found that those oligomenorrhoeic PCOS displayed elevated kisspeptin pulse frequency and loss of temporal coupling between LH and kisspeptin pulses (Katulski et al., 2018).

In terms of ovarian kisspeptin action in PCOS, *KISS1*, neurokinin B and its receptor have also been shown to be downregulated in GLC from women with PCOS (Blasco et al., 2019), however another study showed *KISS1* and *KISS1R* were overexpressed in PCOS GLC (Hu et al, 2019). The actions of kisspeptin and its receptor in the human ovary however remain unknown.

1.16 Summary & hypothesis

PCOS is a complex condition whose aetiology is incompletely understood. GWAS studies have implicated gonadotropins and gonadotropin receptor action in the pathogenesis of the condition. The primary site of gonadotropin and gonadotropin receptor activity are GCs and theca cells. Gonadotropin receptors are predominantly $G_{\alpha s}$ coupled GPCRs. Their signalling and trafficking have been studied mainly in cell lines and have not been elucidated in human GLCs.

Ovaries of women with PCOS display follicles 'arrested' at the small antral stage and do not progress to become ovulatory follicles. The actions of gonadotropins and their receptors, steroid enzymes, insulin, androgens and growth factors have been implicated in the aetiology of follicles arrest, however the exact molecular mechanisms underlying antral follicle arrest in PCOS are unknown.

Androgens have a critical role in follicle development and regulation, but their role around the time of ovulation is unclear. Androgens play a critical role in the pathogenesis of PCOS.

Kisspeptin and kisspeptin receptor are expressed by the human ovary, but their direct role there, if any, is unknown. Kp-54 is effective as an oocyte maturation trigger for IVF and is not associated with significant ovarian hyperstimulation. The indirect effects of kp-54 on GLC function however are unknown.

The overall goal of this thesis is to study gonadotropin receptor expression, signalling, trafficking and functioning in GLCs from women with and without PCOS. My main hypothesis, based on data from GWAS studies and in vitro data, is that LHR and FSHR function are altered in PCOS and this contributes to the aetiology of the condition.

My secondary hypotheses are;

- i. Follicle arrest in PCOS is characterised by a distinct profile of dysregulation of key genes in GCs including gonadotropin receptors, steroid enzymes, inhibins and growth factors. This is based on data that shows that LHR action occurs prematurely and FSHR action is augmented in GCs from small antral follicles in PCOS,
- ii. Androgens contribute to gonadotropin receptor signalling and function in GLCs. This is based on data from animal studies.
- iii. Kisspeptin receptor is functioning in human ovaries and may also contribute to gonadotropin receptor action in GLCs. This is based on data from animal studies.

1.17 Aims

- i. Establish normal gonadotropin receptor signalling pathways and receptor trafficking in GLCs.
- ii. Examine LHR and FSHR signalling, trafficking and function in GLCs from women with and without PCOS.
- iii. Identify mechanisms underlying follicle arrest in PCOS by studying gonadotropin dependent gene expression in GCs from unstimulated small antral follicles from women with and without PCOS.
- iv. Examine the effect of androgen treatment on GLC function and gonadotropin signalling.
- v. Establish the direct and indirect gonadotropic effects of kisspeptin on GLC function.

Chapter 2

Materials and methods

2.1 Patient recruitment

Granulosa lutein cells (GLCs) were obtained with informed written consent from women undergoing oocyte retrieval for IVF/ICSI/oocyte cryopreservation in the Hammersmith Hospital Wolfson IVF unit. Women were provided with information about the project at their IVF coordination meeting. They were then asked if they wished to participate on the morning of the procedure. PCOS was defined based on the Rotterdam criteria (see 1.6.1).

2.2 Ethical approval

2.2.1 Granulosa lutein cell collection

Ethical approval was obtained from the Hammersmith Research Ethics Committee (Feb 2009-08/H0707/152).

2.2.2 Kisspeptin-54 as an oocyte maturation trigger

The Hammersmith and Queen Charlotte's Research Ethics Committee approved this study (Reference: 10/H0707/2). The study was carried under a license from the United Kingdom Human Fertilization and Embryology Authority. Written informed consent was obtained from all subjects. Approval of this study as a Clinical Trial of an Investigative Medicinal Product was granted by the Medicines and Healthcare Products Regulatory Agency, UK. The study was registered on the National Institutes of Health Clinical Trials database ([ClinicalTrials.gov](https://clinicaltrials.gov) NCT01667406). The study was performed in accordance with the Declaration of Helsinki.

2.2.3 Unstimulated granulosa cell collection

This study was carried out in accordance with the recommendations of the ethical committee of the municipalities of Copenhagen and Frederiksberg with written informed consent from all subjects. All subjects gave written informed consent in accordance with the Declaration of

Helsinki. The protocol was approved by the ethical committee of the municipalities of Copenhagen and Frederiksberg (journal number; H-2-2011-044).

2.3 Patient clinical data recording and storage

Clinical information regarding the women was collected and stored in an anonymised, password protected database. The clinical parameters in Table 2.1 were recorded.

Table 2.1 Clinical parameters recorded in database

Clinical parameters
Age
Height (cm)
Weight (Kg)
Body mass index
Previous IVF cycles
PCOS diagnosis (Y/N)
Ovarian Morphology (Normal/PCO)
Antral Follicle Count
Baseline FSH
Baseline LH
AMH
Menstrual cycle regularity
IVF Protocol
Days of stimulation
Medication used for stimulation
Cumulative dose of FSH (iu)
Maturation trigger

Number of follicles >14mm
Number of eggs collected
Indication for IVF/ICSI/Oocyte cryopreservation

2.4 Granulosa cells from small antral follicles

GCs were collected from hSAFs collected at the time of cryopreservation of ovarian tissue for fertility preservation. This cryopreservation usually involved surgical removal of an entire ovary prior to cancer treatment which is potentially gonadotoxic. The procedure was done at random times during the menstrual cycle. Follicular fluid was aspirated from small antral follicles not required for cryopreservation. GCs were then extracted from follicular fluid using centrifugation. This procedure was carried out by the team of Claus Andersen, and the frozen GCs were sent to us on dry ice.

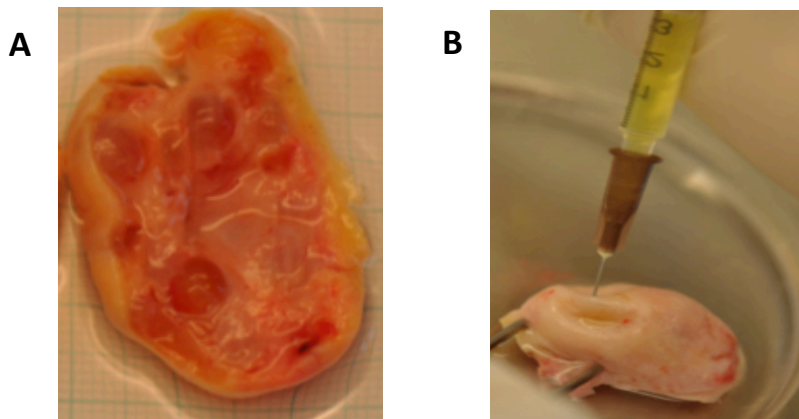


Figure 2.1 Images from an ovary being prepared for cryopreservation. A An ovary cut in half. **B** Follicular fluid being aspirated using a 23G needle from the outside of the ovary or within the medulla.

2.5 Follicular fluid collection and granulosa lutein cell isolation

Transvaginal ultrasound guided oocyte retrieval was carried out for IVF/ICSI/oocyte cryopreservation at the Hammersmith Hospital Wolfson IVF unit. Follicular fluid aspirates from both ovaries were pooled and collected in sterile 50ml conical centrifuge tubes. All samples were processed under sterile conditions in a fume hood using aseptic cell culture technique.

Samples were centrifuged (Thermo Fisher Scientific Megafuge 16) at 1000rpm for 5 mins to separate cells from follicular fluid. The follicular fluid supernatant was discarded and 8ml pre-warmed Dulbecco's phosphate buffered saline (DPBS) was added to the cell pellet. A percoll density gradient was then used to separate red blood cells from GLCs. A 45% Percoll solution was pre-prepared containing 8.9ml Percoll, 1ml 10xM199, 10ml 1xM199 and 100ul sodium bicarbonate. The cells were gently layered onto 4ml pre-shaken percoll mixture in a 15ml centrifuge tube using a 10ml stripette and then centrifuged at 1600rpm for 30 mins (Thermo Fisher ALC PK110). Red blood cells separate and sink. The GLCs were then collected from the PBS/Percoll interface using a 10ml stripette and washed twice in DPBS. Further processing of the cells then depended on the planned experiment.

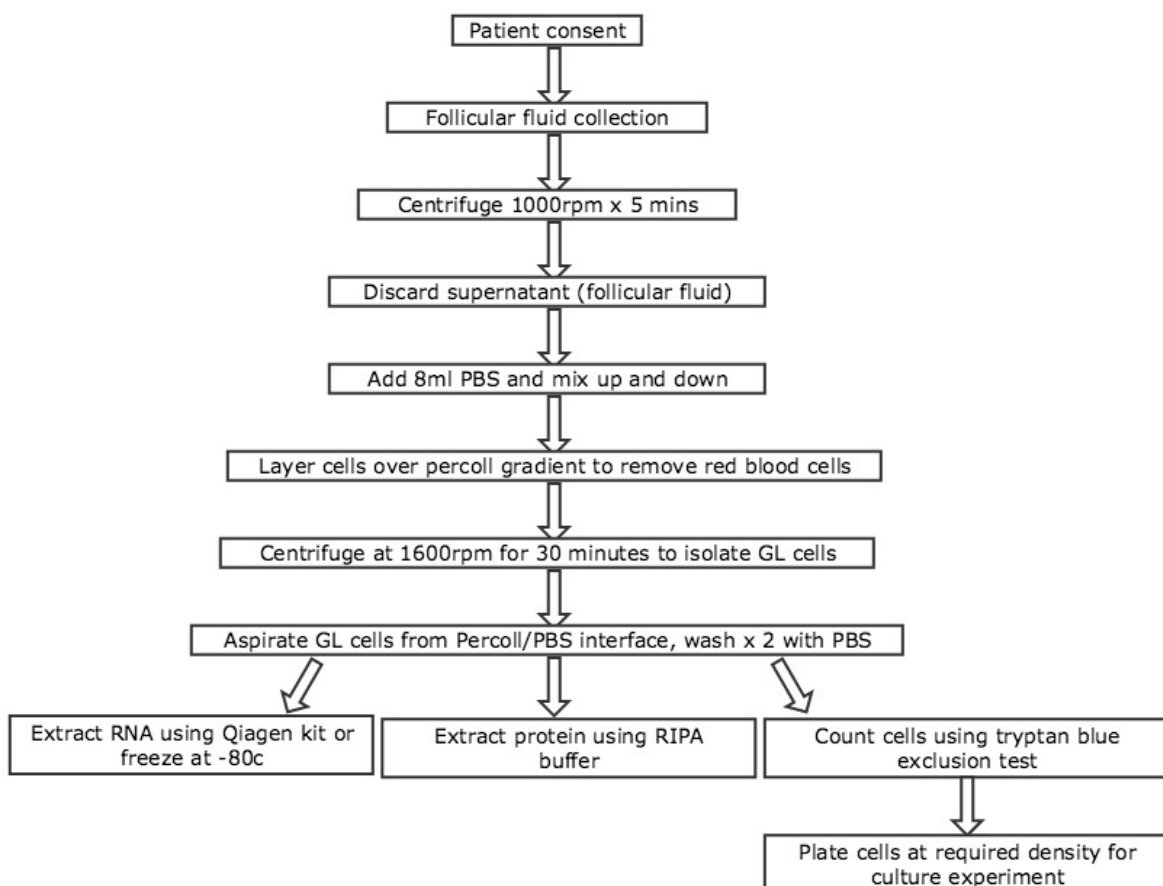


Figure 2.2 Flow diagram of protocol for isolation of human granulosa lutein cells and preparation for experimentation.

2.6 GLC culture

After GLC isolation as described above, cell number and viability were determined using a Trypan Blue exclusion kit. Cells were then plated at a density of 2×10^5 per ml of medium (see individual experimental protocol for well size, volume of culture media and duration of culture). Culture medium reagents are listed in Table 2.10. Cells incubation was at 37 °c in 5% CO₂. Medium was refreshed every 48 hours. Serum free medium was used overnight prior to experiments.

2.7 Transfection of plasmid DNA (APPL1-GFP)

GLCs plated into 6 well plates aiming for 80% confluence were transfected 24 hours after plating in media supplemented with 10% (v/v) foetal bovine serum (FBS). APPL1-GFP was used for this as this plasmid has been transfected into HEK293 cells successfully by previous members of the lab group. For each well of transfection, plasmid DNA (0.5 – 3 µg) was combined with 250 µL of Opti-MEM and 5 µL of Lipofectamine 2000 was combined with 250 µL of Opti-MEM in separate tubes and incubated for 5 mins at room temperature. These were then mixed and incubated for a further 20 mins at room temperature. The mix was then added in drop-wise manner to the cells and incubated at 37°C with 5% CO₂.

Both Viomer yellow and red transfection protocols were also attempted. GLCs were plated in 24 well plates aiming for approximately 80% confluence. Plasmid DNA (0.5 – 3 µg) was added to Buffer Yellow or Red. Buffer Red or Yellow was added to Viomer Red or Yellow and vortexed for 3-5 seconds. The plasmid/buffer mix was added to the Viomer solution and incubated for 15 mins. The transfection complexes were then added to the cells. A low, standard and high transfection scale was attempted.

After approximately 16 hours, the medium was changed back to general culture medium and GLCs were imaged at confocal microscopy. GFP was visualised using 488nm laser light source.

2.8 Protein extraction

GLCs either in plates after culture or in pellets, were harvested on ice in radio-immunoprecipitation assay buffer (RIPA) containing protease and phosphatase inhibitor cocktail. They were then centrifuged at 4 °C for 15 mins at 13,000rpm. Cell lysate was collected and debris discarded.

2.9 Protein quantification

Protein was quantified using the Pierce BCA (bicinchoninic acid) Protein Assay Kit. This combines the reduction of Cu^{2+} to Cu^{1+} by protein in an alkaline medium with colorimetric detection of Cu^{1+} by bicinchoninic acid, which is then read at 562 nm wavelength. Protein samples prepared for cAMP assay were assayed using the Bradford assay kit which contains Coomassie Blue dye. When protein is added, the reagent binds to the arginine and hydrophobic amino acid residues of the protein, causing a colour change which is then read using a spectrophotometer at 595 nm wavelength. Both assays measure total protein concentration compared to a bovine serum albumin (BSA) protein standard provided with the kit and assays were completed as per manufacturer's instructions.

2.10 Western immunoblotting

2.10.1 SDS polyacrylamide gel electrophoresis (SDS-PAGE)

Samples were prepared by combining with 4x Bolt LDS sample buffer and 10x Bolt reducing agent and then heated to 70°C for 10 mins to denature proteins. Reduced samples were loaded into individual wells in precast Bolt 4 - 12% Bis-Tris (10 or 15 well) gels and subjected to sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE) at 200 V with a starting current 160mA, ending current 70mA for 45 mins in 1x Bolt MES SDS running buffer in the Mini Gel Tank. Seeblue plus 2 pre-stained ladder was used as a molecular weight marker.

2.10.2 Protein transfer to nitrocellulose membranes

Proteins were then transferred onto a nitrocellulose blotting membrane using the sub blot mini wet transfer system in transfer buffer at 100 V, 450 mA at 4°C for 1 hour. The Ponceau stain was used to assess membrane transfer.

2.10.3 Antibody application/detection/quantification

The membrane was then blocked for 1 hour at room temperature using 5% BSA or skim milk powder in Tris- buffered saline (TBS) with 1% Tween-20 (v/v) (TBST) before incubation with primary antibody overnight at 4°C in 1% BSA/skim milk. After 3 × 15 minute washes in TBST the membrane was incubated in the appropriate horseradish peroxidase (HRP)- conjugated secondary antibody for 1 hour. See tables 2.3 and 2.4 for antibodies used. Following 3 × 15-minute washes in TBST, protein was visualised by chemiluminescent detection. The membrane was incubated with chemiluminescent detection reagent and the light signal detected using x-ray film or digital chemi-imaging. GAPDH, total ERK and total AKT were used as loading controls.

The bands were quantified using Image Studio Lite software (LI-CORE Biosciences, version 5.2.5). The relative adjusted signal was calculated by normalising to the loading control and then compared between groups (e.g. Control vs PCOS). This relative adjusted signal was used for statistical analysis. Results are plotted as fold change compared to the control or untreated samples, depending on the experiment.

2.10.4 Membrane stripping

When two proteins of the same or similar molecular weights needed to be visualised on the same membrane, primary and secondary antibodies were stripped off the membrane using Western Stripping buffer (see Materials section). The buffer was first heated to 60°C, fresh β -mercaptoethanol was added and membranes were incubated for 30 mins at room temperature on a roller. The membrane was washed with TBS-T (3 short washes then 4 washes of 15 min

each) to remove any remaining stripping buffer and was re-blocked with the appropriate blocking buffer, primary antibody was then added as above.

2.11 Cell fixing/staining/Immunohistochemistry

Sterile coverslips were added to 12 well culture plates prior to seeding with cells. Medium was removed and cells were washed with PBS. Cells were fixed by applying 4% PFA for 20 mins at room temperature. Cells were blocked for 1 hour in PBS with 2% FBS, then permeabilised by adding PBS with 2% FBS 0.02% triton X, then washed 4 times with PBS 2% FBS. They were then incubated in primary antibody overnight at 4 degrees. The next day cells were washed 3 times with PBS + 2% FBS, then incubated with secondary antibody 1:1000 in PBS 2% FBS for 1 hour at room temperature. Cells were then washed with PBS and mounted on glass slides. ProLong gold antifade mountant with DAPI was used to stain nuclei and mount samples onto slides. The slides were stored in the dark at 4 degrees until imaged. They were imaged using a TCS-SP5 confocal microscope (Leica) with a 63× objective and 1.4 numerical aperture. Leica LAS AF image acquisition software was used. Image files were analysed using ImageJ or LAS AF Lite (Leica). Primary and secondary antibodies are listed in Table 2.3 and 2.5.

2.12 Measuring co-localisation

In order to measure the percentage of co-localisation of EEA1 or APPL1 antibodies with LH488 within endosomes, cells were stained as above and imaged using confocal microscopy. The number of endosomes (stained red with EEA1 or APPL1) were counted per cell. The number staining yellow were counted to represent co-localisation, i.e. a combination of red and green. This was then expressed as a percentage of the total number of endosomes. They were then averaged out for the total number of cells counted. Image files were analysed using ImageJ or LAS AF Lite (Leica).

2.13 Gene expression

2.13.1 RNA extraction

Total RNA was extracted from GLCs using the Qiagen RNeasy micro or mini kit, depending on the cell count, and according to the manufacturer's instructions. Fresh samples or samples that had been snap frozen were disrupted using RLT buffer + Beta mercaptoethanol (10 µl β-mercaptoethanol per 1 ml of RLT buffer). The lysate was homogenised using QIA shredder column and centrifuged for 2 mins at maximum speed. One volume of 70% ethanol was added and samples were transferred to RNeasy spin columns and centrifuged at 10,000 rpm for 15 seconds. The RNA was then purified. First 350µl of RW1 was added to the column and samples centrifuged at 10000 rpm for 15 seconds. DNase digestion was then carried out by incubating each sample with a mixture of 10µl DNase I and 70µl of RDD buffer for 15 mins at room temperature. Columns were then washed with 350µl RW1 and 500µl RPE twice and centrifuged at 10000 rpm for 15 seconds. Samples were centrifuged at 10,000 rpm for 2 mins without the addition of any buffer to dry the membrane. The RNA was eluted into 30µl of RNase-free water. RNA was snap frozen at -80 or used immediately for cDNA synthesis.

2.13.2 Determination of RNA quantity, quality and integrity

The concentration and quality of RNA extracted was determined using a Nanodrop Spectrophotometer. The integrity of the RNA extracted was determined using the Agilent Technologies Tapestation. A RIN score >5 was considered suitable for RT-qPCR.

2.13.3 cDNA synthesis

The Superscript IV first strand synthesis kit was used to generate cDNA using random hexamer primers and SuperScript®IV reverse transcriptase in accordance with manufacturer's guidelines. Firstly, the primer was annealed to the template RNA. Components in table 2.11 were mixed, briefly centrifuged and heated to 65°C for 5 mins. The annealed RNA was then added to the reverse transcriptase mix (Table 2.12). The combined reaction mix was incubated at 23°C for 10

mins, 55°C for 10 mins and 80°C for 10 mins using the TC3000 PCR machine, before being diluted to 10ng/ul and stored at -20°C or being used immediately for RT-PCR.

2.13.4 Quantitative real time PCR RT-PCR

2.13.4.1 Primer design and optimisation

Primers were designed or purchased predesigned (Sigma Aldrich; KiCqStart Primers) toward desired transcripts (Table 2.17). Primer efficiency was assessed for all primers using a log standard curve. The efficiency of the primer was calculated as a percentage using the slope of the regression between the log values and the average Ct values. For genes with a low relative expression where a log standard curve was not possible, LinRegPCR (Ramakers et al., 2003, Ruijter et al., 2009) software was used. This uses non-baseline corrected data, performs a baseline correction on each sample separately, determines a window-of-linearity and then uses linear regression analysis to determine the PCR efficiency per sample from the slope of the regression line. The mean PCR efficiency per amplicon is calculated. Efficiency of between 90-110% was considered acceptable.

To assess for a single specific product at qPCR both a melt curve and visualisation of a single band on agarose gel electrophoresis were used. If a second small product was visible, i.e. a primer dimer, primer concentration was then halved and qPCR repeated. Gel electrophoresis was carried out using 2% agarose gel supplemented with GelRed made up in 100 ml TAE buffer at 100V for 1 hour. GelRed bound DNA was visualised under Ultra Violet light in an Alpha Imager mini (Alpha Innotech).

2.13.4.2 Quantitative PCR- $\Delta\Delta C_t$ method

Quantitative PCR was performed using SYBR green detection to measure transcript concentrations. In order to quantify gene expression relative to experimental controls the Delta delta/ $\Delta\Delta C_t$ method was used. 5 μ l/10 μ l of Power SYBR green PCR mastermix was added to each well of a 384- or 96- well microamp optical plate, along with 0.15/0.3 μ l/0.6 μ l primer mix (10 μ m), cDNA and or H₂O, to a final volume of 10 μ l/20 μ l depending on plate size. Housekeeper

genes and samples were both run in duplicate. Adhesive PCR plate seals were applied and the plate was spun down at 1400 rpm for 2 mins. Quantitative PCR cycling was performed on an Applied Biosystems 7900HT Fast instrument. Reaction mix was subjected to an initial 95°C activation step for 3 mins, then 40 cycles: denaturation (95°C for 3 seconds), primer annealing (60°C for 20 seconds), extension (72°C for 1 second) and a fluorescence acquisition reading (74°C for 10 seconds). B2M and GAPDH were both used as internal reference genes and the geomean determined. Expression levels were normalised to the geomean of the internal reference genes and calculated as fold change relative to experimental control using the $2^{-\Delta\Delta C_T}$ method (Livak and Schmittgen, 2001). Data is either presented as $\Delta\Delta C_T$ values, $\Delta\Delta C_T$ values 1000x or displayed as fold change from the experimental control.

2.13.4.3 Housekeeper gene selection

To ensure the generation of accurate and robust RT-qPCR data from GL cells, the geNorm software package was used to identify the most stable reference genes when comparing cells from women with (n=5) and without PCOS (n=5) (Vandesompele et al., 2002). The expression of 12 candidate genes was measured by quantitative real-time PCR and the data analysed by the geNorm software, according to manufacturer's instructions. Lysophylised primers were resuspended and combined with Primerdesign PrecisionPLUS mastermix and RNase/DNase free water. This was pipetted into each well along with 5µl of cDNA 5ng/µl. The final volume in each well was 20µl. Analysis was completed using qbase+ software. Amplification conditions are listed in table 2.2.

Table 2.2 Amplification conditions for GeNorm

	Step	Time	Temperature (degrees Celsius)
Cycling x 40	Enzyme activation	2 mins	95
	Denaturation	10 seconds	95
	Data collection	60 seconds	60

The geNorm algorithm determines the medium reference target stability measure (M-value), as the average pair-wise variation of each reference gene in relation to all the other reference genes enabling the elimination of the least stable gene. This is followed by recalculation of the M values resulting in ranking of the most stable genes, i.e. the lower the M value, the higher the gene stability. The software indicates that a good stable reference gene should have an average geNorm M value ≤ 1.0 (Hellemans et al., 2007). GAPDH and B2M had the lowest M-values and were the most stably expressed gene pair, while 18S and SDHA had the highest M-values and were the least stable genes across all samples (Figure 2.3)(Lerner et al., 2019). We also computed the optimal number of reference genes based on the pairwise variation value (V-value). geNorm defines a pairwise variation of 0.15 as the cut-off value, below which the inclusion of an additional reference gene for normalisation is not needed. As shown in Figure 2.3, the V2/3 value is far below the cut-off of 0.15, indicating that the top two ranked reference genes GAPDH and B2M (with the lowest M-values) are suitable for gene expression normalisation in GLCs from women with PCOS (Lerner et al., 2019).

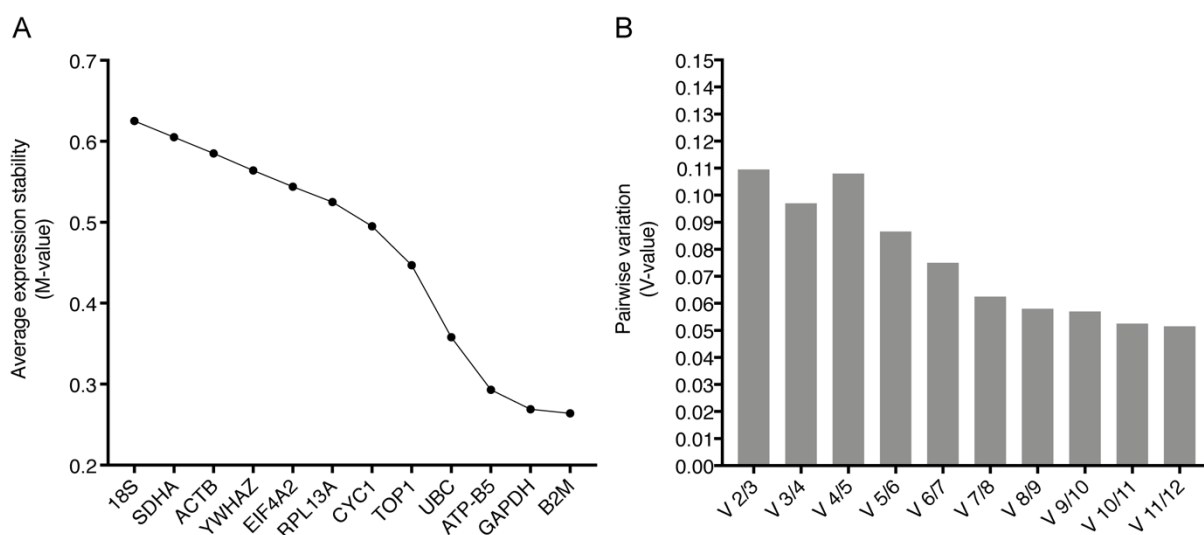


Figure 2.3 Reference gene selection and steroidogenic gene relative abundance.

Gene expression stability analysis by GeNorm. **A** The mean expression stability value (M) of the 12 candidate reference genes. **B** Determination of the optimal number of reference genes for normalisation using pairwise variation analysis by GeNorm. Figure published in (Lerner et al., 2019).

2.14 RNA sequencing

2.14.1 Sample preparation

RNA was extracted from frozen GCs isolated from hSAFs (see 2.2.4). RNA was quantified using nanodrop and qualitative analysis completed using TapeStation, as described in 2.2.12. RNA was then diluted to 15ng/μl and 11 μl of each sample was loaded onto a 96 well plate (Thermo Fisher PCR Plates #AB0800).

Samples were then transferred on dry ice to the Oxford Genomics Centre for sequencing and analysis. There the material was quantified once again using RiboGreen (Invitrogen) on the FLUOstar OPTIMA plate reader (BMG Labtech) and the size profile and integrity analysed on the 2200 or 4200 TapeStation (Agilent, RNA ScreenTape).

2.14.2 Sequencing (completed in Oxford Genomics Centre)

The QuantSeq 3' mRNA-Seq Library Prep Kit FWD (Lexogen) was used for sequencing, on an Illumina HiSeq4000 instrument, as per manufacturer's instructions. The QuantSeq kit is a library preparation protocol designed to generate Illumina compatible libraries of sequences close to the 3' end of polyadenylated RNA. 12 PCR cycles were used for library amplification. Gene expression levels were calculated using standard Illumina RNA-seq methods. The human genome (GRCh37.EBVB95-8wt.ERCC) was used as a reference genome.

2.14.3 Analysis

Pathway analysis was carried via the InnateDB (www.innatedb.com) website using the KEGG and REACTOME databases and the Hypergeometric algorithm and correcting for multiple comparisons using the Benjamini-Hochberg method (Moriya et al., 2007, Fabregat et al., 2018, Reiner et al., 2003). Gene ontology analysis was also carried out via the InnateDB (Innatedb.com) website (Breuer et al., 2013). Protein-protein interactions were conducted to identify the hub

genes by STRING: Functional protein association networks (<https://string-db.org/>) (Szklarczyk et al., 2019) .

2.15 Phospho-kinase array

This array was completed as per manufacturer's instructions. Cells were plated in 6 well plates. Cells were serum starved overnight prior to ligand treatment. After treatment media was removed and cells were washed with PBS. Cells were solubilised by adding 400µl of lysis buffer 6 per condition and lysates were rocked gently at 4 degrees for 30 mins. Protein content was quantified using BCA assay as described previously. 230 mcg of protein was used per array set. Lysates were stored at -80 degrees until use. Membranes were blocked in array buffer 1 for 1 hour on a rocking platform shaker. 334µl of lysate was made up to 2ml with array buffer 1 and this was added to two membranes A and B which were incubated overnight at 4 degrees on a rocking platform shaker. On day 2 membranes were washed in 1x wash buffer for 3 x 10 mins. Membranes were then incubated with detection antibody cocktail A or B and 1x array buffer 2/3 for 2 hours at room temperature on a rocking platform shaker. Membranes were washed in 1 x wash buffer for 3 x 10 mins. Streptavidin-HRP in 1X Array Buffer 2/3 was added and membranes were incubated for 30 mins at room temperature on a rocking platform shaker. Membranes were washed in 1xwash buffer for 3 x 10 mins. 1 mL of the prepared Chemi Reagent Mix was added evenly onto each set of membranes. Membranes were exposed to film. To analyse the results the average signal of the duplicate spots was calculated. An average background signal was assessed from the negative control spots and subtracted. Reference spots were used as loading controls. Fold change relative to untreated lysates was calculated.

2.16 Signalling assays

2.16.1 Cyclic AMP accumulation assay (Cisbio)

Cells were seeded in 24 well plates and cultured in media with FBS. Cells were serum starved overnight prior to the assay. Depending on the experiment, cells were pre-treated with 0.5mM IBMX. After the appropriate ligand treatment and duration, cells were quickly washed with cold PBS and then lysed in 35 ul Cisbio cAMP lysis buffer before being incubated for 15 mins at room temperature on a shaking platform. Plates were then scraped and lysates transferred to 1.5 ml

tubes and centrifuged at 13000 rpm for 5 mins. The supernatant was then either stored at -20°C or analysed immediately following manufacturer's instructions. A 384 well white Greiner microplate was used for the analysis. 10 µl of sample, 5 µl of dye d2 and 5 µl of anti-cAMP cryptate conjugate was added to each well. The reaction was incubated at room temperature for 1 hour, then readings were taken using a PHERAstar FSX plate reader equipped with a Homogenous Time Resolved Fluorescence (HTRF) 337 optic module. Results are calculated from the 665nm/620nm fluorescence ratio, using a standard curve and were normalised to protein concentration. Protein concentration was quantified using the Bradford assay, as described previously. All experiments were conducted in triplicate.

2.16.2 IP1 assay

Cells were seeded in 24 well plates and cultured in media with FBS. Cells were serum starved overnight prior to the assay. Media was removed and stimulation buffer was added in combination with ligands. Treatments were carried out in triplicate. Dye d2 and anti-IP1 cryptate Tb, both diluted in lysis buffer, were added to wells. Cells were then incubated for 15 mins at room temperature on a shaking platform. Plates were then scraped and lysates transferred to 1.5 ml tubes and centrifuged at 13000 rpm for 5 mins. The supernatant was then either stored at -20°C or analysed immediately following manufacturer's instructions. The reaction was incubated at room temperature for 1 hour, then readings were taken using a PHERAstar FSX plate reader equipped with a Homogenous Time Resolved Fluorescence (HTRF) 337 optic module. Results are calculated from the 665nm/620nm fluorescence ratio, using a standard curve and were normalised to protein concentration. Protein concentration was quantified using the Bradford assay, as described previously. All experiments were conducted in triplicate.

2.16.3 Calcium assay

To assess changes in intracellular Ca²⁺ levels, Fluo-4 Direct Calcium Assay Kit (Thermo Fisher) was used, following the manufacturer's protocol. Cells were seeded in glass bottomed dishes (Greiner). A 50% calcium assay reagent was made with probenacid and calcium assay buffer with 50% DMEM. Cells were incubated with this reagent for 30 mins at 37°C followed by an additional

30 min at room temperature. When inhibitors were used, these were added at the appropriate time before the imaging started and within the 1 hour of incubation with the dye. Following incubation, agonist-dependent Ca^{2+} mobilisation was assessed using a TCS-SP5 confocal microscope (Leica), with $\times 20$ dry objective, a 488 excitation laser, capturing 1 frame per second. Baseline Ca^{2+} was assessed for 1 minute prior to ligand treatment, and the effects of ligand assessed for a further 10 mins. All experiments were conducted in duplicate. The inbuilt Leica LASAF analytical software and Image J with a plugin Time Series Analyser were used to analyse the resulting time-lapse series. The maximal intensity was obtained after subtracting the average background intensity (recorded before LH/kisspeptin addition) for each cell and averaged across 10 cells per condition.

2.17 Conjugation of LH to AF488

LH was conjugated to AF488 from the Alex Fluor 488 antibody labelling kit utilising the manufacturer's instructions. 100ul of 1mg/ml ligand diluted in PBS was combined with a reactive dye and incubated for 1 hour at room temperature. The labelled ligand was then purified by loading it onto a purification resin in a spin column and collected by centrifugation for 5 mins at $1100 \times g$. After centrifugation, the collection tube contained labelled protein in approximately 100 μL of PBS, pH 7.2, with 2 mM sodium azide, and free dye remains in the column bed. To calculate the concentration the absorbance with a 1-cm path length at 280 nm (A_{280}) and the absorbance maximum (λ_{max}) for the respective dye (A_{dye}) was measured ($[A_{280} - (A_{\text{dye}} \times CF_{280})] \times \text{dilution factor} / 203,000$ (molar extinction coefficient (ϵ) in $\text{cm}^{-1} \text{M}^{-1}$)). The degree of labelling was calculated using $A_{\text{dye}} \times \text{dilution factor} / \epsilon_{\text{dye}} \times \text{protein concentration (M)}$. Acceptable degrees of labelling were 4-9. Cells were then pre-incubated with labelled ligand at 37 degrees. Media was changed and ligand removed prior to live imaging.

2.18 Statistical analysis

Statistical analysis was completed using Graphpad Prism version 6 and 7. All data were assessed for normal distribution using D'Agostino & Pearson omnibus normality test. Normally distributed data were analysed by unpaired t test or analysis of variance (ANOVA). For data which were not

normally distributed or the n number was too small to test distribution, Kruskal-Wallis or Mann-Whitney tests were used. Post hoc tests were used where appropriate. Spearman or Pearson correlation were used depending on distribution of the data. A p-value of <0.05 was considered significant. Data are presented as Mean $\pm$ SEM or Median $\pm$ 95% confidence interval.

2.19 Tables of antibodies

Table 2.3 Primary antibodies used for western immunoblotting/immunohistochemistry

Primary antibody	Species	Antibody concentration	Dilution WB	Dilution immuno-histochemistry	Supplier/Catalogue no.
APPL1	Rabbit	10 μ g/ml	1:1000	1:500	Cell signalling CS 3858S
Beta arrestin 1/2	Rabbit	125 μ g/ml	1:1000	1:500	Cell signalling CS 4674S
EEA1	Rabbit	44 μ g/ml	1:1000	1:500	Cell signalling CS 3288S
FSHR	Rabbit	1mg/ml	1:1000	1:500	ThermoFisher PA5-28764
FYN	Rabbit	390 μ g/ml	1:500	N/A	Cell signalling CS 4023T
GAPDH	Mouse	1 mg/ml	1:5000	n/a	Merck MAB374
GIPC	Rabbit	200 μ g/ml	1:250	N/A	Santa Cruz Sc-25556
LHCGR	Mouse	Not known	1:1000	1:500	Given by Katja Teerds, Wageningen University, The Netherlands

P44/42 MAPK (total ERK 1/2)	Rabbit	502 µg/ml	1:1000	N/A	Cell signalling CS 4370S
pAMPKα	Rabbit	27 µg/ml	1:1000	N/A	Cell signalling 4535
Phospho 44/42 MAPK (pERK1/2)	Rabbit	19 µg/ml	1:1000	N/A	Cell signalling CS 9102S
Phospho- AKT	Rabbit	91 µg/ml	1:1000	N/A	Cell signalling 4060S
Phospho- CREB	Rabbit	1 mg/ml	1:1000	N/A	Merck 06-519
Phospho- EGFR	Rabbit	423 µg/ml	1:1000	N/A	Cell signalling CS 3777S
Total AKT	Rabbit	34 µg/ml	1:1000	N/A	Cell signalling CS 9272

Table 2.4. Secondary antibodies used for western immunoblotting

Secondary antibody	Dilution	Supplier/Catalogue no.
Goat anti-Rabbit IgG	1:10000	Dako/P0448
Goat anti-Mouse IgG	1:10000	Dako/P0447

Table 2.5. Secondary antibodies used for immunofluorescence

Secondary antibody	Dilution	Supplier/Catalogue no.
Goat anti-mouse IgG Alexa Fluor 488	1:1000	Invitrogen A-11029

Goat anti-rabbit IgG Alexa Fluor 488	1: 1000	Invitrogen A-11008
Goat anti-rabbit IgG Alexa Fluor 555	1: 1000	Invitrogen A-21428
Rabbit anti-mouse IgG Alexa Fluor 555	1: 1000	Invitrogen A-21427

Table 2.6. IgG controls used for immunofluorescence

IgG	Concentration (µg/ml)	Supplier/Catalogue no.
Mouse IgG1	Same as primary antibody concentration	Dako X0931
Mouse IgG2a	Same as primary antibody concentration	Dako X0943
Rabbit IgG	Same as primary antibody concentration	Sigma-Aldrich I8140

2.20 Ligands and inhibitors

Table 2.7 Ligands

Ligand	Target	Supplier	Catalogue number	Working concentration
5αMAndrostanM17β MolM3Mone (DHT)	Androgen receptor	Sigma	A8380	10nM- 1µm

FSH	FSH receptor	National hormone peptide program, UCLA (NHPP)	N/A	10nM
hCG	LH/hCG receptor	NHPP	N/A	10nM
Kisspeptin-54	Kisspeptin receptor	Bachem (given by Professor Waljit Dhillon)		10-1000nM
Kisspeptin-10	Kisspeptin receptor	Bachem (given by Professor Waljit Dhillon)		10-1000nM
LH	LH/hCG receptor	NHPP		10nM

Table 2.8 Inhibitors

Inhibitor	Target	Supplier	Catalogue number	Working concentration
Dynngo-4a	Dynamin	Abcam	ABI20689	50 μ M
IBMX	PDEs	Sigma	I5879	0.5 mM
UBO-QIC	G α _q /11	University of Bonn	n/a	1 μ M
Flutamide	Androgen	Sigma	F9397	20 μ M
IBMX	PDES	Sigma	I5879	500 μ M

2.21 Reagents, kits, miscellaneous materials, solutions and buffers

Table 2.9 Reagents

Reagent	Supplier
Agarose	Sigma
Bolt 4 - 12% Bis-Tris (10/15 well) gels	Invitrogen
Bolt LDS sample buffer 4x	Invitrogen
Bolt MES SDS running buffer	Invitrogen
Bolt reducing agent	Invitrogen
Bovine serum albumin (BSA)	Sigma
Chemiluminescent detection reagent	Thermo Fisher Scientific
Chloroform	Sigma
Dimethyl sulfoxide (DMSO)	Sigma
Dithiothreitol (DTT)	Sigma
DNase I and RDD buffer	Qiagen
Dulbecco's phosphate buffered saline (DPBS)	Gibco
Ethidium bromide	Sigma
Ethylenne diamine tetraacetic acid (EDTA)	Sigma
GelRed	Biotium
M-100 10x	Gibco
M-199 1x	Gibco
Methanol	VWR

Molecular grade ethanol	VWR
Paraformaldehyde powder	Sigma
Percoll	GE Healthcare
Phosphate buffered saline (PBS)	BD Healthcare
Power SYBR green PCR mastermix	Thermo Fisher Scientific
ProLong gold antifade mountant with DAPI	Thermo Fisher Scientific
Protease and phosphatase inhibitor cocktail	Thermo Fisher Scientific
RIPA buffer	Thermo Fisher Scientific
Seeblue plus 2 prestained ladder	Invitrogen
skim milk powder	Oxoid
Sodium azide	Sigma
sodium bicarbonate	Gibco
Sodium chloride	Sigma
Sodium dodecyl sulphate (SDS)	BD Healthcare
Tris base	Sigma
Tris HCl	Sigma
Triton X-100	Sigma
Trizol	Life Technologies
β -mercaptoethanol	Sigma

Table 2.10 Cell culture reagents

Reagents	Catalogue Number	Final Concentration
DMEM/F-12 HAM (1:1)	Gibco 31330-038	-
L-glutamine (200mM)	Gibco 31330-038	2mM
FBS	Life technologies 10082147	10%
Penicillin-Streptomycin solution	Sigma #P0781	
Penicillin (10,000 unit/mL)	Sigma #P0781	10 unit/mL
Streptomycin (10 mg/mL)	Sigma #P0781	0.1 mg/mL
Amphotericin B (250 ug/mL)*	Sigma #A2942, 100 mL	2.5 ug/mL
ITS+1 liquid media supplement (×100):	Gibco #51500-056	×1
1.0 mg/mL insulin		10 ug/mL
0.55 mg/mL transferrin		5.5 ug/mL
0.67 ug/mL sodium selenite(anhydrous)		6.7 ng/mL
0.2 mg/mL Ethanolamine		2.0 ug/mL

Table 2.11 Mix for annealing primer to template RNA

Component	Volume
50ng/μl random hexamers	1 μl
10mM dNTP mix	1 μl
Template RNA	11 μl

Table 2.12 Reverse transcriptase reaction mix

Component	Volume
5x SSIV buffer	4 µl
100 mM DTT	1 µl
Ribonuclease inhibitor	1 µl
SuperScript®IV reverse transcriptase (200U/ µl)	1 µl

Table 2.13 Kits

Kit	Supplier
Alexa Fluor 488 antibody labelling kit	Invitrogen
Bradford protein assay	Thermo Fisher Scientific
cAMP Dynamic 2	Cisbio
Fluo-4 Direct Calcium Assay	Thermo Fisher Scientific
geNorm reference gene selection kit	Primer Design
IP1 Gq kit	Cisbio
RNeasy micro kit	Qiagen
RNeasy mini kit	Qiagen
Phospho-kinase antibody array	RND systems
Pierce BCA (bicinchoninic acid) Protein Assay Kit	Thermo Fisher Scientific
Superscript IV first strand synthesis kit	Invitrogen
Trypan Blue exclusion kit	Sigma

Table 2.14 Miscellaneous materials/machinery

Material/machine	Supplier/provider
Adhesive PCR plate seals	Applied Biosystems
Agilent Technologies 2200 Tapestation	Agilent
Eppendorf centrifuge 5415R	Eppendorf
Eppendorf centrifuge 5415R	Eppendorf
ImageQuant LAS 4000	GE Healthcare
Microamp optical 96 or 384 well reaction with barcode	Applied Biosystems by Life Technologies
Nanodrop Spectrophotometer Nanodrop ND-1000	Thermo Fisher Scientific
Nitrocellulose blotting membrane	GE Healthcare
PCR machine TC3000	Techne
PHERASTAR FSX plate reader	BMG Labtech
QIA shredder column	Qiagen
sterile 50ml conical centrifuge tubes	VWR
Sub blot mini wet transfer system	Appleton Woods
x-ray film	GE Healthcare
PCR machine TC3000	Techne

Table 2.15 Solutions/buffers

Solution/buffer	Components
Immunofluorescence Blocking Solution / primary and secondary antibody dilution solution	2% (v/v) FBS in PBS with Ca ²⁺
Immunofluorescence permeabilisation solution	2% (v/v) FBS in PBS with Ca ²⁺ 0.2% (v/v) Triton X-100
Percoll density gradient (45%)	44.5% (v/v) Percoll 50% v/v 1xM199 5% 10xM199 0.5% Sodium Bicarbonate
Phosphate buffered saline (PBS)	140 mM NaCl 2.5 mM KCl 1.5 mM KH ₂ PO ₄
Ponceau stain	Ponceau S Red powder 5% (v/v) acetic acid
TAE buffer	40 mM Tris Base, 20mM glacial acetic acid 1mM EDTA pH 8.0
Tris buffered saline (TBS)	130 mM NaCl 20 mM Tris pH 7.6

TBS-Tween 20 (TBS-T)	0.1% Tween 20 in TBS
Western blot running buffer	Bolt MES SDS Running Buffer 20x + deionized H ₂ O
Western blot Transfer Buffer	70% (v/v) distilled water 20% (v/v) methanol 10% (v/v) 10x TB (250 mM Tris Base, 1.92 M Glycine) pH 8.3
Western blot Stripping Buffer	100 mM β -mercaptoethanol 2% (v/v) SDS 62.5 mM Tris HCl, pH 6.8
Western blot Blocking Solution / Primary Antibody Dilution Solution	5% (w/v) skimmed milk or BSA in TBS-T
4% paraformaldehyde	4% (w/v) PFA in PBS, pH 7.2-7.4

2.21. Analysis and imaging software

Table 2.16 Analysis/imaging software

Software	Use
Image Studio Lite software (LI-CORE Biosciences, version 5.2.5)	Western blot band signal quantification
Graphpad prism versions 6 and 7	Graphs, statistical analysis
Image J	Image analysis

2.22 Primer sequences

Table 2.17 Primer sequences

Gene	Gene ID	Exon Location	Primer sequence (5'3')	Product size(bp)
ACTB	60	Fwd 3 Rev 3-4	Fwd GACGACATGGAGAAAATCTG Rev ATGATCTGGGTCATCTTCTC	131
AMH	268	Fwd 5 Rev 5	F- GAGACTTACCAGGCCAACAA R- TCCTCCGACAGGCTGATG	185
AMHR2	269	Fwd 3 Rev 5	F- ACTTCTGCAATGCCAATTAC R- CTGTAGTTCTTTCGCTGTAG	187
APPL1	26060	Fwd 2 Rev 4	Fwd AAGGACTTGGAGAACAAG Rev ATTCCAAGGGGGAATATCTAC	184
AR	367	Fwd 8 Rev 8	F- CAGCATTATTCCAGTGGATG R- TCTTGAGCAGGATGTGG	124
AREG	374	Fwd 3 Rev 3-4	Fwd AAAGAAAGAAAAAGGGAGGC Rev CATTGTCATGTTACTGCTTC	142
ARRB1	408	Fwd 8 Rev 9	Fwd AGATCTATTACCATGGAGAACC Rev GATCTTGATCTTCTTCACCGGGT	81
ARRB2	409	Fwd 14 Rev 15	Fwd TTTGATACCAACTATGCCAC Rev CATCATAGTCGTCATCCTTC	92
B2M	567	Fwd 2	Fwd AAGGACTGGTCTTTCTATC	118

		Rev 2-3	Rev GATCCCACTTAACTATCTTGG	
CDH5	1003	Fwd 10 Rev 10-11	Fwd CGCAATAGACAAGGACATAAC Rev TATCGTGATTATCCGTGAGG	92
CYP11A1	1583	Fwd 6 Rev 7	Fwd AGCATCAAGGAGACACTAAG Rev GCAGGAATCATGTAATCTCG	95
CYP19A1	1588	Fwd 8-9 Rev 9	Fwd GGTGAGAGAGACATAAAGATTG Rev TTCAGGATAATGTTTGTCCC	170
EREG	2069	Fwd 2-3 Rev 3	Fwd GTTCAGACAGAAGACAATCC Rev GACTCATGTCCACCAGATAG	112
ESR1	2099	Fwd 6-7 Rev 7	Fwd GGAGTGTACACATTTCTGTC Rev CAAAGTGTCTGTGATCTTGTC	90
ESR2	2100	Fwd 7 Rev 7-8	Fwd AAATCTTTGACATGCTCCTG Rev AGGGTACATACTGGAATTGAG	113
FSHR	2492	Fwd 10 Rev 10	Fwd GCGGAACCCCAACATCGTGTC Rev TGAAGAAATCTCTGCGAAAGT	248
FSHRvar1	2492	Fwd 8-10 Rev 10	Fwd GGACCAGTCATTCTCTCTGAGC Rev CTCATTGCATAAGTCATAGTC	181
FSHRvar2	2492	Fwd 20 Rev 22	Fwd AAGTTTTACTATGGCTGAATAAG Rev CCATAGCTAGGCAGGGAATGG	172
FST	10468	Fwd 2-3 Rev 3	Fwd TAAAGAAACGTGTGAGAACG Rev CTTTACATCTTGCCTTTAGGAG	182
GATA4	1010	Fwd 5-6	Fwd AGACGTTCTCAGTCAGTG	183

		Rev 6	Rev ACGCAGTGATTATGTCCC	
GIPC	10755	Fwd 1 Rev 3	Fwd GAGCAGATGAAGAACTGAG Rev GGTCACCAGAAGATCTAAAC	164
HSD3B2	3284	Fwd 2 Rev 2-3	Fwd AAGTGTTTCCTGCTACTTTG Rev CTGGAGCTTAGAAAATTCCTC	181
INHBA	3624	Fwd 1-2 Rev 2	Fwd GAGAATTTGCTGAAGAGGAG Rev CAAAAGTTGTTGTGATTGCC	136
INHBB	3625	Fwd 2 Rev 2	Fwd AACGTTTCAGGGTATGAATG Rev TTTACTCTACCTTCTGGGTG	82
INSR	3643	Fwd 9 Rev 9	Fwd GATCCAATCTCAGTGTCTAAC Rev CCTTTGAGGCAATAATCCAG	152
KISS1	3814	Fwd 2 Rev 3	Fwd CCACTTTGGGGAGCCATTAG Rev CCAGTTGTAGTTCGGCAGGTC	295
KISS1R	84634	Fwd 1-2 Rev 2	Fwd GACCAACTTCTACATCGC Rev GATGTAGTTGACGAACTTGC	136
LHCGR	3973	Fwd 5 Rev 6	Fwd GATGTTACGAAGGTCTTCTC Rev AAAAGCATTTCTGGTATGG	90
LHRa	3973	Fwd 9 Rev 11	Fwd ATTTGTCAATCTCCTGGAGGC Rev CACTCAGTTCACTCTCAGCA	191
LHRb	3973	Fwd 8-10 Rev 11	Fwd AGGGCCGAAAACCTTACAGAA Rev CACTCAGTTCACTCTCAGCA	131

LHRc	3973	Fwd 9 Rev 10-11	Fwd ATTTGTCAATCTCCTGGAGGC Rev CCCCATGCAAAAAGTGTTTTG	167
LHRd	3973	Fwd 8-10 Rev10-11	Fwd AGGGCCGAAAACCTTACAGAA Rev CCCCATGCAAAAAGTGTTTTG	107
SERPINF1	5176	Fwd 4 Rev 4-5	Fwd GAACAGAATCCATCATTACC Rev CTTCTTCTCAAAGACGATCC	146
STAR	6770	Fwd 4 Rev 5	Fwd GACAAAGTGATGAGTAAAGTGG Rev CAGCTCGTGAGTAATGAATG	195
VEGFA	7422	Fwd 4-5 Rev 5 6	Fwd AATGTGAATGCAGACCAAAG Rev GACTTATACCGGGATTCTTG	106

Chapter 3

Gonadotropin receptor signalling and intracellular trafficking in human granulosa lutein cells

3.1 Introduction

Gonadotropins bind to their receptors, which then undergo a conformational change and transmit a signal to intracellular effectors. This activates a G protein, which for gonadotropin (and all glycoprotein receptors) is known to be the classical G α s/cAMP/PKA pathway. LHR, in the presence of high concentrations of ligand and/or receptor, also couples to another G protein, G α q/11. Both gonadotropin receptors activate the MAPK pathway, although through different mechanisms, as discussed in chapter 1.

Internalisation of both LHR and FSHR relies on both β -arrestin and clathrin recruitment (Min et al., 2002, Ghinea et al., 1992). Once internalised most LHR and FSHR is trafficked back to the plasma membrane, determined by sequences in the receptor C-tails (Jean-Alphonse et al., 2014). Work completed by the Hanyaloglu laboratory has shown that interaction of the LHR C-tail with GIPC dictates trafficking of LHR to the very early endosome (VEE), from which it generates a sustained MAPK/ERK signal. Trafficking and signalling from the VEE is tightly regulated by APPL1. This mechanism of trafficking to VEE and the role of GIPC and APPL1 appears to be similar for FSHR.

Gonadotropin receptor signalling and trafficking has predominantly been studied in cell lines transfected with tagged receptor. Therefore, I firstly established techniques for studying these receptors using HEK293 cells transfected with stable LHR. I then aimed to characterise gonadotropin receptor signalling mechanisms in GLCs from women without PCOS, before attempting to study differences that occur in these mechanisms in PCOS, which will be discussed in chapter 4.

GLCs express both gonadotropin receptors and are therefore, theoretically, a good model for studying these receptors in health and disease. They are also the major site of progesterone production, one of the key functions of LHR. GLCs also remain responsive to gonadotropins *in vitro*, as will be demonstrated in this chapter and has been shown by others (Casarini et al., 2012, Nordhoff et al., 2011). One of the limitations of these cells is that they are collected at the time

of oocyte retrieval for IVF/ICSI/egg freezing. As part of this process women undergo ovarian stimulation, which can involve can FSH, hCG, GnRH agonist or antagonist treatment, all of which can impact gonadotropin receptor expression and function. Therefore, I optimised culture conditions and timing in order to minimise the impact of exogenous treatments.

3.1.1 Specific aims

The aims were to address the following questions:

1. Are GLCs a good model for studying gonadotropin receptor signalling and function?
2. How can GLCs be optimised to study gonadotropin receptor signalling and function?
3. What are the mechanisms involved in spatial regulation of gonadotropin receptor signalling in GLCs?

3.2 Methods and Results

3.2.1 Optimising culture of GLCs to study gonadotropin receptor function

Different groups that have studied GLC gonadotropin receptors and steroidogenesis have cultured the cells for varying time points prior to completing experiments. This culture period has varied from 2 (Chang et al., 2014) to 6/7 days (Nordhoff et al., 2011). I therefore studied the effect of time in culture on GLC gonadotropin cAMP response and gene expression, in order to pick the optimal culture period for my experiments.

3.2.1.1 cAMP response to LH and FSH is highest in GL cells on day 4 in culture

GLCs were cultured in 24 well plates in triplicate for 2, 3, 4, or 6 days. Cells were serum starved overnight prior to cAMP assay. Cells were stimulated with LH or FSH 10nM for 5 minutes. cAMP levels were measured and fold change increase in cAMP relative to untreated cells was calculated. The biggest increase in cAMP after LH treatment occurred after 4 days in culture. (Figure 3.1). There was no significant change in cAMP after FSH treatment on the different days, likely due to the variability between samples.

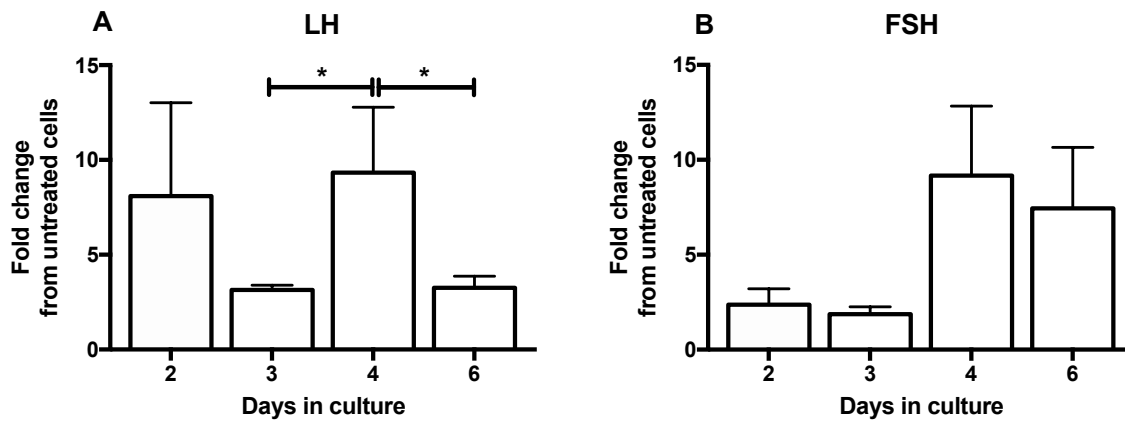


Figure 3.1. Fold-change in cAMP after treatment with LH or FSH after GLCs are left in culture for 2,3,4 or 6 days. Cells were pre-treated with IBMX for 5 mins and then stimulated with LH or FSH 10nM for mins and cAMP assay completed. **A** The biggest mean fold change increase in cAMP after LH treatment was seen after 4 days in culture compared to 3 or 6 days ($n=6$), $p<0.05$. **B** There was no significant difference between mean fold increase in cAMP after FSH treatment, ($n=6$) (ns).

3.2.1.1 GLC gonadotropin receptor and steroid enzyme expression recovers in culture

GLCs were frozen on day of collection (day 1) or cultured in 6 well plates for 2,3,4 or 6 days and then frozen at -80°C. RNA was extracted, quantified and cDNA was synthesised for RT-QPCR. Steroid enzyme and gonadotropin receptor expression in GLCs recovers and increases in culture compared to 'fresh' GLCs, frozen on the day of extraction. While it appeared that gonadotropin receptor and steroid enzyme gene expression increased after 1-2 days in culture, there was no significant difference in expression (Figure 3.2), likely due to the low number of samples used ($n=3$).

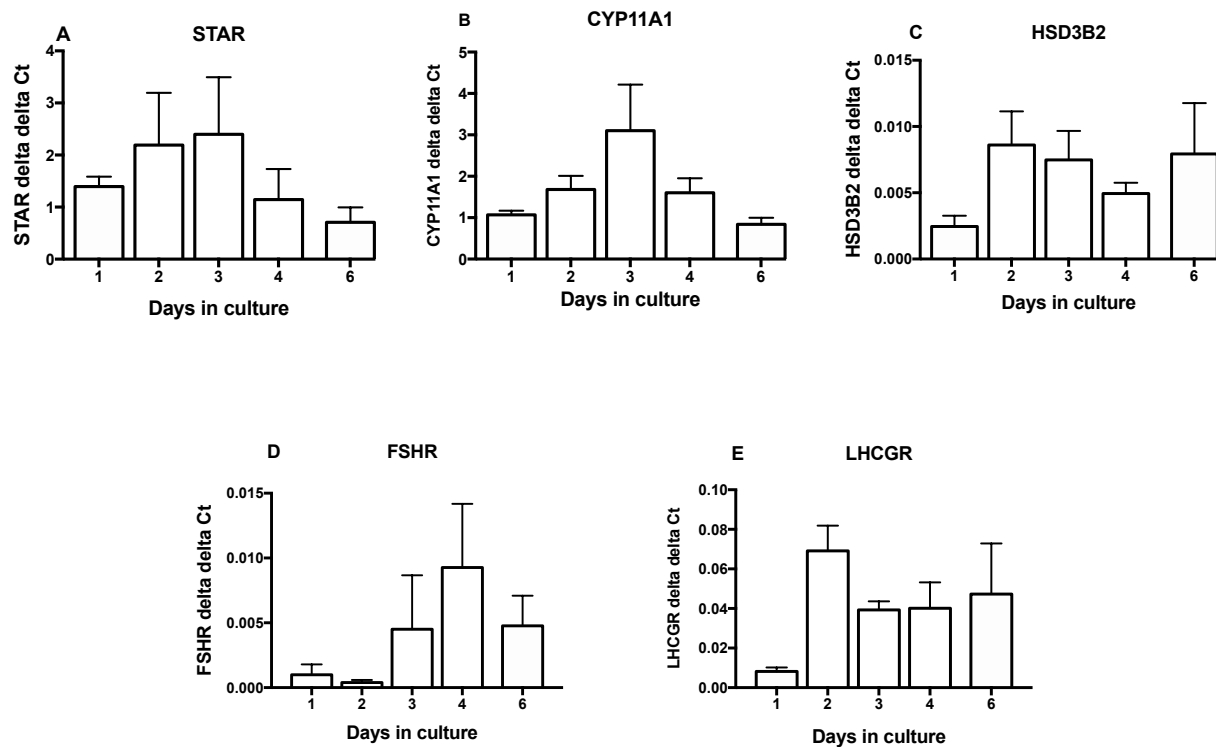


Figure 3. 2 Gonadotropin receptor and steroid gene expression in GLCs in culture for 1-6 days. Values are mean $\pm$ SEM. **A** There was no significant difference in STAR expression over time in culture ($n=3$). **B** There was no significant difference in CYP11A1 expression over time in culture ($n=3$). **C** There was no significant difference in HSD3B2 expression over time in culture ($n=3$) **D** There was no significant difference in FSHR expression over time in culture ($n=3$) **E** There was no significant difference in LHCGR expression over time in culture ($n=3$).

This increase in cAMP generation after 4 days in culture, which is mirrored in the steroid enzyme expression and gonadotropin receptor expression in culture, is likely because of the recovery over time from exogenous gonadotropins and GnRH agonists/antagonists which were given as part of the IVF cycle. Given that cAMP accumulation after LH treatment was highest on day 4, I opted to perform most of the experiments involving gonadotropin receptor activity/steroidogenesis on day 4. Cells were serum starved overnight from day 3.

3.2.2 GLCs remain responsive to gonadotropins in vitro

GLCs are a suitable model for studying gonadotropin receptor action as they express both the LH and FSH receptors. In addition, they remain responsive to gonadotropins *in vitro* and steroidogenesis occurs. To demonstrate this in GLCs obtained from control women, GLCs were cultured for 4 days and treated with LH or FSH. Incubation with LH for 24 hours stimulates progesterone production in culture media (Figure 3.3A) and augments gene expression of *LHCGR* and *STAR* (Figure 3.3D, E). Treatment with LH or FSH for 5 minutes stimulates cAMP production (Figure 3.3B, C). Incubation with FSH for 24 hours downregulates gene expression of *FSHR* (Figure 3.3F).

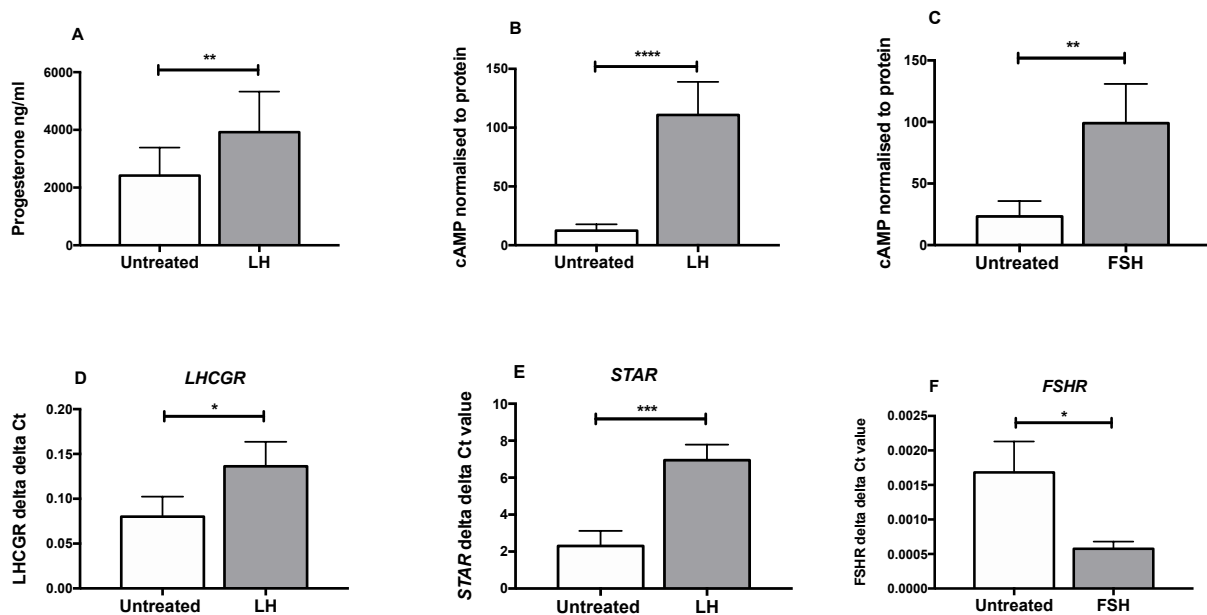


Figure 3.3 GLCs remain responsive to gonadotropins in vitro after 4 days. **A** GLCs incubated for 24 hours with LH 10nM stimulated progesterone production and output into culture media ($n=13$) ($p<0.01$). **B** GLCs incubated for 5 minutes with LH 10nM stimulates cAMP production ($p<0.0001$) ($n=9$). **C** GLCs incubated for 5 minutes with FSH 10nM stimulated cAMP production ($p<0.0001$) ($n=9$). **D** GLCs incubated for 24 hours with LH 10nM stimulated an increase in LHCGR expression ($n=8$) ($p<0.05$). **E** GLCs incubated for 24 hours with LH 10nM stimulated an increase in STAR expression ($n=8$) ($p<0.001$). **F** GLCs incubated for 24 hours with FSH 10nM downregulated FSHR ($n=6$) ($p<0.05$). Mann Whitney test used for A-F, values are mean $\pm$ SEM.

3.2.3 Characterisation of gonadotropin receptor signalling in GLCs

3.2.3.1 Characterisation of LH and FSH induced MAPK and PI3K activation in GLCs

The Ras-RAF ERK pathway can be activated by different stimuli and represents signal generation from distinct G proteins. $G_{\alpha s}$, $G_{\alpha q/11}$, $G_{\beta\gamma}$ and arrestins can contribute to ERK 1/2 phosphorylation. The $G_{\beta\gamma}$ heterodimer couples to PI3K and activates (phosphorylates) AKT. I measured phosphorylation of ERK 1/2 and AKT at different time points after LH and FSH addition in control GLCs, in order to assess which time points should be used to compare signalling in control and PCOS GLCs (Chapter 4) and to compare signalling with and without androgens (Chapter 5). GLCs on day 4 in culture were treated with LH or FSH 10nM for 5, 15, 30 and 60 minutes. Phosphorylation of ERK 1/2 and AKT was determined by western blot. GAPDH was used as a loading control.

A significant increase in pERK 1/2 signal intensity occurred after 5, 15 and 30 minutes following LH and FSH treatment (Figure 3.4 A, B).

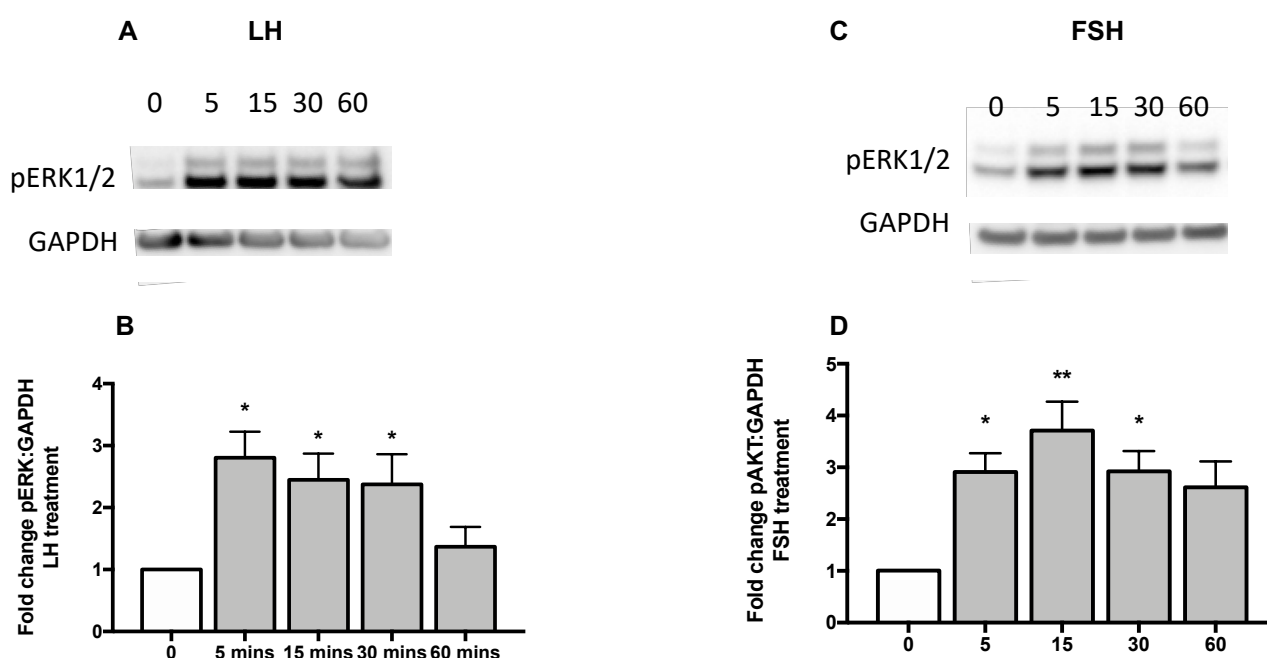


Figure 3.4 Time course of LH and FSH induced ERK 1/2 phosphorylation. **A** Representative western blot stained for pERK 1/2. GAPDH is used as loading control. GLC were treated with LH 10nM for 0, 5, 15, 30, 60 minutes. **B** There is a similar significant increase in pERK1/2 after LH treatment for 5, 15 and 30 minutes. The highest signal intensity is after 5mins LH treatment (ns) ($n=4$, mean $\pm$ SEM). **C** Representative western blot stained for pERK 1/2. GAPDH is used as loading control. GLC were treated with FSH 10nM for 0, 5, 15, 30, 60 minutes. **D** There is a significant increase in pERK1/2 after FSH treatment for 5, 15 and 30 minutes. ($n=5$, mean $\pm$ SEM). Kruskal Wallis test.

Overall LH and FSH treatment did not consistently or significantly increase pAKT signal intensity in GLCs (Figure 3.5). Half of samples treated with FSH (4 of 8) and LH (3 of 6) did show an increase in pAKT signal intensity after LH or FSH treatment but the other half did not. The increase in pAKT in these samples was modest (less than 2-fold increase from basal).

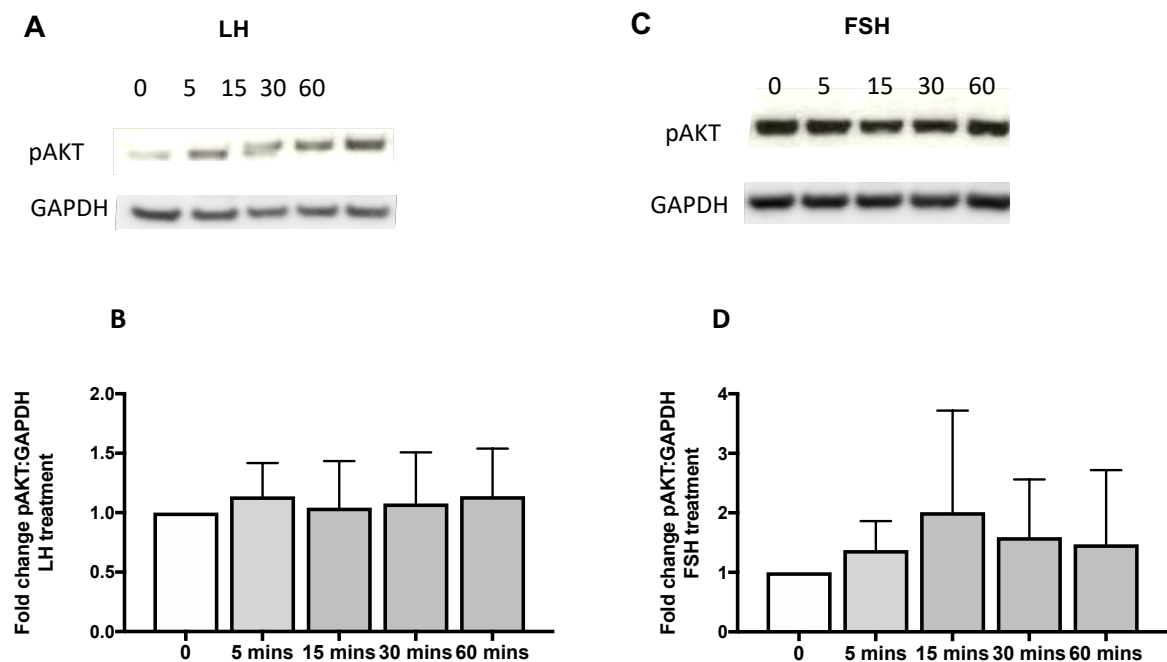


Figure 3.5 Time course of LH and FSH induced AKT phosphorylation. **A** Representative western blot stained for pAKT. GAPDH is used as loading control. GLC were treated with LH 10nM for 0, 5, 15, 30, 60 minutes. **B** There is no significant increase in pAKT after LH treatment for 5, 15 and 30 minutes, ($n=8$, mean $\pm$ SEM). **C** Representative western blot stained for pAKT. GAPDH is used as loading control. GLC were treated with FSH 10nM for 0, 5, 15, 30, 60 minutes. **D** There is no significant increase in pAKT after FSH treatment for 5, 15 and 30 minutes ($n=6$, mean $\pm$ SEM), *kruskal wallis* test.

3.2.3.2 Calcium signalling in GLCs

It is established that one of the key physiological G protein pathways activated by LH/LHR, is G α q/11, leading to activation of PLC and increase in inositol phosphates and intracellular Ca²⁺. LHR induces G α q/11-Ca²⁺ signalling only under high receptor and high hormone concentrations (Breen et al., 2013). GLCs were treated with a Ca²⁺ indicator dye, Fluo4-AM, for 1 hr. and imaged

live via confocal microscopy. LH 100nM was administered live. LH induced calcium mobilisation in a subpopulation of GLCs (Figure 3.6) however this response was not consistent, despite experimental conditions being similar.

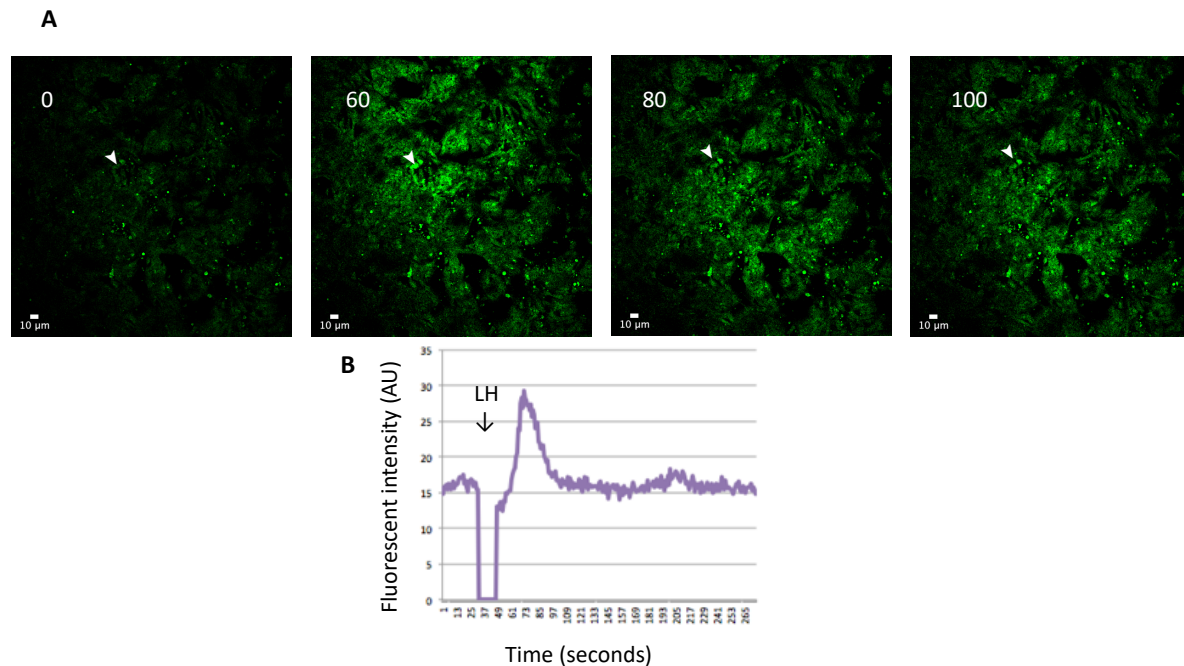


Figure 3.6 GLCs induce intracellular calcium in response to LH. GLCs were cultured in glass dishes. On day 4 in culture they were incubated with fluorescent calcium inhibitor Fluo4-AM for 1 hr and imaged live via confocal microscopy for 30 seconds before LH 100nM was added. A Representative time-lapse confocal images showing ‘wave’ of calcium signalling over 1 minute after LH administration (0, 60, 80, 100 = time in seconds). Arrow highlights fluorescence in individual cells. **B** Fluorescent intensity plot from video A following LH stimulation, expressed in arbitrary units (AU).

Gαq/11 mediated signalling can also be measured by accumulation of inositol monophosphate (IP-1), a downstream metabolite of inositol 1,4,5-triphosphate (IP-3). On day 4 in culture GLCs were treated with LH 100nM for 2 hours. LH consistently resulted in a significant increase in IP-1 (Figure3.7).

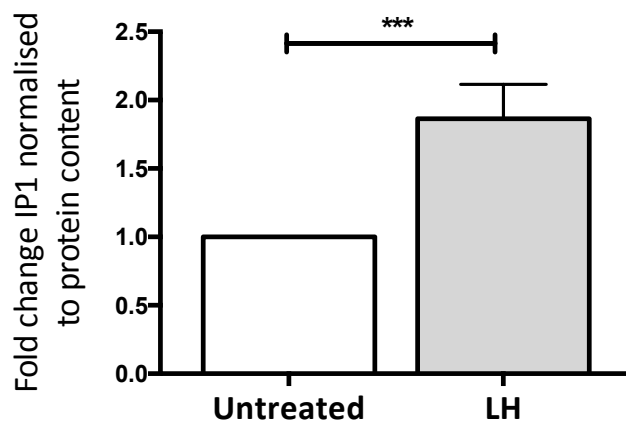


Figure 3.7 IP1 accumulation after incubation with LH 100nM for 2 hours ($n=7$, $p<0.001$, Mann Whitney test, mean $\pm$ SEM).

3.2.4 Approaches to studying gonadotropin receptor trafficking in GLCs

Gonadotropin receptor internalisation and trafficking has been studied in cell lines using a ‘tagged’ receptor model, for example FLAG tagged receptor DNA construct (Jean Alphonse et al, 2014).

3.2.4.1 Transfection of GLCs

In order to study receptor trafficking and internalisation in GLCs, I therefore first aimed to transfect GLCs with APPL1-GFP. This plasmid has been used successfully and easily in distinct cell lines by the Hanyaloglu lab and others (Broussard et al., 2012). I transfected it in GLCs using different methods (see chapter 2), different concentrations of plasmid DNA and using varying cell confluence, however the transfection was consistently unsuccessful.

3.2.4.2 Conjugation of LH to 488 to visualise receptor trafficking

As it was not possible to transfect GLCs, I sought out other tools through which I could visualise the fate of gonadotropin receptors. I used a conjugation kit (AlexaFluor 488) to label LH with a fluorescent dye (see chapter 2 for method). Given that LH is thought to remain attached to its receptor during the trafficking process (Lyga et al., 2016), this would allow visualisation of LHR in GLCs. I tested the LH 488 on HEK293 cells stably expressing LHR. LH488 10nM was administered for between 1 and 4 hours at both 37 and 4 degrees. HEK cells were then fixed, DAPI was used to stain nuclei and cells were imaged using confocal microscopy. HEK293 cells incubated with LH488 at 4 degrees (to prevent receptor internalisation) (Figure 3.8A) showed clear cell membrane localisation of LH. HEK293 cells incubated with LH488 at 37 degrees (Figure 3.8B) showed localisation of LH to both cell membrane and intracellular compartments, likely endosomes (Figure 3.8B). The LH488 was also tested to ensure it remained active after the conjugation process. It was added to HEK293 F-LHR cells for 5 minutes in parallel with regular LH and cAMP was the same for both LH488 and LH, demonstrated that the LH488 is still functional (Figure 3.8C).

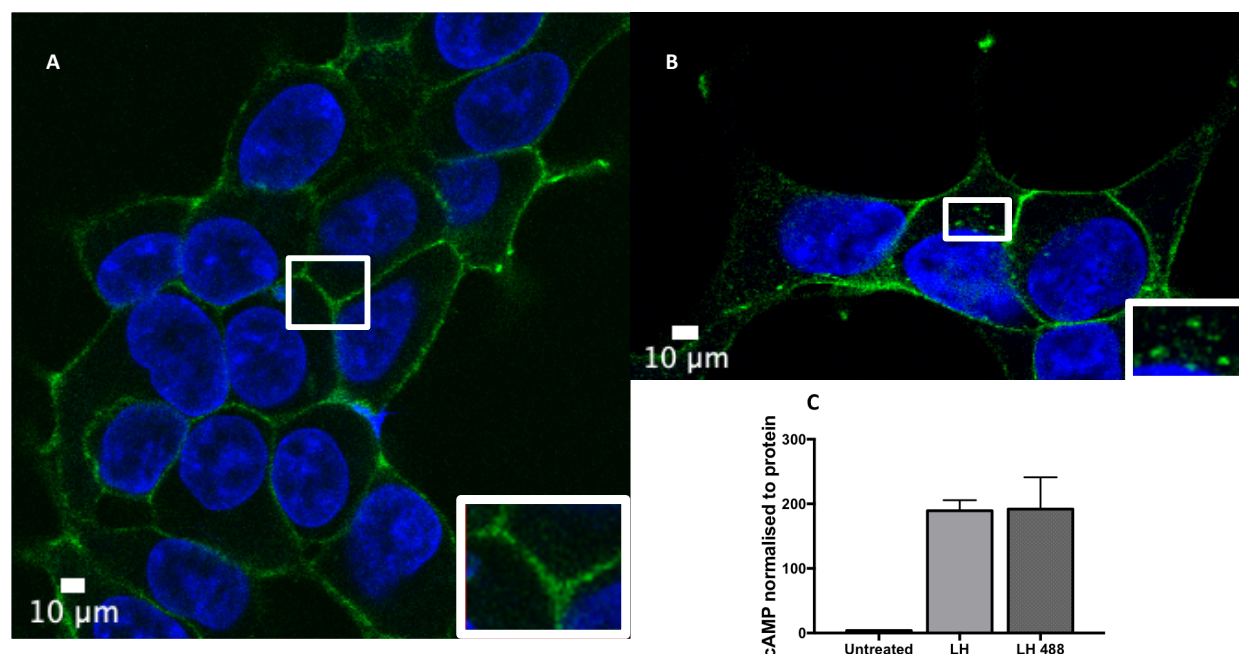


Figure 3.8 LH conjugated to 488 and incubated with HEK293 F-LHR cells. **A** HEK293 F-LHR cells incubated with LH488 10nM for 3 hours on ice. Incubation on ice prevents receptor internalisation of receptor and highlights membrane bound LH. Nuclei stained with DAPI. Scale bar = 10 μ M. **B**

HEK293 F-LHR cells incubated with LH488 for 3 hours at 37 degrees. LH488 is visible at the cell membrane and internalised to endosomes. Scale bar = 10 μ M. C LH 488 is active and incubation with LH488 10nM for 5 mins stimulates cAMP release in HEK293 FLHR cells, the same quantity as LH. Values are mean $\pm$ SEM.

3.2.4.3 LH488 co-localises with markers of very early endosomes (APPL1) and endosomes (EEA1) in HEK293 F-LHR cells.

After testing the activity and location of LH488, I then measured co-localisation of LH488 with VEE marker (EEA1) and EE marker (EEA1) at different time points. Cells were fed live with LH488, fixed, permeabilised and stained with anti-APPL1 or anti-EEA1 primary antibodies followed by anti-rabbit AlexaFluor 555 and DAPI and imaged with confocal microscopy. 15 minutes after LH treatment 30% of LH488 colocalised with APPL1, and this reduced to 20% after 30 minutes and 15% after 45 minutes, suggesting that LH (and LHR) is trafficked to the VEE but dissociates from the VEE over time. After all time points of LH488 treatment, co-localisation with EEA1 remained high (approximately 80%), suggesting that LH may dissociate from LHR, which is known to traffic predominantly to VEE (Sposini et al., 2017).

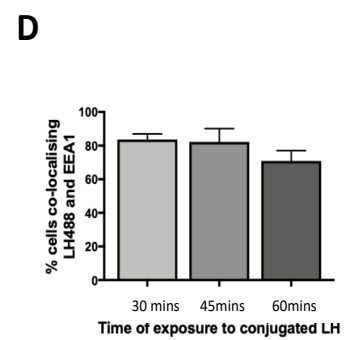
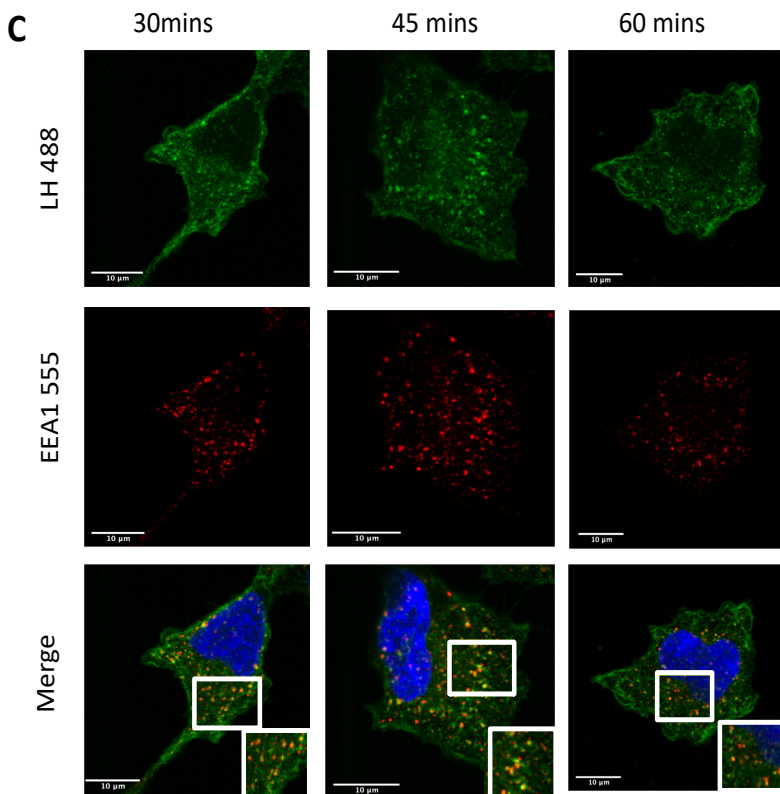
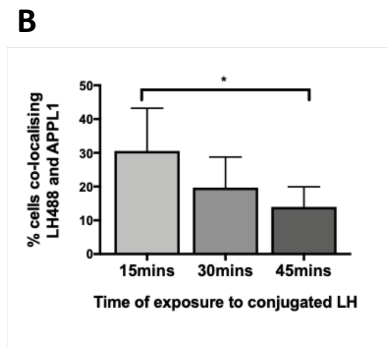
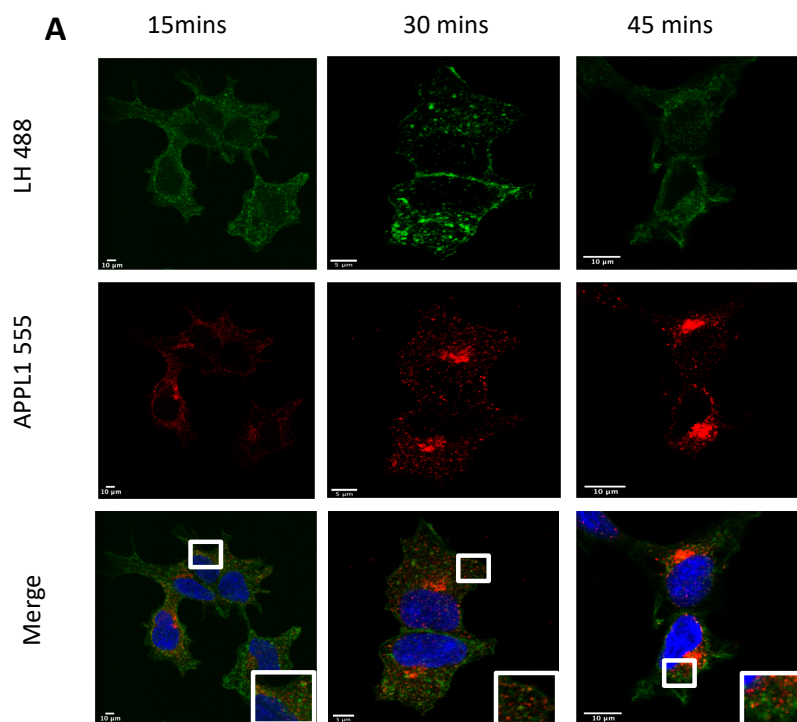


Figure 3.9 LH488 co-localises with EEA1 and APPL1 in HEK293 FLAG-LHR cells. **A** HEK293 cells were incubated with LH488 for 15, 30 and 45 minutes and then fixed permeabilised and stained with APPL1 (and Alexafluor 555) Scale bar = 10 μ M. **B** Quantification of LH488 and APPL1 positive endosomes at 15, 30 and 45 minutes. Co-localisation with APPL1 reduced with longer LH488 incubation time. **C** HEK293 cells were incubated with LH488 for 30, 45 and 60 minutes and then fixed permeabilised and stained with EEA1 (and Alexafluor 555) Scale bar = 10 μ M. **D** Co-localisation with EEA1 was 70-80% and did not change significantly between time points. N=10-15 cells per condition, collected across 2 independent experiments. Mann Whitney test, values are mean $\pm$ SEM.

3.2.4.4 LH488 in GLCs

After optimising LH 488 in HEK293 FLAG LHR cells, it was tested in GLCs. However, unlike in the HEK293 cells, LH was not clearly visible at the cell membrane or intracellularly when imaged with confocal microscopy (Figure3.10A). The GLCs were incubated with LH 488 for between 15 minutes and 4 hours at both 4 and 37 degrees. Cell were imaged both live and after fixation. GLCs were also fixed, permeabilised and stained with anti-APPL1 antibody and imaged via confocal microscopy (Figure3.10B). APPL1 specific staining was clearly visible in GLCs, however co-localisation studies were not possible due to lack of LH staining.

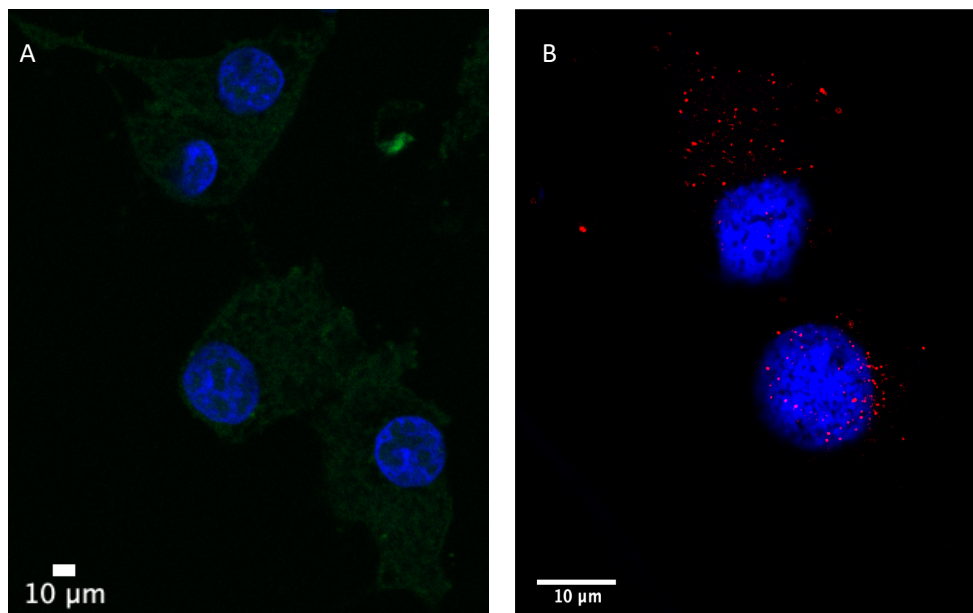


Figure 3.10 GLCs incubated with LH488 or stained with APPL1. **A** GLCs were incubated with LH 488 and fixed and stained with DAPI. LH488 is not clearly visible at cell membrane or in endosomes in GLCs. **B** GLCs were fixed, permeabilised and stained with APPL1 and AlexaFluor 555 and imaged using confocal microscopy. Specific staining for APPL1 is clearly visible. Scale bar = 10 μ M.

3.2.5 Approaches to studying the role gonadotropin receptor internalisation in signalling

3.2.5.1 Gonadotropin receptors in GLCs are largely dependent on internalisation to stimulate cAMP release and for steroidogenesis

Most GPCRs do not depend on receptor internalisation for signal generation and can signal from the plasma membrane. This has not been studied in human ovarian tissue expressing endogenous LHR and FSHR. Dyngo-4a, a dynamin inhibitor, blocks internalisation of various GPCRs (Bowman et al., 2016, Robertson et al., 2014, Tsvetanova and von Zastrow, 2014) and therefore is used to study the necessity for receptor internalisation. On day 4 in culture, GLCs were pre-treated for 45 minutes with Dyngo-4a and then LH or FSH 10nM for 5 minutes. Dyngo-

4a reduced the cAMP signal by 60-70% (Figure 3.11 A, C), suggesting that cAMP production is largely but not completely dependent on receptor internalisation. When cells were also treated with IBMX the reduction in cAMP with Dyngo-4a was only approximately 50%. This difference in Dyngo-4a sensitivity may be due to the plasma membrane derived cAMP accumulating in the presence of IBMX, and so the Dyngo-4a effect is not as apparent.

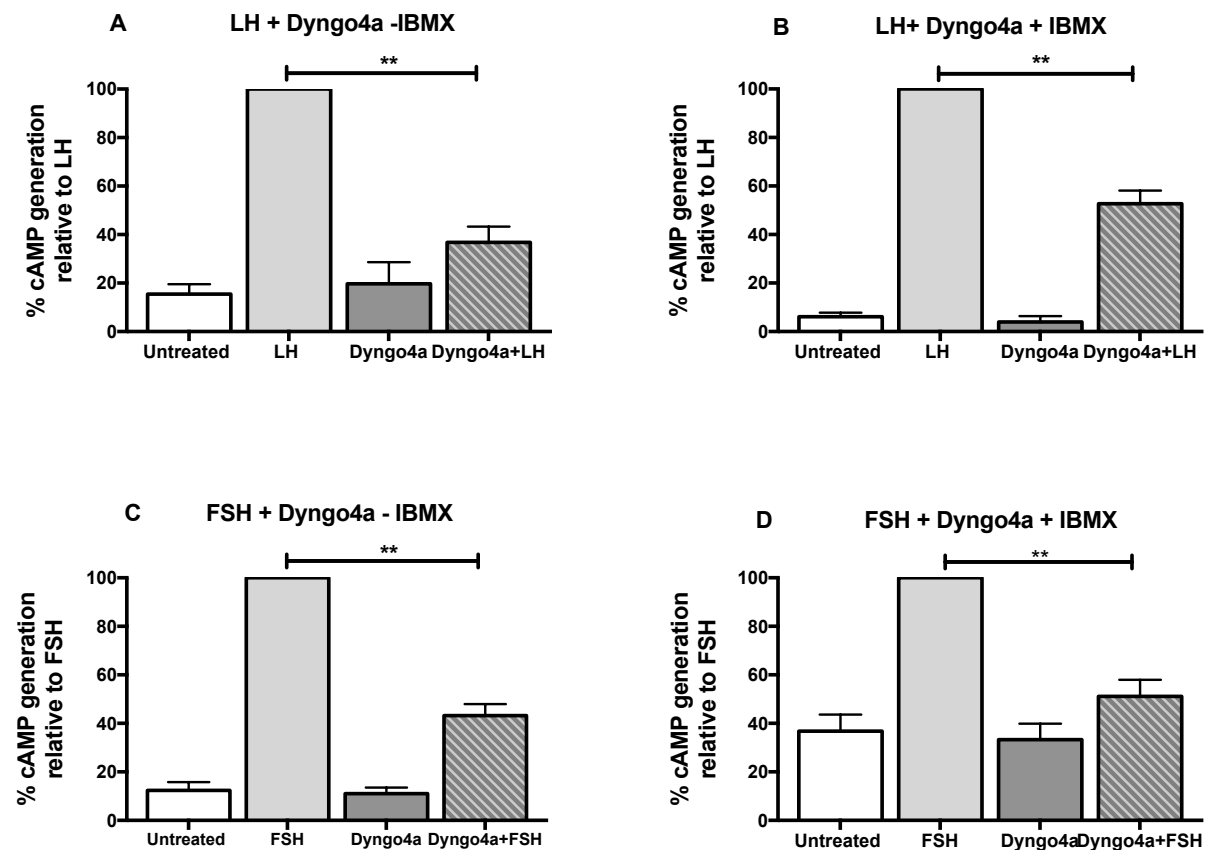


Figure 3.11 Inhibition of receptor internalisation inhibits cAMP generation in response to LH or FSH. A Dyngo-4a reduced cAMP production in response to LH by $74\% \pm 13$ ($n=4$) (<0.01). **B** Dyngo-4a in the presence of IBMX reduced cAMP production in response to LH by $48\% \pm 12$ ($n=5$) (<0.01). **C** Dyngo-4a reduced cAMP production in response to FSH by $57\% \pm 10$ ($n=4$) (<0.01). **D** Dyngo-4a in the presence of IBMX reduced cAMP production in response by $49\% \pm 15$ ($n=5$) (<0.01). Mann Whitney test used for all, values are mean $\pm$ SEM.

Given that Dyngo-4a prevents the majority of receptor internalisation and downstream cAMP synthesis, I examined the downstream effect of this inhibition on steroidogenesis. GLCs were treated on day 4 with Dyngo-4a for 1 or 24 hours, with or without LH 10nM for 24 hours, or LH alone. DMSO was used as a vehicle control. Dyngo-4a alone and in combination with LH appeared to reduce *LHCGR*, *FSHR* and *HSD3B2* (Figure 3.12 A, B, E), however these changes were not significant. There was also a trend towards inhibition of the LH-induced increase in *STAR* and *CYP11A1* (Figure 3.12 C, D). Again, these values are non-significant (the number of replicates for this experiment was small; $n=4$), but there may be a pattern of Dyngo-4a inhibition of steroidogenesis and receptor downstream function.

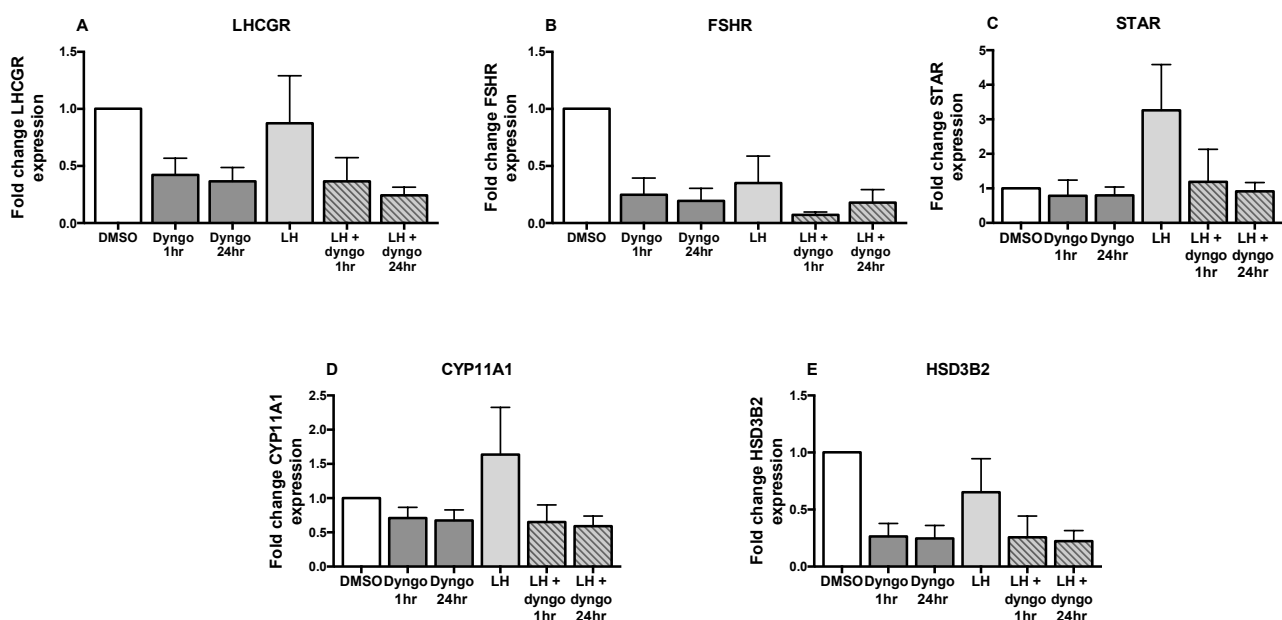


Figure 3.12 Steroid gene expression in GLCs pre-treated with Dyngo-4a for 1 or 24 hours ($n=4$, mean $\pm$ SEM). GLCs were treated with DMSO, LH 10nM, Dyngo-4a for 1 or 24 hours, and Dyngo-4a for 1 or 24 hours with LH 10nM. **A** Dyngo-4a alone did not significantly reduce LHCGR or inhibited the LH induced change in LHCGR at both 1- and 24-hours treatment. **B** Dyngo-4a alone did not significantly reduce FSHR or further reduce the LH induced reduction of FSHR at both 1- and 24-hours treatment. **C** Dyngo-4a alone did not affect STAR expression or significantly inhibit the LH induced increase in STAR at 1 and 24 hours. **D** Dyngo-4a alone did not affect CYP11A1 expression or significantly inhibit the LH induced increase in CYP11A1 at 1 and 24 hours. **E** Dyngo-

4a did not significantly reduce HSD3B2 expression, both alone or in combination with LH. Kruskal Wallis test (ns).

3.3 Discussion

The data presented in this chapter highlights the important utility, but also the limitations, of studying GLCs *in vitro*. It also demonstrates some of the careful consideration and optimisation processes that were necessary to prepare these cells for functional studies. Lastly, it defines some of the important regulatory processes in gonadotropin receptor function and signalling that have been studied previously in cell lines but not in human GLCs.

3.3.1 GLCs as a model for studying gonadotropin receptor function

GLCs have several advantages as a model for studying gonadotropin receptor function. They are easily obtainable as a 'waste product' of oocyte retrieval for IVF. They express both LH and FSH receptors and remain responsive to gonadotropins *in vitro*. As show in Figure 3.3 LH treatment *in vitro* in GLCs stimulates cAMP accumulation, an increase in *LHCGR* and *STAR* expression, and progesterone production. FSH treatment *in vitro* stimulates cAMP production and downregulates *FSHR* expression. However, *FSHR* expression is much lower than in GCs from hSAFs (see Figure 5.2). However it is difficult to obtain tissue from human small antral follicles which are highly FSH responsive, and these cells have much lower expression of *LHCGR* (Figure 5.2) and neither do they have LH receptor activity under normal conditions (Willis et al., 1998).

One of the major limitations to the use of GLCs is that as part of the IVF cycle, women receive multiple treatments which could affect gonadotropin receptor expression and function. These include FSH, hCG, GnRH agonists and antagonists. In addition, due to the risk of ovarian hyperstimulation in PCOS (Delvigne et al., 1993, Delvigne and Rozenberg, 2002a, Delvigne and Rozenberg, 2002b), these women often receive lower doses of FSH and GnRH agonist to trigger oocyte maturation, as opposed to hCG. Therefore, it is important to minimise the possible confounding effects of exogenous treatments.

I have shown that gonadotropin receptor function is restored when GLCs are left in culture. Cyclic AMP response to LH peaks after 4 days in culture (Figure 3.1),). I therefore chose to use day 4 for experiments. Steroid enzyme and gonadotropin receptor gene expression do not significantly change in culture, but this is likely because of the small number of samples used in this experiment. While most studies utilising GLCs do not directly address this in their experimental protocol, it is critical to consider the effect of exogenous medication on gonadotropin receptor function. One previous study compared day 3 with day 6 in culture and found higher cAMP production in response to LH and hCG on day 6 (Casarini et al., 2012). Another study showed that after 3 days in culture, addition of FSH resulted in a dose and time-dependent increase in GLC oestrogen production (Foldesi et al., 1998). Wickings et al showed that progesterone accumulation and aromatase activity in GLCs in response to FSH peaked at day 4 in culture and in response to LH had recovered by day 4 but was maximal at day 6 (Wickings et al., 1986). Overall these studies are consistent with my observation that GLCs recover their responsiveness to gonadotropins over time in culture.

Recombinant FSH has a relatively short half-life (elimination half-life is approximately 1 day (le Contonnec et al., 1994)) and is given only until the oocyte maturation trigger (36 hours before oocyte retrieval), therefore while FSH may have some impact on gonadotropin receptor function, it is likely to have less of an effect than other factors. While GnRH antagonists (median terminal half-life 20-80 hrs (Duijkers et al., 1998) also have a similarly short half-life, ovulation is prevented for an average of 5 days using GnRH antagonist, so those given >36 hours prior to oocyte retrieval may still exert an effect in GLCs for several days. GnRH agonists are used as maturation triggers in those at high risk of OHSS and have an elimination half- life of approximately 80 minutes (Kuhl et al., 1984). Serum gonadotropins return to normal within 24 hours of administering the GnRHa buserelin (Fauser et al., 2002), therefore while it may have some impact on gonadotropin receptor function, it is likely to be relatively less than hCG.

hCG is the biggest concern in terms of its potential to suppress gonadotropin receptor function. It is the most commonly used trigger of oocyte maturation and has an elimination half-life of 30 hours. Serum LH levels only return close to baseline by day 6 after administration of hCG

(embryo transfer day) and are completely back to normal if measured a week later (Fauser et al., 2002). hCG affects both LH and FSH receptor expression, therefore explaining why resensitisation of both receptors takes several days *in vivo*. Day 4 in culture, which is equivalent to day 6 post administration of hCG, was therefore chosen as the day to perform GLCs involving gonadotropins.

Another major limitation for using GLCs as a model for studying receptor internalisation and trafficking is the difficulty in tagging/visualising the receptor. I was unable to establish plasmid transfection in the GLCs, therefore was unable to insert a fluorescent tagged receptor plasmid in order to study receptor trafficking. There are several reasons why transfection may not have been successful. Firstly, GLCs clump *in vitro*, making the confluent cells which are ideal for transfection difficult to obtain. Secondly GLCs are terminally differentiated, i.e. do not proliferate and cannot be used to continuous culture. If the cell does not divide, access of the plasmids to the cell nucleus is largely blocked. There have been minimal studies demonstrating successful transfection in GLCs (Chen et al., 2010, Kiriakidou et al., 1996), likely for the above reasons. Future attempts could perhaps use SiRNA transfection or CRISPR-Cas9 delivery.

I conjugated LH to a 488 fluorescent dye, and was able to visualise cell membrane and endosomal bound LH (Figure 3.6) in HEK293 FLAG-LHR cells, as well as being able to co-localise LH with markers of the VEE (APPL1) and EE (EEA1) at different time points. I showed that LH tracks with the receptor to the VEE and this reduces over time (Figure 3.7). These data are similar to data from the Hanyaloglu lab using Flag to visualise LHR (also in HEK293 FLAG LHR cells). However, EE co-localisation was higher than has been previously shown in HEK293 FLAG LHR cells, suggesting that some LH may dissociate from the LHR and traffic to the EE. It would be fascinating to study this in more detail, however this conjugated LH was poorly visible in GLCs, likely because of the lower endogenous LHR expression in GLCs compared to HEK293 FLAG-LHR, in which the receptor is overexpressed. I also attempted to use antibodies to the LHR and FSHR however neither showed consistent specific staining at IHC and multiple non-specific bands at western blot (data not shown).

3.3.2 Gonadotropin receptor signalling and trafficking in GLCs

I have optimised signalling assays to characterise and measure the pathways involved in gonadotropin receptor signalling in 'control' GLCs, i.e. those from women without PCOS, in order to both contribute to our understanding of gonadotropin receptor signalling and to enable using these assays to compare GLCs from women with PCOS.

I measured gonadotropin induced MAPK signalling over time using pERK1/2 signal intensity on western blot (Figure 3.4). This has been described once previously in human GLCs for LH (Casarini et al., 2012) but not to the best of my knowledge, for FSH. These data will again facilitate a comparison with PCOS and establish kinetic parameters for the phosphokinase array. I also measured gonadotropin induced PI3K/AKT signalling over time using pAKT signal intensity at western blot (Figure 3.5). There was no consistent increase in pAKT after LH or FSH treatment. This may be because there is high basal pAKT, which makes it difficult to visualise ligand induced increases.

While $G\alpha_q/11$ signalling has been demonstrated for LHR I was unable to consistently measure calcium signaling in GLCs. This may be because this pathway depends on a high concentration of receptor (Breen et al., 2013) and endogenous receptor expression will vary from sample to sample. It may also be because it is recommended that cell confluence be approximately 70% for this technique, which as mentioned above is difficult to achieve given the natural clustering of GLCs. Therefore, I used IP-1 accumulation, which was consistently increased in GLCs after incubation with LH, as an alternative assay to demonstrate and measure $G\alpha_q/11$ signalling in GLCs. This assay is not affected by cell confluence and appears to be more consistent and sensitive in measuring LH induced changes in GLCs. FSHR mediated signaling is known to be predominantly through the $G\alpha_s$ /cAMP/PKA pathway, however $G\alpha_q/11$ signalling has been demonstrated for FSHR in cell lines with overexpressed FSHR, not in cells that endogenously express the receptor (Thomas et al., 2011).

The data in Figure 3.11 show that for LH and FSH receptor it appears as though internalisation is required for the majority of the acute cAMP response in GLCs. However it is important to consider that there may be off target effects or toxicity associated with Dyngo-4a which may affect cAMP production. This is the first time this requirement of gonadotropin receptor internalisation has been demonstrated in human ovary samples. In addition to LHR and FSHR, the only other receptor shown to require internalisation for acute cAMP response is the D1 dopamine receptor (Kotowski et al., 2011). Inhibiting receptor internalisation with Dyngo-4a also reduced gene expression of *LHCGR* and LH induced increase in *LHCGR*. In addition, Dyngo-4a treatment reduced *FSHR* and further reduced the LH induced downregulation of *FSHR*. It also inhibited LH induced upregulation of *STAR* and *CYP11A1* and basal and LH-induced increase in *HSD3B2*. This proves the critical importance of receptor internalisation for receptor signalling and downstream function, in this case steroidogenesis. It is unclear why Dyngo-4a would reduce basal (unstimulated) receptor expression. This may be due to an effect on constitutive receptor activity, or it may be a non-specific or off target effect, possibly acting through another receptor.

When gonadotropin receptors are internalised they are trafficked to the very early endosome (VEE), which is regulated by APPL1. Nothing is known about the downstream functions of the VEE. A role for APPL1 in regulating gonadotropin receptor action was demonstrated by Comim et al, who found that knockdown of APPL1 in bovine theca cells enhances LH-mediated androgen production (Comim et al., 2013a). In addition, knockdown of APPL1 in HEK293 cells increases LH-dependent cAMP signalling (Sposini et al., 2017).

3.3.3. Proposed model of gonadotropin receptor signalling in GLCS

Combining the data from this chapter with the known pathways involved in gonadotropin receptor signalling in GLCs and other cell types (Sposini and Hanyaloglu, 2018, Sposini et al., 2017, Jean-Alphonse et al., 2014, Casarini et al., 2012), the model below demonstrates gonadotropin receptor signalling and highlights what I have demonstrated occurs in GLCs. This will be used as a model to compare signalling pathways and mechanisms in PCOS in the next chapter.

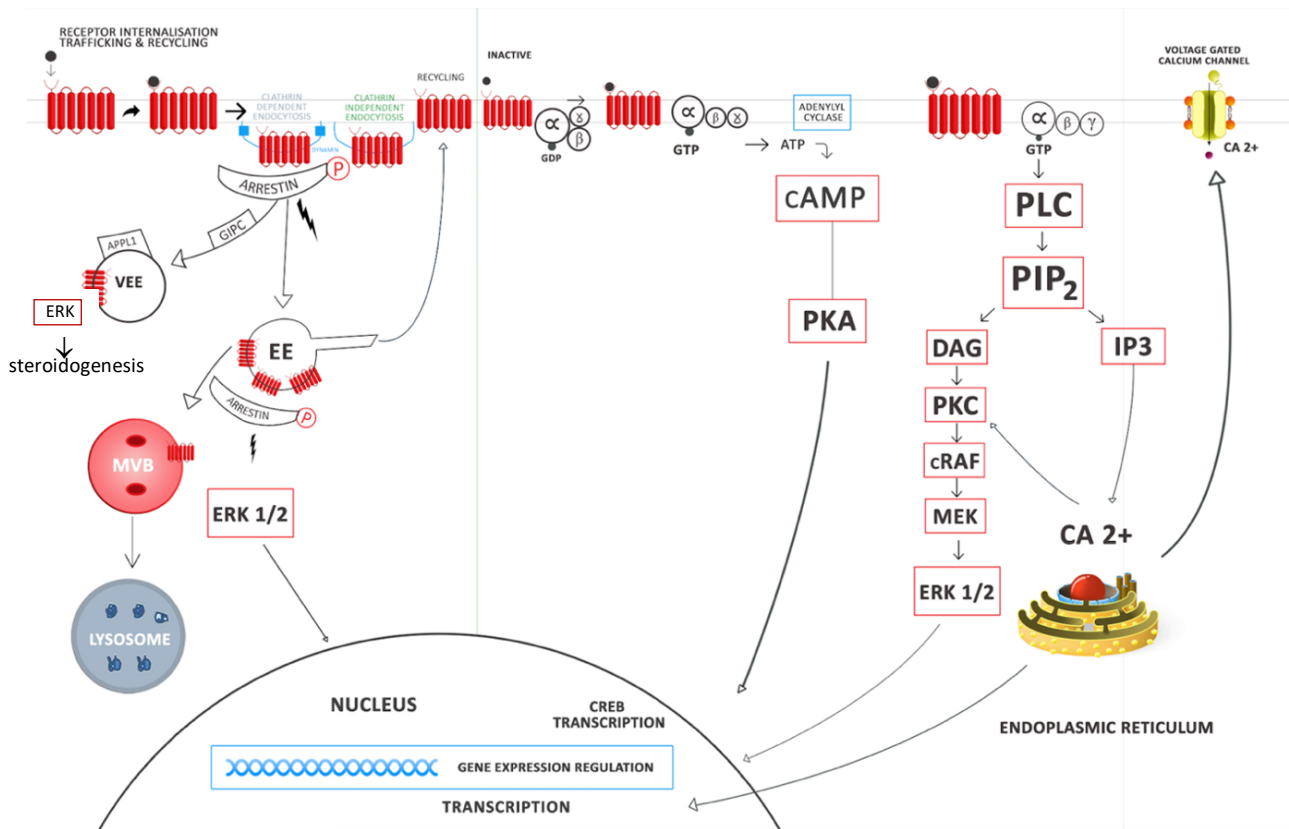


Figure 3.13 Proposed gonadotropin receptor signalling pathway in GLCs. After agonist binding, receptors undergo endocytosis via clathrin dependent or independent mechanisms. Clathrin coated pits (CCPs) allow accumulation of receptor/arrestin complexes that can activate arrestin-mediated signalling. After internalisation, receptors at the CCPs bind to the PDZ protein GIPC and are differentially sorted to distinct endosomal compartments, from where they can continue to activate or reactivate receptor signalling. The VEE, marked by APPL1, both sorts receptors for recycling to the plasma membrane and generates a sustained MAPK signalling response. Other receptors are trafficked to the EE, marked by EEA1, from where they are either recycled to the plasma membrane or degraded, leading to either signal resensitisation or signal termination. Activation of $G_{\alpha s}$ activates the cAMP-PKA pathway, $G_{\alpha q/11}$ activates the calcium signaling pathway (LHR only). $G_{\beta\gamma}$ activation of the PI3K pathway does not appear to consistently occur in GLCs.

3.3.4 Conclusions

GLCs are a good model for studying gonadotropin receptor signalling as they express both LH and FSH receptors and maintain responsiveness to both gonadotropins *in vitro*. Their limitations include the fact that the receptor function is likely to be impacted by exogenous treatment and that they are difficult to transfect or to visualise the receptor in order to study mechanisms such as receptor trafficking.

I have demonstrated for the first time that gonadotropin receptors in human ovary samples are dependent on receptor internalisation for signal generation and steroidogenesis. Both LH and FSH stimulates pERK 1/2 signal generation and LH stimulates calcium/Gαq11 signalling.

Chapter 4

Gonadotropin receptor signalling and function in granulosa lutein cells from women with Polycystic Ovary Syndrome

4.1 Introduction

The aetiology of PCOS remains unclear. Aberrant secretion and action of gonadotropins are implicated in the aetiology of follicle arrest but, to date, we have only limited knowledge about the precise mechanisms that are involved. Recent genome-wide association studies (GWAS) in PCOS women have identified polymorphisms in loci associated with gonadotropins and gonadotropin receptors (Chen et al., 2011, Hayes et al., 2015, Shi et al., 2012, Tian et al., 2016). The functional significance of these polymorphisms is unclear and thus represents a key area for further research. LHR protein and gene expression are increased in theca cells (TCs) from women with PCOS and these show an enhanced response of androstenedione, *in vivo*, to exogenous hCG (Jakimiuk et al., 2001, Comim et al., 2013b). GCs from SAFs lack functional LHR and *in vivo* treatment with LH has no effect on steroid production in GCs from hSAFs. However GCs in a sub-population of hSAFs taken from women with PCOS and treated with LH show “premature” responsiveness and steroid production (Willis et al., 1998). GCs from women with PCOS also show enhanced E2 production in response to FSH (Mason et al., 1994, Willis et al., 1996).

To date there has been no comprehensive study of gonadotropin receptor function and signalling in GLCs from women with PCOS. The complexity of GPCR signalling is increasingly being recognised; including pleiotropic coupling, ligand bias, endosomal signalling, and receptor crosstalk, all of which are relevant to LHR/FSHR function and may play a role in the pathogenesis of PCOS.

Several studies have measured progesterone production in PCOS GLCs, and have had varying results, from showing enhanced progesterone production (Rice et al., 2005), unchanged progesterone production (Adams et al., 2016), to reduced progesterone production (Huang et al., 2016). Studies have also measured steroid enzyme gene expression in fresh GLCs (Lerner et al., 2019) but these have not been measured in GLCs which have been left to recover in culture.

4.1.1 Specific aims

To determine whether there are differences between women with and without PCOS in gonadotropin receptor signalling pathways, receptor expression, steroid enzyme expression and progesterone production in GLCs.

4.2 Methods and Results

Having established the nature of LHR and FSHR signalling pathways in GLCs in chapter 3, I then utilised this data to compare receptor function in GLCs from women with PCOS.

4.2.1 cAMP production is increased in PCOS GLCs after LH but not FSH treatment

cAMP is the main second messenger generated in response to activation of LHR and FSHR. On day 4 in culture GLCs were treated with FSH or LH for 5 mins and cAMP was measured. The amount of cAMP accumulation after LH treatment was significantly higher in PCOS GLCs than in control GLCs (Figure 4.1A). There was no difference between groups in cAMP in GLCs treated with FSH (Figure 4.2B).

I previously showed (Figure 3.11) that FSHR and LHR in GLCs are dependent on receptor internalisation for the majority of cAMP signal generation. In order to assess if the enhancement in cAMP levels in response to LH in PCOS GLCs was related to differences in receptor internalisation I measured the level of inhibition of cAMP response when receptor internalisation was inhibited with Dyngo-4a and compared PCOS with control GLCs. Inhibiting receptor internalisation with Dyngo-4a resulted in a similar inhibition of cAMP generation in response to LH and FSH in both control and PCOS GLCs (60-70% inhibition) (Figure 4.1B, 4.2B).

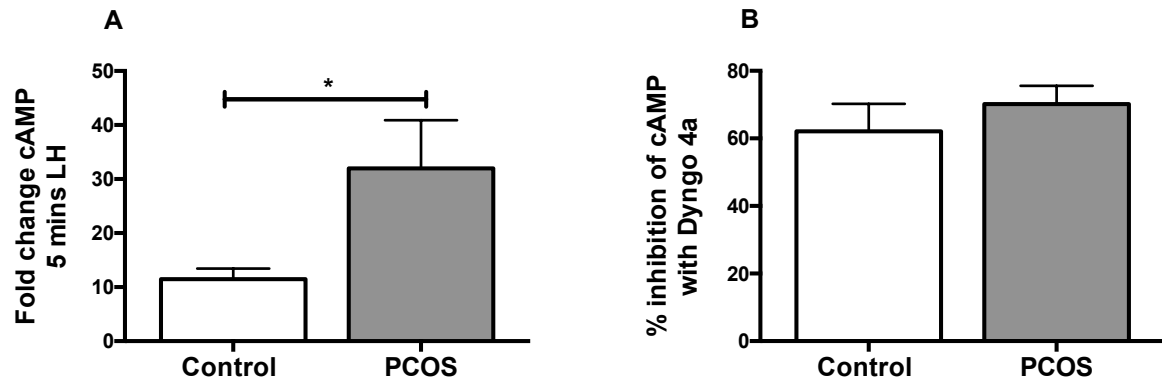


Figure 4.1 cAMP accumulation in control and PCOS GLCs in response to LH, with (B) or without (A) pre-treatment with Dyngo-4a. **A** GLCs were pre-treated with IBMX and then stimulated with LH 10nM for 5 mins. cAMP response was higher in GLCs from PCOS women ($p < 0.05$) (control $n = 16$, PCOS $n = 10$). **B** GLCs were pre-treated with Dyngo-4a 30 μ M for 45mins and then stimulated with LH for 5 mins ($n = 7$). Dyngo-4a inhibited 60-70% of cAMP response to LH and was not different between control and PCOS GLCs. Values are mean $\pm$ SEM.

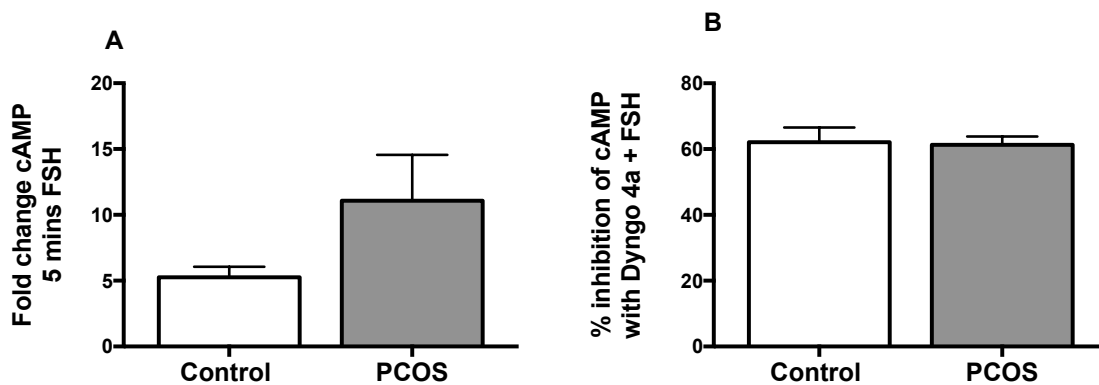


Figure 4.2 cAMP accumulation in control and PCOS GLCs in response to FSH, with (B) or without (A) pre-treatment with Dyngo-4a. **A** GLCs were pre-treated with IBMX and then stimulated with FSH 10nM for 5 mins. cAMP response was not significantly different in GLCs from PCOS or control women ($n = 16$ controls, $n = 7$ PCOS). Unpaired t-test, Values are mean $\pm$ SEM. **B** GLCs were pre-treated with Dyngo-4a and then stimulated with FSH for 5 mins ($n = 5$). Dyngo-4a inhibited 60-70% of cAMP response to FSH and was not different between control and PCOS GLCs. Mann Whitney test, values are mean $\pm$ SEM.

4.2.2 Trafficking protein gene expression is augmented in PCOS

Given that the cAMP response to LH stimulation is higher in PCOS GLCs I measured the gene expression of key proteins involved in trafficking of the LHR and its signalling after ligand binding. As discussed previously, GIPC targets LHCGR to the VEE, APPL1 is essential for LHCGR/FSHR recycling from the VEE and β -arrestins are involved in signal generation, termination, endocytosis and receptor recycling. There is a significantly higher gene expression of β -arrestin 2 (Figure 4.3 A) and GIPC (Figure 4.3B) in GLCs from women with PCOS. There is no significant difference in APPL1 expression in PCOS GLCs compared to controls. (Figure 4.3C).

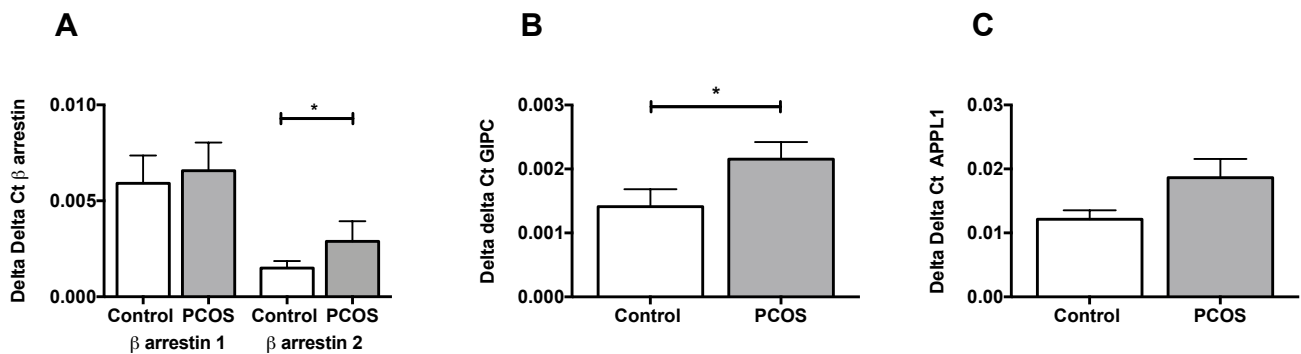


Figure 4.3 Gene expression of key receptor trafficking proteins in control and PCOS GLCs (Mean $\pm$ SEM). A β -arrestin-2 expression is significantly higher in PCOS than control ($p=0.03$). β -arrestin-1 expression is similar between PCOS and controls ($p=0.3$), (Control $n=9$, PCOS $n=12$, unpaired T -test). B GIPC gene expression is significantly higher in PCOS than control GLCs ($p=0.04$) Mann whitney test (Control $n=9$, PCOS $n=12$) C APPL1 gene expression is the same in PCOS and control GLC ($p=0.1$) (Control $n=9$, PCOS $n=12$, Mann-Whitney test).

4.2.3 $G\alpha q11$ signalling is similar between control and PCOS GLCs

LHR activates $G\alpha q11$ signalling in the presence of high physiological concentrations of receptor and ligand (Breen et al., 2013). As discussed in chapter 2, IP1 accumulation was used to measure $G\alpha q11$ signal activation as calcium response was not reliable in GLCs. IP1 is a stable downstream

metabolite of IP₃ induced by activation of a phospholipase C (PLC) cascade. There was no significant difference between control and PCOS GLCs however there was a trend towards lower IP₁ in PCOS GLCs.

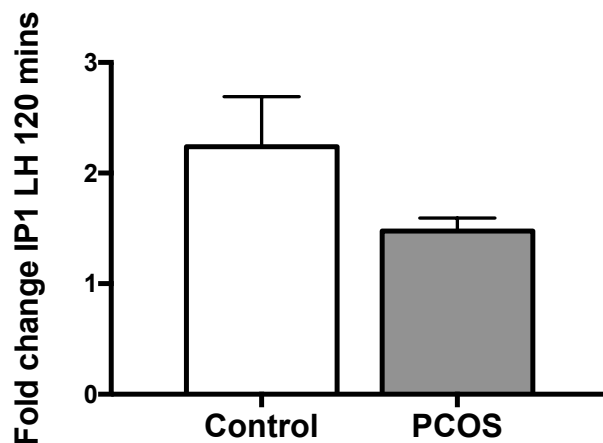


Figure 4.4 IP₁ accumulation in control and PCOS GLCs after LH treatment. GLCs were treated with LH 100nM for 2 hours and IP₁ accumulation measured ($n=9$ controls, $n=5$ pcos). There was no significant difference in IP₁ measured between control and PCOS GLCs. Mann Whitney test. Values are mean $\pm$ SEM.

4.2.4 MAPK/ERK signalling is not different between control and PCOS GLCs

The ERK pathway can be activated by different stimuli. G α s, G α q/11, G β γ and arrestins can contribute to ERK 1/2 phosphorylation, therefore pERK is a downstream marker of activation of multiple possible pathways. Control and PCOS GLCs were treated with LH or FSH and pERK 1/2 signal intensity was measured at western blot. There were no significant differences in the fold change increase in pERK1/2 signal intensity from the baseline intensity to ligand induced pERK1/2 between control and PCOS GLCs, although there was a trend towards higher pERK at 30 and 60mins LH treatment in the PCOS samples.

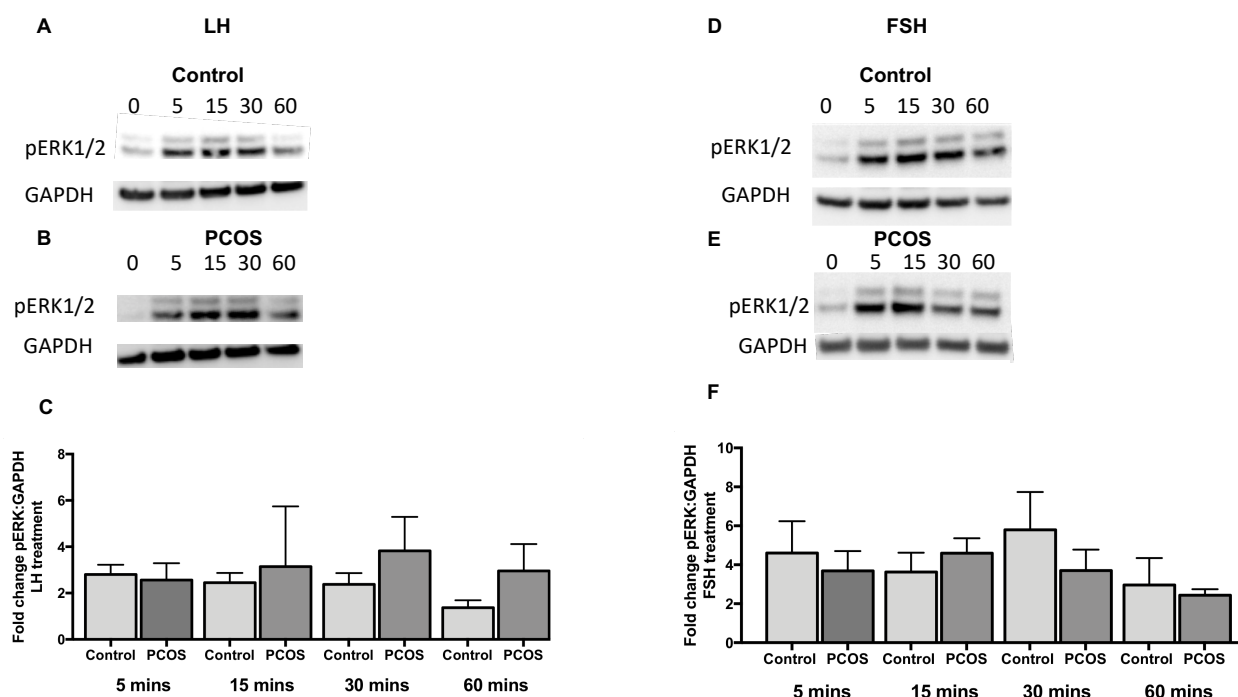


Figure 4.5 pERK signal intensity is similar in control and PCOS GLCs after LH and FSH treatment.

On day 4 in culture GLCs were treated with LH or FSH 10nM for 0, 5, 15, 30, 60 mins. pERK 1/2 signal intensity was measured via western blot. **A** Representative western blots demonstrating pERK1/2 signal intensity after LH treatment in a control GLC sample. **B** Representative western blots demonstrating pERK1/2 signal intensity after LH treatment in a PCOS GLC sample. **C** There were no significant differences in pERK 1/2 signal intensity between control and PCOS GLCs ($n=4$ in each group, Kruskal Wallis test, values are mean $\pm$ SEM) **D** Representative western blots demonstrating pERK1/2 signal intensity after FSH treatment in a control GLC sample. **E** Representative western blots demonstrating pERK1/2 signal intensity after LH treatment in a PCOS GLC sample. **F** There were no significant differences in pERK 1/2 signal intensity after FSH treatment between control and PCOS GLCs ($n=4$ controls, $n=6$ PCOS, Kruskal Wallis test, values are mean $\pm$ SEM).

4.2.5 PI3K/AKT signal activation by LH or FSH is not consistent in either control or PCOS GLCs.

The G $\beta\gamma$ heterodimer couples to PI3K and activates AKT. I showed in chapter 3 that LH and FSH stimulation of GLCs do not clearly increase pAKT production, although this may be because

baseline pAKT is high and therefore it is difficult to see ligand induced changes. In order to assess if there were any visible ligand induced changes in pAKT in PCOS GLCs I measured phosphorylation of AKT after LH and FSH treatment for 0, 5, 15, 30 and 60 minutes in control and PCOS GLCs. In PCOS GLCs there were no differences in fold change increase in pAKT after LH or FSH treatment compared to controls.

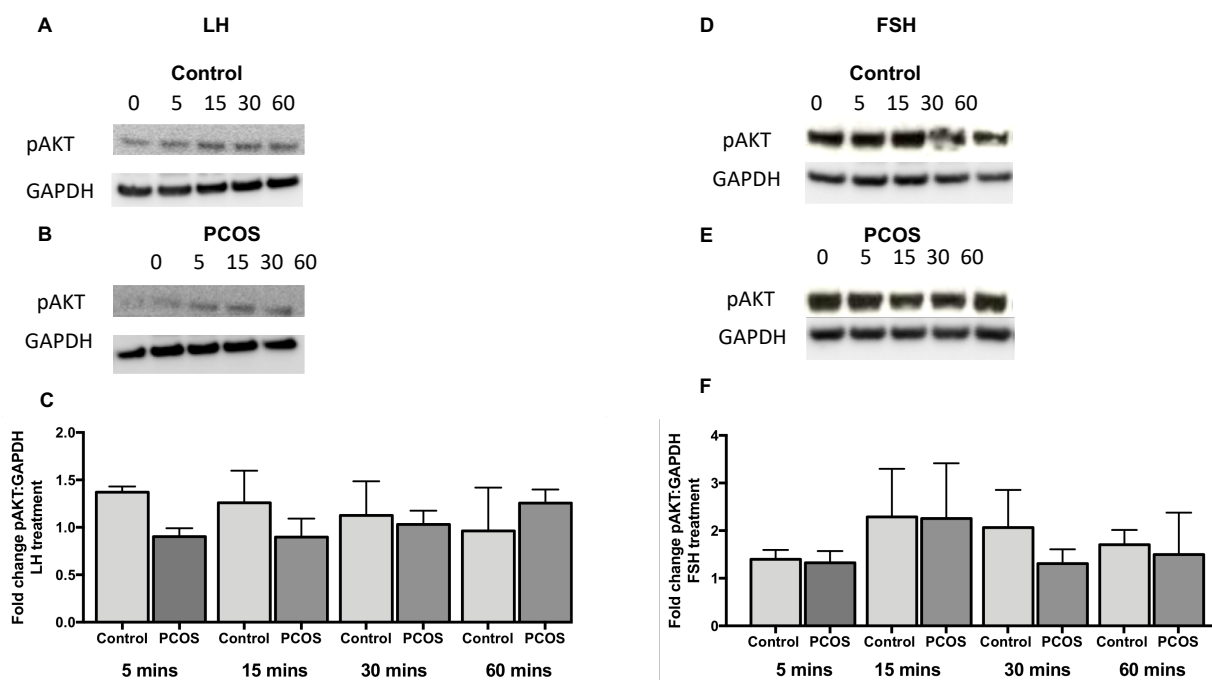


Figure 4.6 pAKT signal intensity is similar in control and PCOS GLCs treated with LH and FSH. On day 4 in culture GLCs were treated with LH or FSH 10nM for 0, 5, 15, 30, 60 mins. pAKT signal intensity was measured at western blot. **A** Representative western blots demonstrating pAKT signal intensity after LH treatment in a control GLC sample. **B** Representative western blots demonstrating pAKT signal intensity after LH treatment in a PCOS GLC sample. **C** There were no significant differences in pAKT signal intensity between control and PCOS GLCs ($n=4$ in each group, Kruskal Wallis test, values are mean $\pm$ SEM) **D** Representative western blots demonstrating pAKT signal intensity after FSH treatment in a control GLC sample. **E** Representative western blots demonstrating pAKT signal intensity after LH treatment in a PCOS GLC sample. **F** There were no

significant differences in pAKT signal intensity between control and PCOS GLCs ($n=4$ controls, $n=5$ PCOS, Kruskal Wallis test, values are mean $\pm$ SEM.).

4.2.6 Phosphokinase arrays in PCOS GLCs demonstrate altered protein phosphorylation at basal conditions and after LH and FSH treatment.

I have shown above that there is augmented cAMP response to LH in PCOS GLC, and augmented signalling/trafficking molecule expression. In order to assess what downstream effects this may be having, control and PCOS GLCs were treated with LH (and FSH) for 0, 5 and 30 minutes. Lysates from 3 samples were pooled and then used to probe phospho-kinase array membranes that detect 43 different phospho-kinases. Images of the membranes are shown in Appendix 1 (Figure A1.1 and A1.2). Fold change over untreated cells for control and PCOS at each time point was calculated and compared. An arbitrary 2-fold ratio change was chosen as the cut off to determine an increase or decrease in phosphorylation level.

In untreated GLCs from the 2 separate experiments there was a higher activation of P53 (3 different phosphorylation sites), P70 S6 kinase, P27 and AKT 1/2/3 (2 different phosphorylation sites), in PCOS GLCs compared to controls, and a lower expression of FYN, HCK and LYN (Figure 4.7).

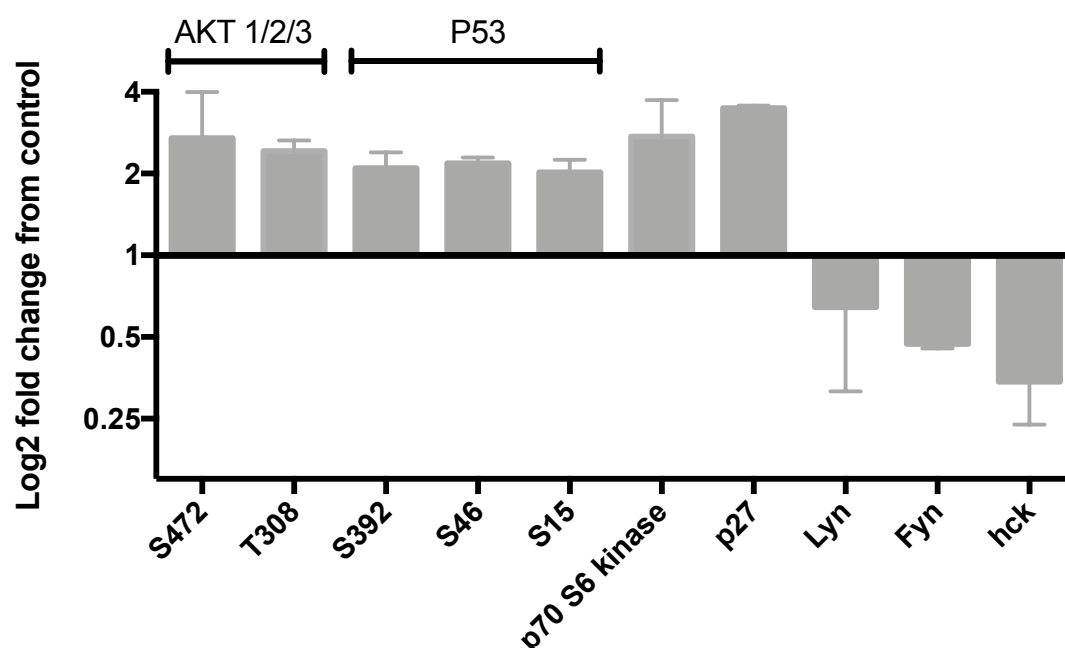


Figure 4. 7 Basal changes in phospho-protein levels in PCOS GLCs compared to controls.

Untreated GLCs from 2 separate experiments were used to probe phospho-kinase array membranes. There was a higher basal AKT 1/2/3 (On S472 and T308) p53 (On S392, S46, S15), p70 s6 kinase, P27 in PCOS GLCs compared to controls. There was a lower expression of FYN, HCK and LYN in PCOS GLCs compared to controls. Statistical analysis was not possible n numbers =2.

LH treatment in PCOS samples resulted in augmented expression of multiple downstream proteins. After both 5 and 30 mins 9 phospho-proteins had a higher increased over basal in PCOS compared to controls (Figure 4.8 A). Following 30 mins LH treatment 11 phospho-proteins increased over basal in PCOS compared to controls (Figure 4.8B). No phospho-proteins had a higher expression in PCOS at 5 mins only, i.e. they were higher at both 5 and 30 mins.

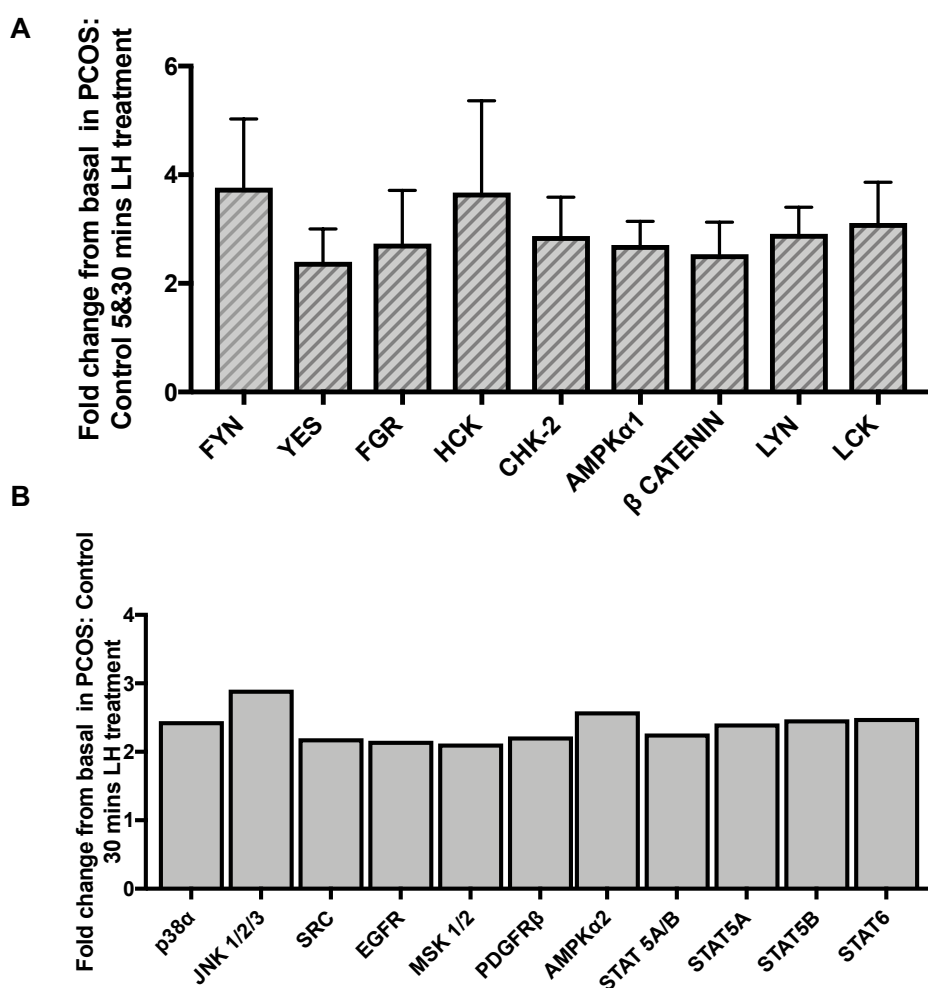


Figure 4.8 Fold change in phospho-expression after LH treatment for 5 mins and 30 mins over basal in PCOS GLCs compared to controls. On day 4 in culture GLCs were treated with LH 10nM for 0, 5 or 30 mins. Lysates from 3 control and 3 PCOS samples were pooled and were used to probe phospho-kinase array membranes. Signal intensity from 43 phospho-proteins was measured and compared to basal (untreated) cells. This fold change value was then used to compare PCOS with control. Statistical analysis was not completed as n numbers were 1-2. **A** 9 phosphoproteins (FYN, YES, FGR, HCG, CHK-2, AMPK α 1, β -catenin, LYN, LCK) had a higher expression over basal in PCOS samples compared to controls and both 5 and 30 mins LH treatment. Mean $\pm$ SEM. **B** 11 phospho-proteins (P38 α , JNK 1/2/3, SRC, EGFR, MSK 1/2, PDGFR β , AMPK α 2, STAT 5A, STAT 5B, STAT 5AB, STAT6) had a higher expression over basal in PCOS samples compared to controls after 30 mins LH treatment (but not 5 mins).

FSH treatment in PCOS samples resulted in both higher and lower expression of downstream proteins compared to control. After 5 mins FSH treatment 12 phospho-proteins had a higher increase from basal in PCOS compared to controls and 8 phosphoproteins had a bigger reduction from basal in PCOS compared to control (Figure 4.9). At 30 minutes FSH treatment there were no significant differences between PCOS and control samples.

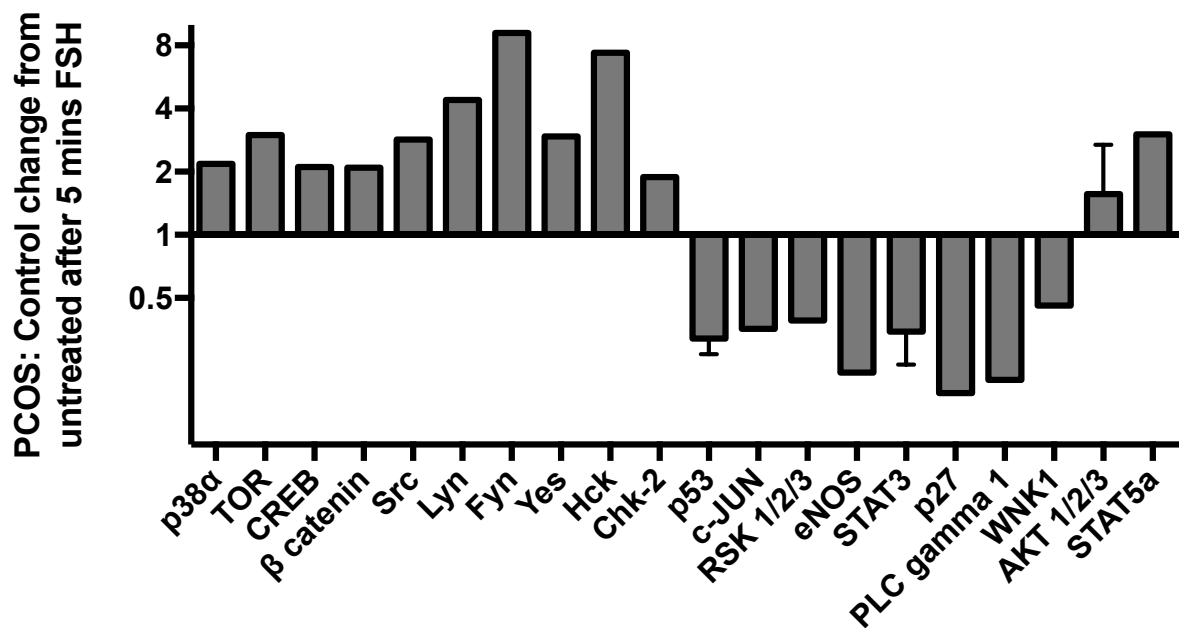


Figure 4. 9 Fold change in phospho-expression after FSH treatment for 5 mins over basal in PCOS GLCs compared to controls. On day 4 in culture GLCs were treated with FSH 10nM for 0 or 5 mins. Lysates from 3 control and 3 PCOS samples were pooled and were used to probe phospho-kinase array membranes. Signal intensity from 43 phospho-proteins was measured and compared to basal (untreated) cells. This fold change value was then used to compare PCOS with control. Statistical analysis was not completed as n numbers were 1-2. 12 phospho-proteins had a higher increase from basal in PCOS compared to controls (p38α, TOR, CREB, β catenin, SRC, LYN, FYN, YES, HCK, CHK-2, STAT5a, AKT 1/2/3) and 8 phosphoproteins had a bigger reduction from basal in PCOS compared to control (p53, c-JUN, RSK 1/2/3, eNOS, STAT3, p27, PLCγ1, WNK1).

Western blot was used to validate the above results, as the phosphoarrays were completed in pooled samples without repeats. Sample validation blots are shown in Figure 4.10. Basal levels of FYN were lower in PCOS samples compared to controls (Figure 4.10B), and the LH induced change from baseline was higher in PCOS GLCs compared to controls (Figure 4.10C). AMPKα was higher at baseline in PCOS compared to control and there was an LH induced increase, where there was none in the control sample (Fi. The WES automated capillary western immunoblot was kindly completed by Robin Willows in David Carling's group at Imperial College London, as this group study AMPK and had an optimised system for detecting and quantifying AMPK.

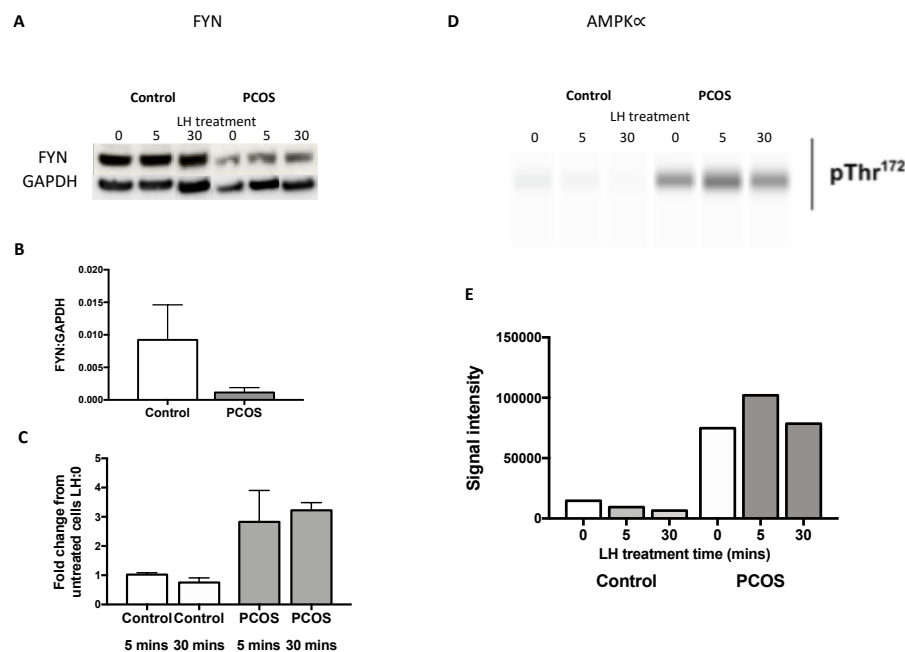


Figure 4. 10 FYN and AMPK α protein expression in control and PCOS GLCs. **A** Representative western blot showing higher FYN expression in untreated GLCs, and an increase in FYN expression with LH treatment in the PCOS but not the control sample. GAPDH is used as a loading control **B** FYN expression relative to GAPDH in control GLCs appears higher than in PCOS but the difference is not significant ($n=3$, Mann Whitney test, values are mean $\pm$ SEM). **C** LH treatment increases FYN expression from basal in PCOS but not control GLCs ($n=2$, ns, values are mean $\pm$ SEM). **D** AMPK α expression as measured via WES (capillary automated western immunoassay). AMPK α expression is higher in PCOS than control sample in untreated conditions and after 5 and 30 mins LH treatment. **E** AMPK α was higher at baseline in PCOS compared to control and there was an LH induced increase, where there was none in the control sample ($n=1$).

4.2.7 Gonadotropin receptor expression in control and PCOS GLCs on day of collection and after 4 days in culture

It has been reported that there is a higher protein expression of LHCGR in PCOS GLCs (measured after 4 days in culture) (Kanamarlapudi et al., 2016) but also has been reported that there is a lower *LHCGR* gene expression (measured after 1 day in culture) (Yang et al., 2015). While I did not see a significant increase in *LHCGR* in either fresh GLCs or those maintained in culture for 4 days (Figure 4.11), there was a consistent trend towards a higher expression in PCOS GLCs. The median in PCOS GLCs after 4 days in culture was double that of control GLCs. LH treatment *in vitro* stimulated an increase in *LHCGR* in control GLCs but a significant decrease in *LHCGR* in PCOS GLCs. This further suggests that activation and regulation of LHR signalling is altered in PCOS.

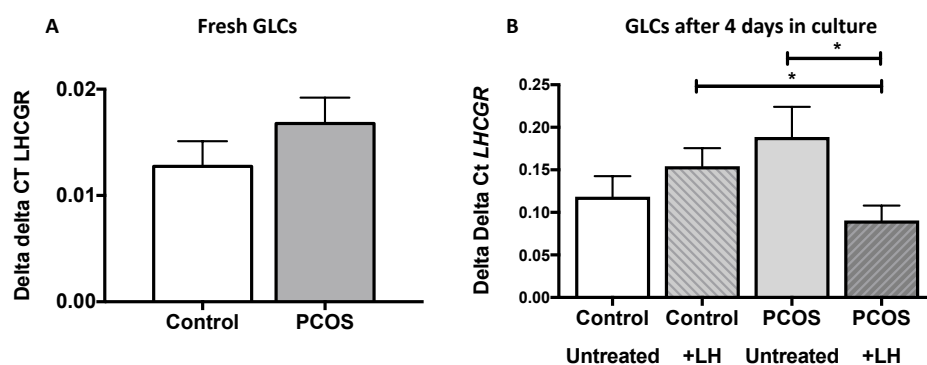


Figure 4. 11 LHCGR in GLCs on the day of collection (fresh GLCs) and after 4 days in culture.

GLCs were either frozen on day of collection (A) or were cultured and then treated with LH 10nM on day 4 for 24 hours (B). RT-qPCR was used to analyse gene expression. **A** There is no significant difference in LHCGR expression, $p=0.10$ ($n=10$ controls, $n=12$ PCOS) Mann Whitney test. **B** There was no significant difference in LHCGR between untreated control and PCOS GLCs ($p=0.10$), ($n=11$ controls, $n=7$ PCOS). LH treatment did not change LHCGR in control GLCs. LH treatment in PCOS GLCs significantly downregulated LHCGR ($n=11$ controls, $n=7$ PCOS) ($p<0.05$) Kruskal Wallis test. Values are mean $\pm$ SEM.

There are 4 known splice variants of the LHR. LHR-A is the full length receptor, LHR-B is lacking exon 9, LHR-C is lacking part of exon 11 and LHR-D is lacking both exon 9 and part of exon 11 (Minegishi et al., 2007, Nakamura et al., 2004). They are implicated in certain diseases but have not been examined in PCOS. There were no significant differences in *LHCGR* splice variant expression between control and PCOS GLCs at either time point. Interestingly, allowing GLC to recover *in vitro* resulted in an approximately 10-fold increase in *LHCGR b* and *c*, suggesting that these splice variants are suppressed more by exogenous treatments than the other variants.

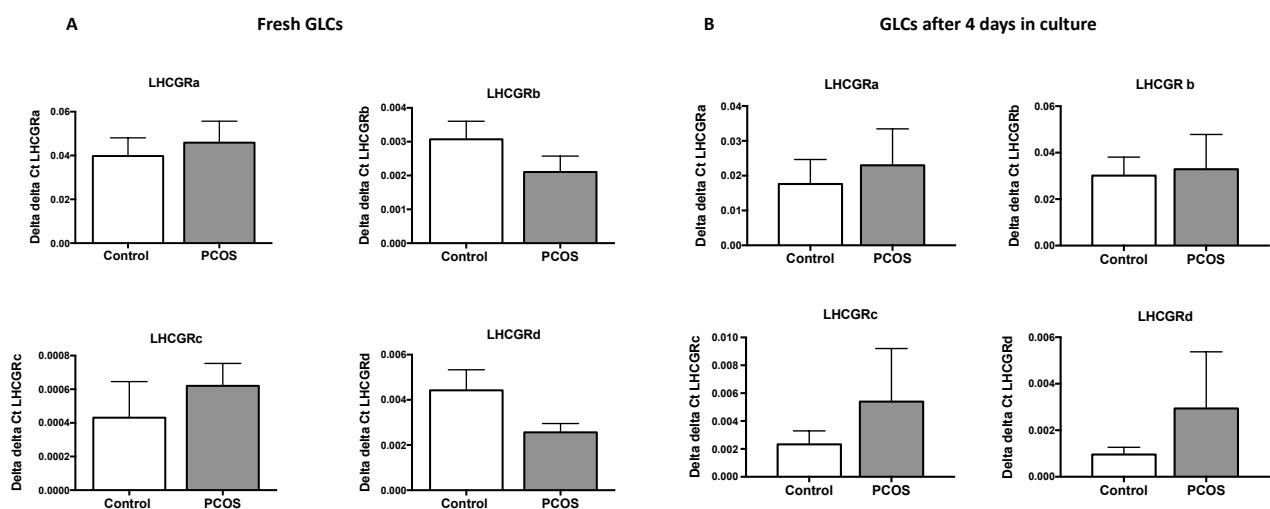


Figure 4. 12 LHCGR splice variant gene expression on the day of collection (fresh GLCs) and after 4 days in culture. GLCs were either frozen at -80 on day of collection (A) or were cultured for 4 days prior to freezing (B). RT-qPCR was used to analyse gene expression. There were no significant changes in gene expression of any of the 4 LHCGR splice variants either in fresh GLCs (A) ($n=10$ controls, $n=12$ PCOS), or those in culture for 4 days (B) ($n=11$ controls, $n=7$ PCOS, Mann-Whitney test, mean $\pm$ SEM).

It has been reported in fresh GLCs that FSHR expression is augmented in PCOS GLCs (Catteau-Jonard et al., 2008). FSHR was significantly higher in PCOS GLCs than control on day 1 but was

not significantly different after 4 days in culture. LH treatment downregulated *FSHR* in both control and PCOS GLCs.

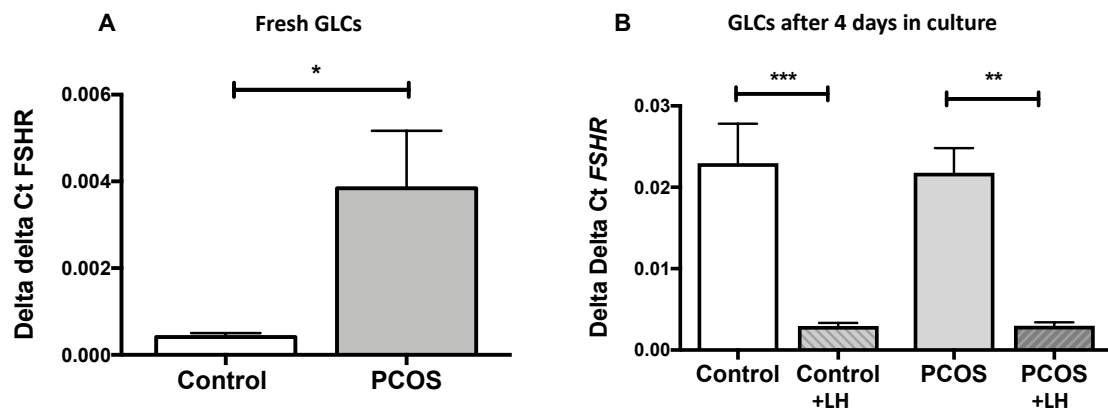


Figure 4. 13 FSHR expression in GLCs on the day of collection and after 4 days in culture, with and without LH treatment. A FSHR is higher in fresh GLCs in PCOS women than control women ($n= 8$ controls, $n=12$ PCOS) ($p=0.01$) Mann Whitney test. **B** There is no difference in FSHR expression between control and PCOS GLCs after 4 days in culture. LH 10nM treatment for 24 hours suppressed FSHR in both control ($p<0.001$) and PCOS GLCs ($p<0.01$). One way ANOVA Values are mean $\pm$ SEM.

4.2.8 Steroid enzyme expression in control and PCOS GLCs on day 4 in culture (basal and LH stimulated)

We have previously studied steroid enzyme gene expression in GLCs from control and PCOS women and found reduced expression of *CYP11A1* (Lerner et al., 2019). However this study was completed in fresh GLCs, i.e. those frozen on the day of collection. I measured gene expression of steroid enzymes in GLCs cultured *in vitro* for 4 days, with and without LH treatment for 24 hours. These enzymes are critical for GLC steroidogenesis. There was no significant difference in *STAR*, *HSD3B2*, *CYP11A1* or *CYP19A1* expression between untreated control and PCOS GLCs (Figure 4.14). LH stimulated a significant increase in *STAR* expression in both control and PCOS GLCs (Figure 4.14A). LH stimulated a significant increase in *CYP11A1* and *HSD3B2* in control but not PCOS GLCs (Figure 4.14B, C). LH did not change *CYP19A1* expression (Figure 4.14D).

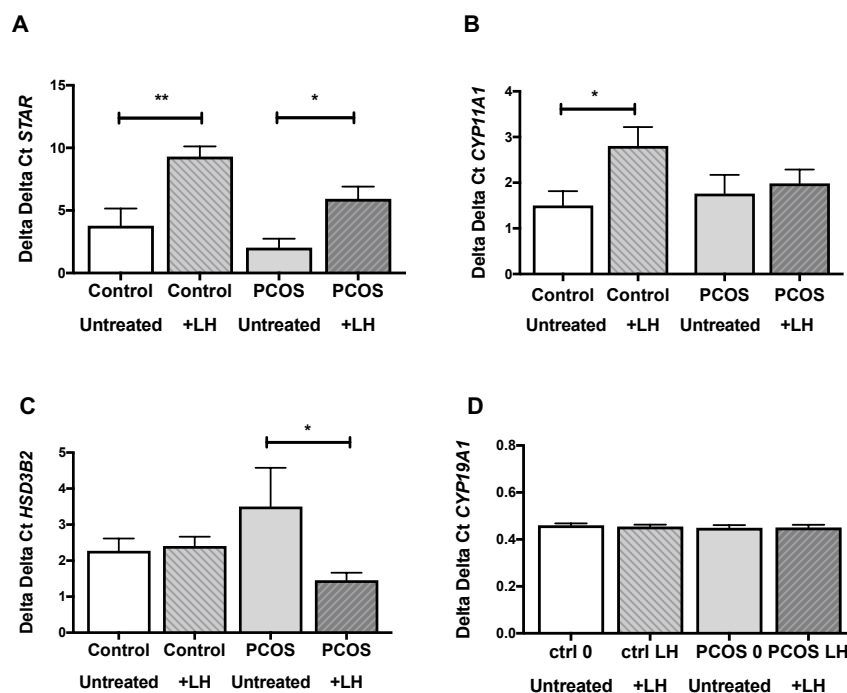


Figure 4. 14 Steroid enzyme gene expression in GLCs *in vitro* with and without LH treatment. On day 4 in culture GLCs were treated with LH10nM for 24 hours ($n=11$ control, $n=7$ PCOS). **A** There was no significant difference in *STAR* expression between control and PCOS GLCs and LH stimulated a significant increase in *STAR* expression in both control ($p<0.01$) and PCOS GLCs ($p<0.05$). **B** There was no significant difference in *CYP11A1* expression between control and PCOS

GLCs and LH stimulated a significant increase in CYP11A1 in control ($p<0.05$) but not PCOS GLCs. **C** There was no significant difference in HSD3B2 expression between control and PCOS GLCs and LH stimulated a significant increase in HSD3B2 in control ($p<0.05$) but not PCOS GLCs **D** There was no significant difference in CYP19A1 expression between control and PCOS GLCs and LH did not change CYP19A1 expression/ Kruskal Wallis test. One way ANOVA used for all, Values are mean $\pm$ SEM.

4.2.9 Amphiregulin and epiregulin expression are not different between control and PCOS in GLCs and expression is increased by LH treatment in vitro.

AREG and EREG are members of the EGF family of growth factors. Given the increase in EGFR seen in the phospho-array, and the fact that AREG and EREG expression are known to be regulated by LH (Freimann et al., 2004, Sekiguchi et al., 2004), I measured expression of these factors in control and PCOS GLCs with and without LH treatment. There were no differences in AREG between PCOS and control GLCs. LH stimulated an increase in expression of both factors (Figure 4.15).

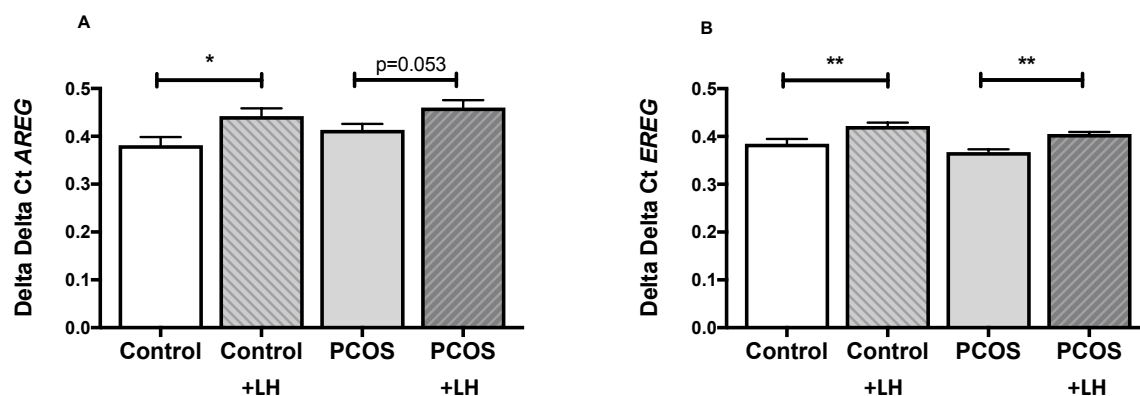


Figure 4. 15 Gene expression of AREG and EREG in control and PCOS GLCs cultured with and without LH. On day 4 in culture GLCs were treated with LH 10nM for 24 hours ($n=11$ control, $n=7$ PCOS, mean $\pm$ SEM). **A** There was no difference in AREG expression between PCOS and control

GLCs. LH stimulated an increase in AREG expression in both control ($p<0.05$) and PCOS ($p=0.053$) GLCs. **B** There was no difference in EREG expression between PCOS and control GLCs. LH stimulated an increase in AREG expression in both control ($p<0.01$) and PCOS ($p<0.01$) GLCs. One way ANOVA

4.2.10 Basal and LH stimulated progesterone output into conditioned media was not different between control and PCOS GLC

Due to the alterations in LH receptor signalling I have discussed above, and the contradictory results from previous studies, I measured basal and LH stimulated progesterone output into culture media from GLCs from women with and without PCOS. On day 4 in culture GLCs from 7 control women and 6 with anovulatory PCOS were treated with LH 10nM for 24 hours. Culture media was collected and progesterone output from GLCs was measured. Progesterone levels did not differ in culture media from control and PCOS GLCs, either from basal (untreated) or LH stimulated GLCs (Figure 4.16).

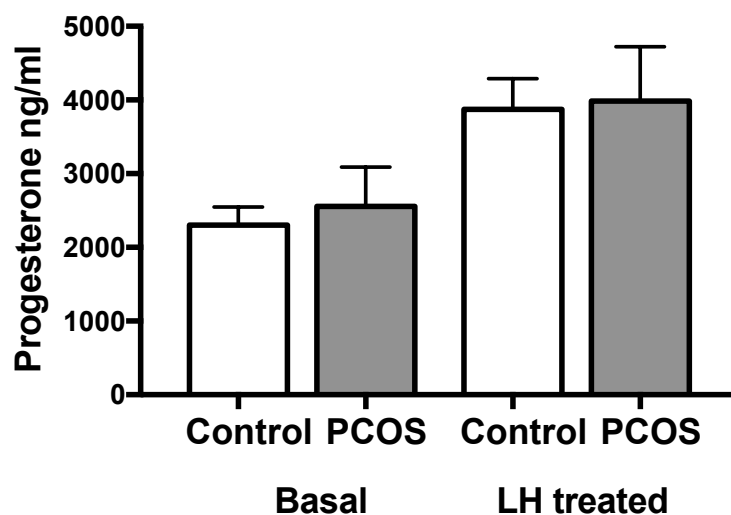


Figure 4. 16 Progesterone accumulation in culture media of control and PCOS GLCs. Basal progesterone on day 5 in culture did not differ between GLCs from control ($n=7$) and anovulatory PCOS ($n=6$). GLCs were treated on day 4 culture in culture with LH 10nM for 24 hours. LH stimulated progesterone output from GLCs into conditioned media was not different between control ($n=7$) and PCOS ($n=6$). Unpaired t-test, Values are mean $\pm$ SEM.

Table 4.1 Summary results- Gonadotropin receptor signalling, expression and steroidogenesis in PCOS GLCs

Test/Assay	Result in PCOS compared to controls ↑- increase, ↓- decrease, ↔- no difference
cAMP LH	↑
cAMP FSH	↔
β-arrestin 1 mRNA	↔
β-arrestin 2 mRNA	↑
GIPC mRNA	↑
APPL1 mRNA	↔
pERK 1/2 LH	↔
pERK 1/2 FSH	↔
IP-1	↔
pAKT LH	↔
pAKT FSH	↔
Phosphoarray basal changes: P53, P70 S6 Kinase, AKT 1/2/3, P27, Fyn, Hck, Lyn	↑ ↓
Phosphoarray LH (5+30 mins): Fyn, Yes, Fgr, Hck, Chk-2 AMPKα1, β catenin, Lyn, Lck	↑ ↑
Phosphoarray LH (30 mins): P38α, JNK1/2/3, Src, EGFR, MSK 1/2, PDGFRβ, AMPKα2, STAT5A, 5B, 5A/B, 6	↑ ↑
Phosphoarray FSH (5 mins):	↑

P38 α , β catenin, TOR, CREB, Src, Lyn, Fyn, Yes, Hck, Chk-2, STAT5A, AKT 1/2/3	↑ ↓
P53, c-JUN, RSK 1/2/3, eNOS, STAT3, P27, PLC γ 1, WNK1	↓
LHCGR mRNA Fresh GLCs	↔
LHCGR 4 days in culture	↔
LHCGR 4 days in culture +LH	↓
LHCGR a-d mRNA	↔
FSHR mRNA Fresh GLCs	↑
FSHR 4 days in culture	↔
FSHR 4 days in culture +LH	↔
STAR mRNA	↔
STAR + LH	↔
CYP11A1 mRNA	↔
CYP11A1+ LH	↓
HSD3B2 mRNA	↔
HSD3B2 + LH	↓
CYP19A1 mRNA	↔
CYP19A1 + LH	↔
AREG mRNA	↔
AREG + LH	↔
EREG mRNA	↔
EREG + LH	↔
Progesterone	↔
Progesterone + LH	↔

4.3 Discussion

In order to investigate gonadotropin receptor signalling and function in GLCs from women with PCOS, I comprehensively studied receptor expression, signalling protein expression, second messenger generation, downstream phosphoprotein expression, steroid enzyme expression and steroidogenesis. This has highlighted multiple differences in receptor function in PCOS GLCs, predominantly related to the LHR.

Ligand binding and subsequent signal transduction to Gs activates adenylyl cyclase to produce the second messenger signal, cAMP from ATP (Marsh et al., 1973). I found that activating LHR but not FSHR in PCOS GLCs resulted in a higher accumulation of cAMP than control GLCs (Figure 4.1, 4.2). Only one study has examined cAMP accumulation in GLCs and found higher cAMP levels in GLCs from women with PCOS, which they attributed to a higher expression of ARF6-GTP, a small GTPase known to play a role in receptor internalisation, although a causal relationship was not shown (Kanamarpudi et al., 2016). This augmented LH response may be for a number of reasons. Firstly I found a consistent trend towards an increase in expression of *LHCGR* in cultured PCOS GLCs, but not *FSHR* (Figure 4.11). Increasing receptor expression has been shown to increase agonist stimulated cAMP accumulation (George et al., 1988). Secondly, there was an increase in expression of trafficking protein GIPC in PCOS GLCs (Figure 4.3), which traffics the receptor-ligand complex to the VEE, from where it can continue to generate cAMP (Sposini and Hanyaloglu, 2018, Sposini et al., 2017). Enhanced internalisation can augment cAMP activity, as has been shown in mutations in the LHR which enhance internalisation of free and agonist bound receptors result in an increase in basal cAMP. A gain of function mutation in LHR associated with leydig cell hyperplasia showed constitutive activity towards the cAMP response (McGee and Narayan, 2013). Thirdly, I also found a higher expression of β -arrestin 2 in PCOS GLCs (Figure 4.3). Interaction with β -arrestins promotes the transfer of ligand-bound receptor from the cell surface to early endosomes via dynamin- and clathrin-dependent endocytosis (Goodman et al., 1996). It has been shown in other GPCRs receptors that β -arrestins promote rather than attenuate cAMP production in response to ligand and arrestin-mediated activation of ERK1/2 signalling causes inhibition of PDEs and permits sustained cAMP signalling (Vilardaga et al., 2014). Therefore PCOS GLCs may have augmented LHR cAMP signalling due to increased endosomal trafficking via GIPC, augmented arrestin activity and increased receptor expression.

Knockdown of APPL1 in HEK293 cells consistently augments cAMP accumulation in response to LH, which is not due to altered surface expression of LHR (Sposini et al., 2017). Loss of APPL1 has been shown to enhance LH-mediated androgen production in bovine theca cells (Comim et al., 2013a). While there is no difference in APPL1 mRNA in PCOS GLCs, there may be differences in post translational modification of APPL1, or another adaptor protein affecting APPL1. Interestingly, the phosphorylation status of APPL1 on serine 410 is critical in regulating LHR trafficking and signalling (Sposini et al., 2017) thus the potential role of APPL1 PKA phosphorylation in LHR recycling and cAMP signalling in control and PCOS GL cells could be explored in future work, using APPL1 phospho-deficient and phospho-mimetic mutant constructs and a specific phospho-APPL1 antibody. Future work could study LHR function in other cells which endogenously express LHR, such as human theca cells, which are also implicated in the aetiology of PCOS, although these cells are difficult to obtain. This could also be studied in GCs cultured from a PCOS mouse model with transfection of a tagged receptor and signalling proteins, however receptor trafficking is likely to be different, and PCOS is heterogeneous, therefore it is difficult to generate a model that exactly reproduces the phenotypes observed in PCOS.

Activation of the ERK cascade in granulosa cells is critical for multiple downstream cellular functions, which include survival, differentiation and steroidogenesis (Dewi et al., 2002, Peter and Dhanasekaran, 2003), (Wortzel and Seger, 2011). $G_{\alpha s}$, $G_{\alpha q/11}$, $G_{\beta\gamma}$ and arrestins can contribute to ERK 1/2 phosphorylation (Lefkowitz and Shenoy, 2005). I did not see any significant difference in the LH stimulated ERK signalling in PCOS GLCs. This may be for a number of reasons. Firstly, even though there is augmented $G_{\alpha s}$ signalling and arrestin expression in PCOS GLCs, other signalling pathways also simultaneously contribute to pERK expression. Secondly a relatively small number of samples was used ($n=4$ in each group), due to sample availability and size. There was a possible trend towards increased pERK after 30 and 60 minutes LH treatment in PCOS GLCs. Future work could look at these time points with a bigger sample number. Since the ERK cascade is central to cell proliferation and survival, and lower apoptotic rates were found in granulosa cells from small follicles from women with PCOS (Das et al., 2008),

one might predict augmented pERK generation in PCOS samples. However, work by AH lab has identified conditions that result in increased cAMP without affecting pERK. Knockdown of APPL1 in HEK293 cells, which augments cAMP production in response to ligand, does not affect pERK1/2 generation (Sposini, 2017). There was also no difference in pERK generation in PCOS GLCs after FSH stimulation, which was predictable given the similar levels of receptor expression and cAMP generation in both groups after FSH stimulation.

The PI3K/AKT pathway is important in follicle competence. Levels of pAKT in bovine granulosa and cumulus cells correlate positively with oocyte quality (Andrade et al., 2017). This pathway also prevents apoptosis of bovine follicle cells (Hu et al., 2004a). I did not see consistent activation of pAKT in GLCs in control or PCOS samples, however this is possibly due to high basal levels of pAKT making it difficult to measure ligand related differences. Future work could assess PI3K by measuring enzymatic activity using immunoprecipitation (Ciraolo et al., 2014).

While the effects of gonadotropins are mediated mostly by their activation of the $G_{\alpha s}$ /cAMP/PKA pathway, additional pathways such as the $G_{\alpha q11}$ pathway are also activated by LH. As discussed in Chapter 3, gonadotropin receptor activation of $G_{\alpha q11}$ /phospholipase C is detectable only when cells express a high receptor density or high concentrations of ligand. Activation of this pathway has been demonstrated for FSHR only in cell lines with over-expressed receptor (Thomas et al., 2011), however I have shown that this pathway is activated by LH/LHR in GLCs. This pathway is important peri-ovulation, when LHR expression reaches its peak (Breen et al., 2013). IP-1 accumulation did not differ between control and PCOS samples, however the number of PCOS samples was small (n=5). There was a trend towards lower IP-1 accumulation in PCOS GLCs, which could be due to bias for $G_{\alpha s}$ over $G_{\alpha q}$ in PCOS. Work by AH lab has shown that there may be crosstalk between $G_{\alpha s}$ and $G_{\alpha q}$ in HEK293 F-LHR cells, where inhibition of PKA activation affects LH- induced calcium response and cAMP levels in cells stimulated with LH are strongly reduced following $G_{\alpha q}$ inhibition (Sposini, 2017).

The phospho-kinase array highlighted the downstream effect of augmented signal generation in response to LH in PCOS GLCs (Figure 4.8). Overall the pattern was that of increased phospho-protein activity from basal after LH treatment in PCOS GLCs. While the function of some of these proteins is unclear in GLCs, several members of the SRC family of tyrosine kinases (LYN, LCK, CHK-2, HCK, FYN) have an increased expression in PCOS GLCs in response to LH. β -arrestin 2 is essential for activation of FYN, and FYN is a mediator of activation of the LHR ERK cascade (Galet and Ascoli, 2008). β -arrestin 2 is over-expressed in PCOS GLCs (Figure 4.3A) and may be contributing to this increased activation of FYN. These Src proteins also play an important role in ovarian cancer progression (Kim et al., 2011). Women with PCOS are at increased risk of endometrial cancer (Dumesic and Lobo, 2013), and certain studies have suggested that they are at increased or decreased risk of ovarian cancer, although this has not been proven (Schildkraut et al., 1996). AMP kinases regulate cellular energy balance and are known to have a role in negatively regulating steroidogenesis (Hirsch et al., 2012, Tosca et al., 2007). Therefore a higher expression of AMPK in PCOS GLCs may reduce progesterone synthesis as has been demonstrated in some studies, although I did not see a defect in progesterone production.

The phospho-kinase array highlighted differences between control and PCOS GLCs at basal conditions (Figure 4.7). Two of the upregulated proteins, P53 and P27 are both cell cycle regulators. P27 knockout mice display premature activation of the primordial follicle pool and premature ovarian insufficiency (Rajareddy et al., 2007). The ovaries of women with PCOS do have an increased density of primordial and primary follicles (Webber et al., 2003), so dysregulation of ovarian cell cycle proteins may contribute to this. P53 has previously shown to be overexpressed in ovarian epithelial cells in women with PCOS, however other cell cycle and apoptotic markers were not overexpressed (Mahdi et al., 2014).

The phospho-kinase array also highlighted some differences in downstream signalling in PCOS GLCs after FSH treatment. This is despite no difference in *FSHR* expression or cAMP accumulation. This may relate, as for LHR, to the augmented expression of signalling proteins which may alter the receptor trafficking and signal propagation in PCOS GLCs, however the profile of differentially regulated phosphoproteins is different to that of LH treated cells. 12

phosphoproteins were upregulated after FSH treatment compared to untreated GLCs in PCOS samples and 8 phosphoproteins were relatively downregulated by FSH.

There was a trend towards a higher expression of *LHCGR* in PCOS GLCs both in fresh GLCs and those maintained in culture (Figure 4.11). The underlying cause of this is unclear but may be due to increased GnRH pulsatility favouring predominantly LH secretion in PCOS (Chaudhari et al., 2018). Insulin has also been shown to increase LHR expression and action, and hyperinsulinaemia is a key feature of PCOS (Willis et al., 1996).

Interestingly, the ability of LHR to alter its own expression in response to LH was altered in PCOS. *LHCGR* in GLCs from control samples increased after exposure to LH *in vitro* (Figure 4.11), whereas *LHCGR* was downregulated after LH treatment in PCOS GLCs. These novel data further highlight the different downstream effects of LH treatment in PCOS GLCs. This may be a negative autoregulatory mechanism in response to the augmented signalling of certain pathways activated by LHR in PCOS GLCs. Altered signalling is likely to result in differential regulation of downstream gene expression. It is known that GPCRs reduce receptor expression at the plasma membrane in order to downregulate a signal, but acutely, at protein level (Goh and Sorkin, 2013, Irannejad and von Zastrow, 2014). Future work could also measure LHR protein expression, as protein and gene expression may differ, however I attempted to optimise an LHR (and FSHR) antibody and neither worked reliably (Chapter 3).

There are 4 known splice variants of the LHR. LHR-A is the full length receptor, LHR-B is lacking of exon 9, LHR-C is lacking part of exon 11 and LHR-D is lacking both exon 9 and part of exon 11 (Minegishi et al., 2007). LHR-B is known to be signalling deficient and reduces expression of both LHR-A and FSHR (Nakamura et al., 2004, Yamashita et al., 2005). LHR-D alters LHR-A signalling activity (Dickinson et al., 2009). Aberrant splice variants, which result in impaired ligand binding, have been observed in individuals suffering from Leydig cell hypoplasia, hypospadias and micropenis (Han et al., 2012). There was no significant difference in splice variant gene expression in PCOS GLCs in fresh or cultured GLCs, so alternate splicing does not appear to contribute to altered LHR function in PCOS. Interestingly, variants b and c increased more in

culture than the other variants, suggesting that these splice variants are suppressed more by exogenous treatments.

While expression of *FSHR* in fresh GLCs was higher in PCOS compared to controls, as has been published previously, there was no difference in expression after cells were left in culture for 4 days (Figure 4.13). This difference may be because women with PCOS receive a lower dose of FSH as part of their stimulation protocol, due to their increased risk of OHSS (Delvigne et al., 1993, Delvigne and Rozenberg, 2002a, Delvigne and Rozenberg, 2002b), therefore having relatively less *FSHR* downregulation by exogenous FSH. After several days the control GLCs have recovered from stimulation with higher doses of FSH, upregulating their *FSHR* to similar expression levels to GLCs from women with PCOS. Future work on *FSHR* signalling in PCOS should study receptor function in GCs from hSAFs. Expression is higher in these less mature cells (Figure 5.2) when *FSHR* function is at its peak. Expression is also lower in PCOS GCs than control (Figure 5.2). They may also be easier to transfect with tagged receptor, as they are not terminally differentiated and divide and grow *in-vitro*.

The importance of the relationship between *FSHR* and *LHR* is increasingly recognised as central to their function. I have shown that LH downregulates *FSHR in vitro* in GLCs in equal measure in control and PCOS samples (Figure 4.13), and FSH upregulated *LHR* (Figure 6.4). Studies from the Hanyaloglu lab have shown that heterodimerisation of gonadotropin receptors transfected into HEK293 cells (Jonas et al., 2018) affects signal generation. While I had intended to study heterodimerisation of these receptors in GLCs, technically this was not possible. Given that GLCs express both receptors they would be ideal primary cell in which to study heterodimerisation, but this has thus far not been possible.

Gonadotropin receptor expression and function can also impact the function of other receptors in GLCs, through receptor crosstalk. *EGFR* is known to be under the control of LH (Nielsen et al.). I have shown in the phospho-array (Figure 4.8) that *EGFR* is upregulated to a higher degree after LH treatment in PCOS GLCs. The role of EGFs in PCOS has not been explored, however expression

of EGFs epiregulin and amphiregulin, and their upregulation in response to LH stimulation did not differ (Figure 4.15).

We have previously shown a significant reduction in *CYP11A1* in fresh PCOS GLCs (Lerner et al., 2019). However there have been no comprehensive studies of steroid enzyme gene expression in cultured GLCs from women with GLCs. There was no significant difference in basal expression of *STAR*, *HSD3B2*, *CYP11A1* and *CYP19A1* in PCOS GLCs (Figure 4.14). LH treatment increased expression of *CYP11A1* and *STAR*. *HSD3B2* expression was reduced after LH treatment only in PCOS GLCs (Figure 4.14). RNA sequencing of fresh GLCs (Lerner et al, unpublished) revealed a downregulation of genes involved in cholesterol metabolism, including *HMGCS2*, *MVD* and *ACAT2*. This suggests a reduction in steroid synthesis from cholesterol in PCOS GLCs. However, I measured progesterone output into conditioned media from untreated and LH treated GLCs (Figure 4.15) and found no significant difference in PCOS GLCs. It is unclear therefore if *in vitro* steroidogenesis does not represent *in vivo* steroidogenesis, which given the interplay with theca and GLCs which is not represented *in vitro* is possible. Theca cells from women with PCOS display increased expression of *CYP17* and augmented response to androgen treatment (Comim et al., 2013b, Jakimiuk et al., 2001), so altered steroidogenesis in PCOS follicles may not be reflected *in vitro* in GLCs alone.

Previous studies have measured progesterone synthesis in PCOS GLCs and results have differed. This is likely to be due to differences in experimental conditions, including dose and duration of gonadotropin treatment used for these cultures. Rice et al incubated GLCs with LH for 48 hours after 2 days in culture and showed dose-dependent enhanced progesterone production in conditioned media from GLCs from 7 women with anovulatory PCOS (Rice et al., 2005). Adams and colleagues found no difference in basal progesterone production in GLCs from 4 women with PCOS after 48 hours in culture (Adams et al., 2016). Huang and colleagues reported reduced progesterone production in response to 48 hours hCG treatment, which commenced 24 hours after isolation, from 8 women with PCOS, along with a reduction in *STAR* expression (Huang et al., 2016). There is some evidence that women with PCOS have lower luteal phase progesterone

production, which would fit with a reduced capacity of GLCs to secrete progesterone (Filicori et al., 1989).

4.3.1 Conclusions

PCOS GLCs have an augmented cAMP response to LH, which I have shown may be due to differences in receptor expression, receptor trafficking to endosomal compartments and arrestin mediated signalling. It may also be related to APPL1 or another, thus far unknown, adaptor protein. There appears to be LHR signal bias in PCOS GLCs, with bias for $G_{\alpha s}$ over $G_{\alpha q}$ which mirrors previous work from the AH lab. This results in increased activation of downstream phosphoproteins, but does not appear to affect steroidogenesis. This may be due to a negative feedback mechanism of LHR in PCOS GLCs resulting in downregulation of receptor expression by LH treatment, or due to the experimental conditions of progesterone measurement.

Chapter 5

Gene expression in granulosa cells from small antral follicles from women with or without polycystic ovaries

5.1 Introduction

In PCOS evidence of disordered ovarian follicular development can be observed even at the early preantral stages (Webber et al., 2003). The later, gonadotropin dependent, stages of follicle development are characterised by arrest at the small-medium sized antral follicle stage and failure to progress to ovulation (Hughesdon, 1982, Webber et al., 2003, Maciel et al., 2004). The exact cause of aberrant follicle development is unknown.

Factors implicated in the aetiology of PCOS and follicle arrest include abnormalities of LHR and FSHR function. As discussed previously GWAS studies have found significant hits close to loci encoding these receptors (Shi et al., 2012, Cui et al., 2013, Tian et al., 2016, Hayes et al., 2015). In addition, GCs from follicles from women with anovulatory PCOS display inappropriate LHR and FSHR activity, with increased oestradiol and premature progesterone production in response to FSH and LH respectively (Willis et al., 1998, Mason et al., 1994). AMH has been shown to inhibit FSH-induced oestradiol production by GCs *in vitro* (Pellatt et al., 2011) and, as AMH production is higher in women with PCOS (Cook et al., 2002, Seifer and Maclaughlin, 2007), it has been postulated that the higher ovarian levels of AMH impair antral follicle function and contribute to follicle arrest. AMH gene expression has not, to my knowledge, been measured in GCs from individual, matched, unstimulated follicles in women with and without PCO.

Other members of the TGF- β superfamily are plausible candidates for disrupted follicle function in PCOS. Follistatin (FST), produced predominantly in GCs (Glister et al., 2006), inhibits FSH secretion by binding and neutralising activin action (Nakamura et al., 1990, Ueno et al., 1987), and has been shown to be elevated in serum of women with PCOS (Pigny et al., 2003).

Conversely, inhibins A and B, which also suppress FSH secretion, were found, in one study, to be lower in follicular fluid in large size-matched follicles from women with PCOS (Welt et al., 2005).

Androgens and insulin may also be important in the mechanism of follicle arrest in PCOS. Insulin can stimulate GC steroidogenesis and enhance GC responsiveness to LH (Willis et al., 1996) and increased androgen synthesis may augment FSH receptor expression, both of which may

increase local production of cyclic AMP and encourage terminal differentiation of GCs (Franks et al., 2008). However it is unclear whether insulin and androgen receptors themselves are differentially expressed in PCOS GCs.

Studies in human small antral follicles (hSAFs) in PCOS are rare, given the difficulty in accessing this tissue. Our collaborators in Copenhagen collected and froze GC samples from women with and without PCO at the time of cryopreservation of ovarian tissue for fertility preservation. I took two approaches to maximise the use of this precious tissue, a targeted approach studying factors linked to follicle arrest in PCOS based on work by our lab and others, and a non-targeted approach using RNA seq.

5.1.1 Specific aims

The aims were to address the following questions:

1. Are there differences in gene expression of key factors implicated in follicle arrest in GCs from women with and without PCO?
2. How does expression of these factors compare between GCs from hSAFs and GLCs?
3. Are there differences in gene expression in control and PCO GCs using RNA seq?
4. Does gene expression measured using RNA seq change with increasing follicle size?

5.2 Methods and Results

5.2.1 Patient demographics and clinical information

GCs were collected from 98 unstimulated hSAFs from 31 women, 10 with PCO and 21 with normal ovaries and regular cycles. The women were aged between 16 and 36 and their mean age was 25 (Table 5.1). Mean age was similar between women with PCO and without. Their underlying medical diagnoses are listed in Table 5.1. Polycystic ovaries were defined primarily on the basis of ovarian volume ($>10 \text{ mm}^3$). Because these samples were taken from women with cancer and who were about to undergo cancer treatment, data on menstrual history or hirsutism

were not recorded. However, biochemical indices of PCOS were measured and reported in Table 5.1.

GLCs were collected from 12 women, 6 with PCOS and 6 without. The PCOS women were younger and received a lower dose of FSH (Table 5.2).

Table 5.1 Patient clinical information (granulosa cell samples from hSAFs)

	Control (n=21)	PCOS (n=10)	p-value
Age (years) (mean± SD)	25.7 ± 5.9	25.9 ± 5.8	0.92
Number of follicles included per woman (mean± SD)	2.5 ± 1.5	4.9 ± 1.7	0.0004
Total number of follicles aspirated per woman (range)	1-6	7-14	
Ovarian volume (mean± SD)	7.1 ± 2	14 ± 2.8	<0.0001
Serum LH (mean± SD) (IU/L)	5.9 ± 4.4	9.2 ± 6.8	0.10
Serum FSH (mean± SD) (IU/L)	5.4 ± 2.8	5.5 ± 2	0.94
Serum AMH (mean± SD) (pmol/L)	17.8 ± 10.8	42± 2.9	0.001
Serum Testosterone (mean± SD) (nmol/L)	0.5 ± 0.49	0.7 ± 0.37	0.22
Follicle size (mm) (mean± SD)	5.8 ± 1.4	5.9 ± 1.5	0.51
Diagnosis	6 Breast cancer 5 Lymphoma 4 Brain cancer 1 medulloblastoma 1 Aplastic Anaemia 1 Sickle cell anaemia 1 Ovarian Cancer 1 Colorectal cancer	4 Breast cancer 4 Sarcoma 1 Colorectal cancer 1 Lymphoma	

	1 Diamond Blackfan anaemia		
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Table 5.2 Patient clinical information (granulosa lutein cell samples)

	Control (n=6)	PCOS (n=6)	p-value
Age	36 ± 3.8	32 ± 1.4	0.056
Indication for IVF	Unexplained 5 Egg freezing 1	Anovulatory PCOS	
FSH dose	3438± 1259	2075± 700	0.06
Maturation trigger	hCG	hCG	
IVF protocol	5 Antagonist 1 Flare protocol	Antagonist	

5.2.2 Sample description

The diameter of the hSAFS from which the GCs were extracted ranged from 2.4 to 12.4mm and the mean follicle size was 5.9mm (Figure 5.1). The mean was similar for both control and PCOS samples (Figure 5.1). The number of follicles included per woman ranged between 1 and 9 (Figure 5.1B).

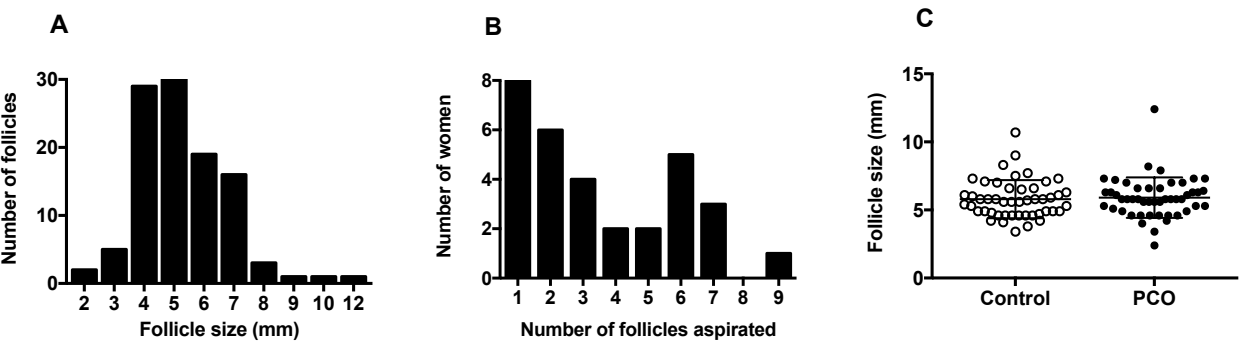


Figure 5.1 Number and size of follicles. A. The number of follicles included of each follicle diameter (mm). **B.** The number of follicles included per woman. **C.** The distribution of follicle sizes

between control and PCOS samples. The mean $\pm$ SD follicle size of control samples was 5.8 ± 1.4 mm and of PCOS samples was 5.9 ± 1.5 mm.

5.2.3 Receptor expression

Gonadotropin, androgen and insulin receptors all play a part in the aetiology of PCOS, therefore I compared gene expression of these receptors in control and PCO GCs and GLCs using RT-qPCR. *LHCGR* expression was detectable in all samples. Median *LHCGR* expression was 30 times higher in GLCs than in GCs. *LHCGR* was higher in PCOS GLCs than in control ($p=0.051$) (Figure 5.2A). *LHCGR* expression was low in GCs compared to GLCs. There was a wider distribution of *LHCGR* expression in the PCO samples compared to control but there was no overall significant difference. By contrast, median *FSHR* expression in GC SAF was 8-fold higher than in GLCs (Figure 5.2C). *FSHR* expression was lower in GC from PCO women compared to control samples, whereas there was no significant difference between control and PCOS GLCs. *AR* expression in control GCs was 3.5 times higher than control GLCs (Figure 5.2D), but was not different between PCO GCs and GLCs. *AR* was lower in PCO compared to control GCs. (Figure 5.2D). There was no correlation between *LHCGR* and *AR* or *FSHR*. There was a strong positive correlation between *AR* and *FSHR* (Figure 5.2E). Insulin receptor (*INSR*) expression was similar in GCs and GLCs and between control and PCOS women (Figure 5.2F).

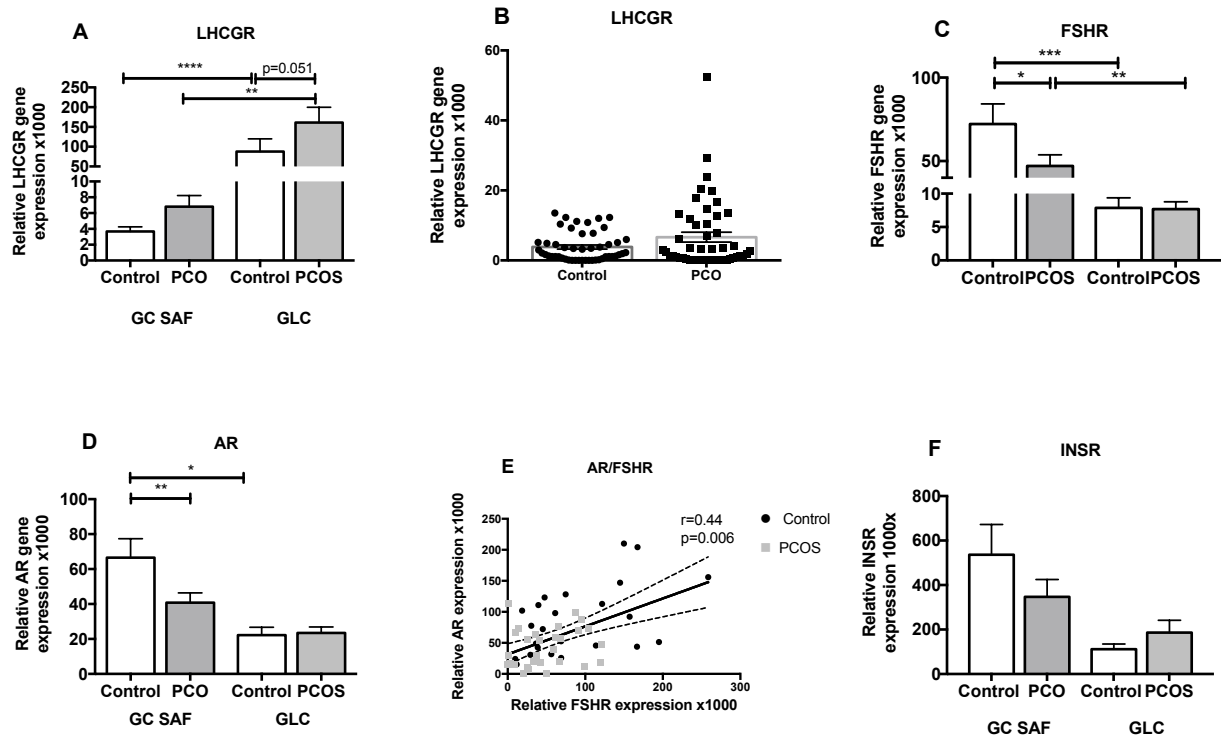


Figure 5. 2 Gonadotropin, Androgen and Insulin Receptor expression. **A** LHCGR was higher in GLC (n=6) than in GCs (control p<0.0001, PCO p<0.01) (n=49, n=49) (Median $\pm$ 95% CI). LHCGR was higher in PCOS GLC compared to control (p=0.051). There was no significant difference in LHCGR between PCOS and control GCs however in the PCOS group a subset of samples (10 of 49) that had an expression between 5 and 20 times higher than the median expression of LHCGR. **B.** Scattergram of LHCGR in control and PCO GCs. **C** FSHR expression was higher in GCs than in GLC (control p<0.001, PCO p<0.01). FSHR expression was lower in PCO GCs than control (p=0.03). FSHR expression was the same in control PCOS GLC. **D** Androgen receptor expression (AR) was 3.5 times lower in control GLC compared to GCs (p<0.001). AR in PCOS GCs is half that of control samples (p<0.01). AR in PCOS GLC is similar to control GLC. **E** AR correlates with FSHR (Spearman correlation, r=0.44, p=0.006) **F** Insulin receptor (INSR) is the same in control and PCO GCs and GLCs. Median + 95% CI. Kruskal Wallis and Mann Whitney tests used.

5.2.3 Steroid enzyme gene expression

Steroid enzymes are critical for ovarian steroid production, which is altered in PCOS GCs (Mason et al., 1994, Willis et al., 1996, Willis et al., 1998). The cause of this is unknown, therefore I compared steroid enzyme gene expression in control and PCO GCs. *CYP19A1* gene expression in GCs was higher in GCs than in GLCs (Figure 5.3A). Median *CYP19A1* was higher in PCO GCs than in controls. *CYP11A1* expression in GCs was in higher in GCs than in GLCs (Figure 5.3B). *CYP11A1* was higher in control GCs compared to PCO. *HSD3B2* expression in GLCs was higher than in GCs (Figure 5.3C). There was a non-significant trend towards higher *HSD3B2* in PCO than control GCs ($p=0.069$) but similar in PCOS and control GLCs. *STAR* expression in GLCs was higher in GLCs than in GCs (Figure 5.4D). *STAR* was higher in PCOS than control GCs (Figure 5.3D). Therefore, it appears that the augmented oestradiol synthesis and premature progesterone synthesis in GCs is associated with augmented aromatase and STAR expression in GCs, a trend towards augmented HSD3B2 and LHCGR expression and lower FSHR and CYP11A1 expression.

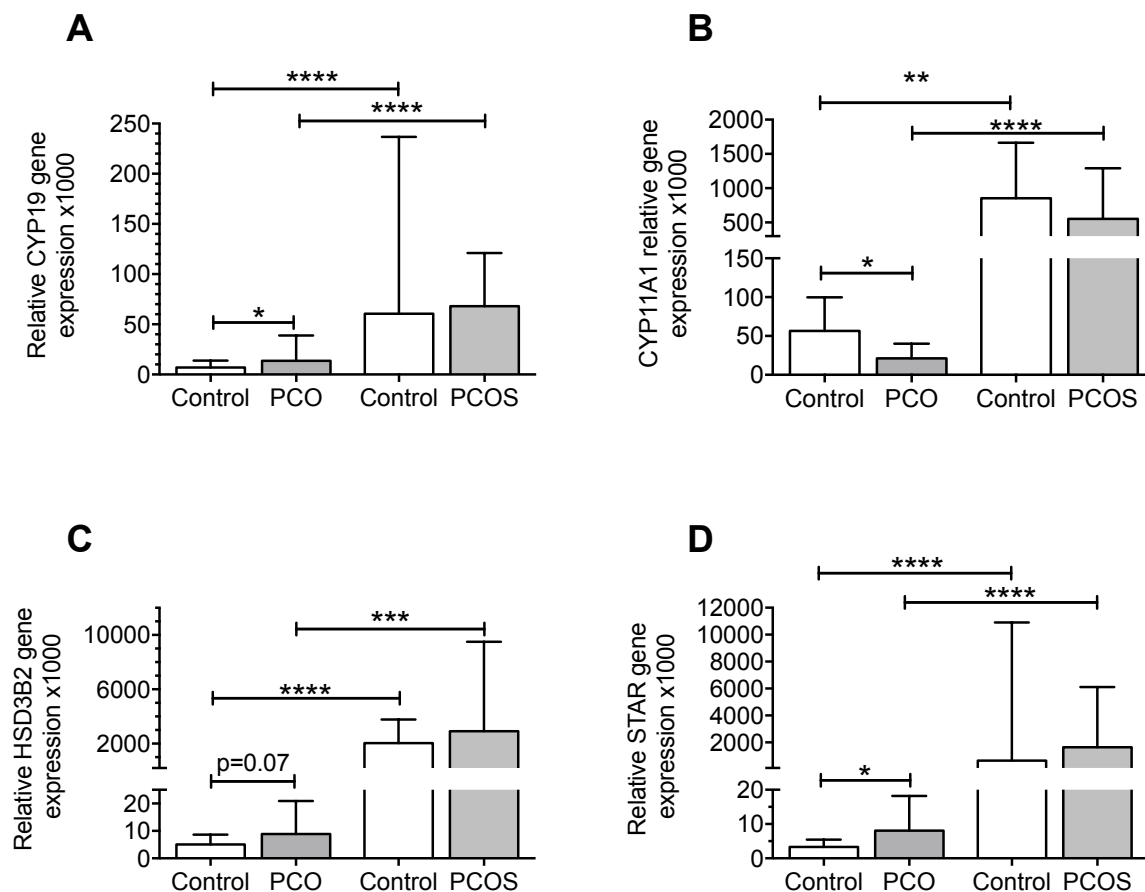


Figure 5.3 Gene expression of steroid enzymes in control and PCO GCs and GLCs, (Median + 95% CI). **A** CYP19A1 gene expression in GCs was higher than in GLCs ($p<0.0001$). Median CYP19A1 expression is 2-fold higher in PCO than control GCs ($p<0.05$). **B** Median CYP11A1 expression in control GCs is 4.3-fold higher than in PCO samples ($p<0.05$) but not in GLCs. Media CYP11A1 was higher in GLC than in GCs (control $p<0.01$, PCO $P<0.0001$). **C** HSD3B2 expression was 350 times higher in GLC compared to GCs (control $p<0.0001$, PCO $p<0.001$). There was a non-significant trend towards higher HSD3B2 in PCO than control GCs (ns, $p=0.07$). **D** STAR expression in control and PCOS GLCs was 194 and 203 times higher than in control and PCO GCs respectively ($p<0.0001$). Median STAR expression is 2.5-fold higher in PCO GCs compared to control ($P<0.05$).

5.2.4 Growth factor gene expression

AMH is implicated in the pathogenesis of PCOS. AMH, follistatin and inhibins are critical regulators of follicle growth and feedback to the pituitary. While expression levels of AMH, FST and INHBB were all significantly higher in GC compared to GLCs, they did not differ between PCO and control GC (Figure 5.4). There was a correlation between AMH and AMHR2 (Figure 5.4C). INHBA expression was higher in PCO GCs than in control samples (Figure 5.4E).

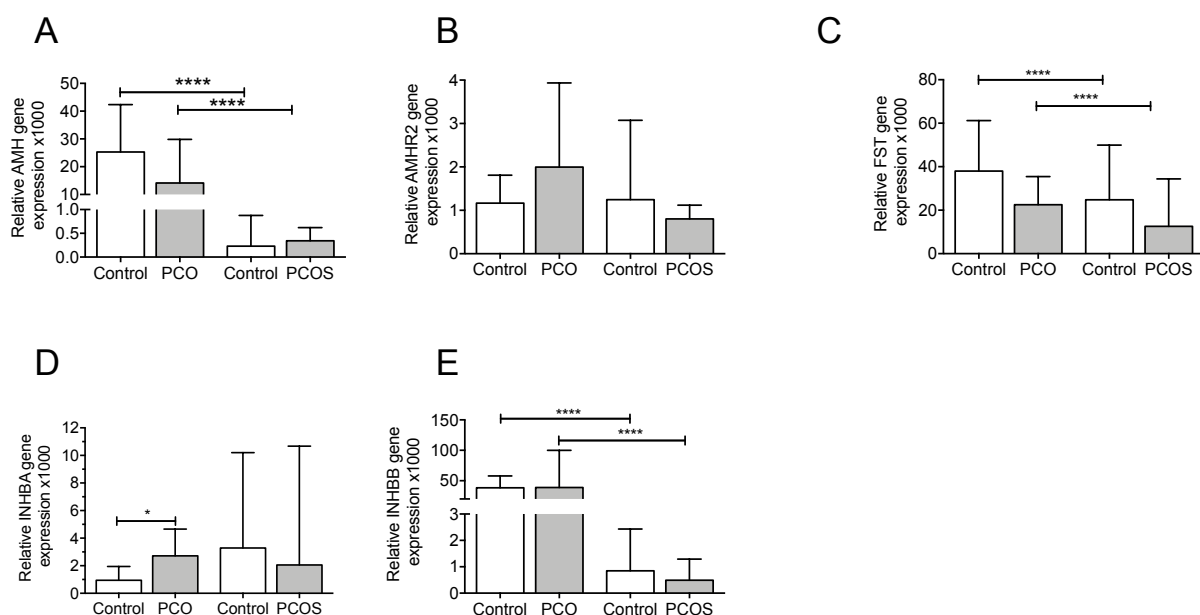


Figure 5.4 Gene expression of growth factors in GLC and GCs (Median + 95% CI). **A** AMH expression is not different between PCOS and control GCs. AMH is negligible in GLCs. **B** AMHR2 expression is not different between PCOS and control GCs. **C** FST (follistatin) expression is not different between PCOS and control GCs. FST is negligible in GLCs. **D** INHBA is higher in PCOS GCs compared to control ($p<0.05$). **E** INHBB is higher in control and PCOS GCs compared to GLCs ($p<0.0001$).

5.2.5 Effect of follicle size on gene expression.

In order to study if differences between control and PCO GC samples were dependent on follicle size or if changes occurred at specific follicle sizes I examined the impact of follicle size on expression of all genes studied. There were no differences in the distribution of gene expression in different follicle sizes between control and PCO samples, i.e. over the range of sizes of SAFs from either control subjects or PCO women, follicle diameter had no significant effect on gene expression (Figure 5.5). However, most follicles were between 4 and 7mm and there were few samples small or large follicles to analyse.

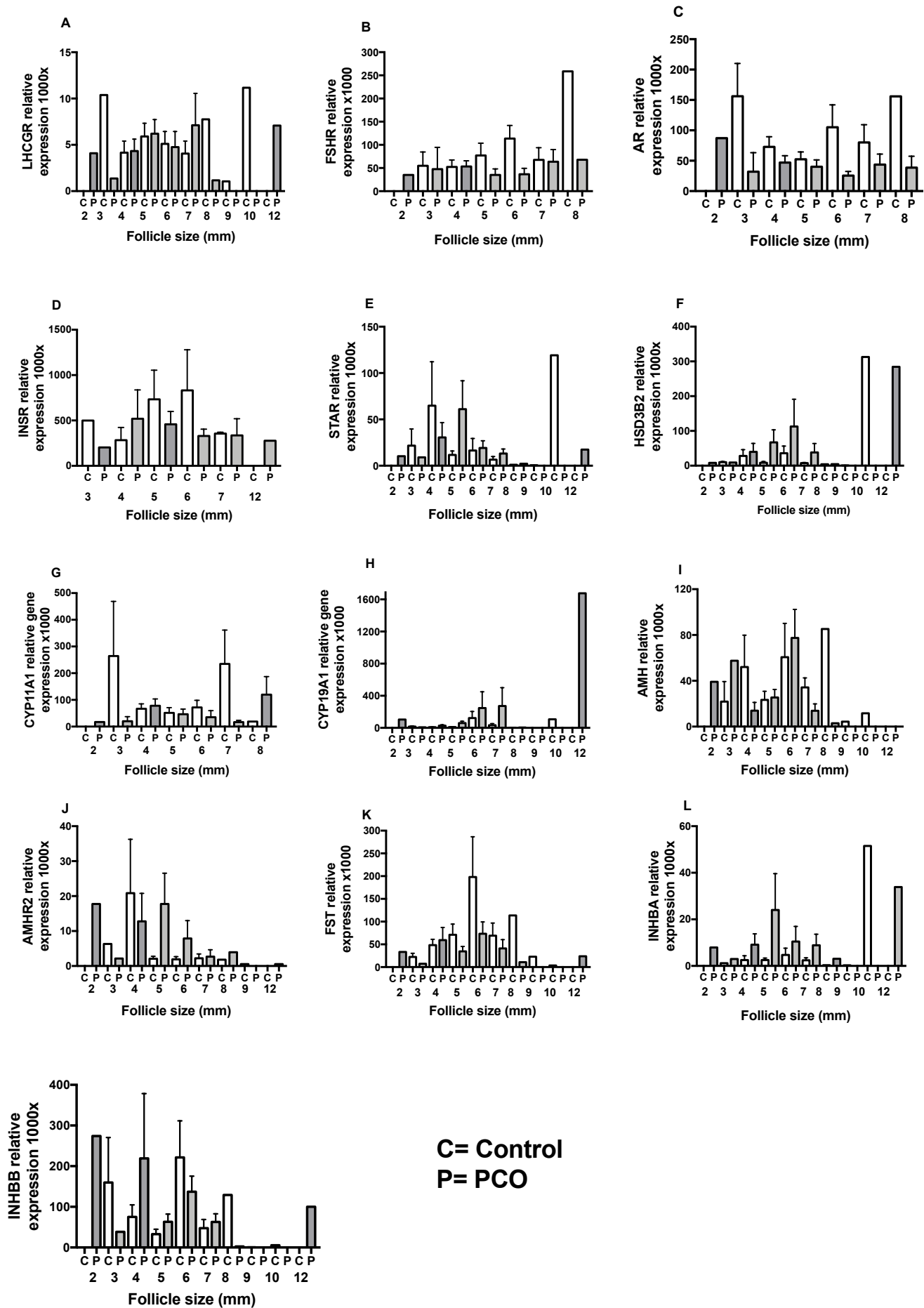


Figure 5.5 Gene expression for each follicle diameter (mm). There were no significant differences between gene expression in control and PCO samples at any particular follicle size in **A** LHCGR, **B** FSHR, **C** AR, **D** INSR, **E** STAR, **F** HSD3B2, **G** CYP11A1, **H** CYP19A1 INHBA, **I** AMH, **J** AMHR2 FST, **K** FST, **L** INHBA, **M** INHBB. Mean $\pm$ SEM, Kruskal Wallis test.

5.2.6 RNA sequencing

5.2.6.1 Differential gene expression in PCOS GCs

RNA sequencing was used to assess the transcriptional profile of GCs from control and PCO women. I found that 23 transcripts were differentially expressed between the two groups, 4 were over-expressed in PCO and 19 were under-expressed, after correction for the false detection rate (FDR) (Figure 5.6). The differentially expressed genes are listed in Table 5.3 and 5.4. The total number of gene transcripts was 13,286. The relative expression of each gene is given in the table as the log normalised counts per million (LogCPM), a value across all samples that gives an indication of overall expression (>5 = high expression).

Table 5.3 Genes over-expressed in PCO

Gene	Gene ID	Fold-change	FDR p-value	LogCPM
MT-ND1 pseudogene 23	MTND1P23	28.2	1.83E-08	9
MT-ND6 pseudogene 4	MTND6P4	17.9	1.83E-08	3.6
Kruppel-like factor 2	KLF2	4.1	0.0116	5.4
Serpin peptidase inhibitor, clade F (alpha-2 antiplasmin, pigment epithelium derived factor), member 1	SERPINF1	4.1	0.0271	3.9

Table 5.4 Genes under-expressed in PCO

Gene	Gene ID	Fold change	FDR p-value	LogCPM
Tubulin, beta 8 class VIII pseudogene 7	TUBB8P7	-85.5	1.83E-08	5.8
Actin-like 8	ACTL8	-53.6	0.0002	4.1
Zygote arrest 1-like	ZAR1L	-54.8	0.0013	2.8
NLR family, pyrin domain containing 4	NLRP4	-97.1	0.0039	3.1
Developmental pluripotency associated 3	DPPA3	-60.1	0.0039	0.6
H1 histone family, member O, oocyte-specific	H1FOO	-92.5	0.0039	2.2
NLR family, pyrin domain containing 7	NLRP7	-46.6	0.0116	1.3
KH domain containing 3-like, subcortical maternal complex member	KHDC3L	-27.8	0.0271	1.6
Oviductal glycoprotein 1, 120kDa	OVGP1	-11.1	0.0271	3.8
Tubulin, beta pseudogene 5	TUBBP5	-8.6	0.0271	3.9
NLR family, pyrin domain containing 9	NLRP9	-21.2	0.0324	1.9
Eukaryotic translation initiation factor 4E family member 1B	EIF4E1B	-53.8	0.0325	1.2
Purkinje cell protein 4 like 1	PCP4L1	-60.4	0.0356	0.6
Ladinin 1	LAD1	-27.4	0.037	1.45
Zygote arrest 1	ZAR1	-54.8	0.0373	1.4
NLR family, pyrin domain containing 13	NLRP13	-62.2	0.0374	1.5

NLR family, pyrin domain containing 11	NLRP11	-15.6	0.0387	2.3
DIRAS family, GTP-binding RAS-like 3	DIRAS3	-5	0.0397	3.2
WEE1 homolog 2	WEE2	-9.7	0.0398	2.6

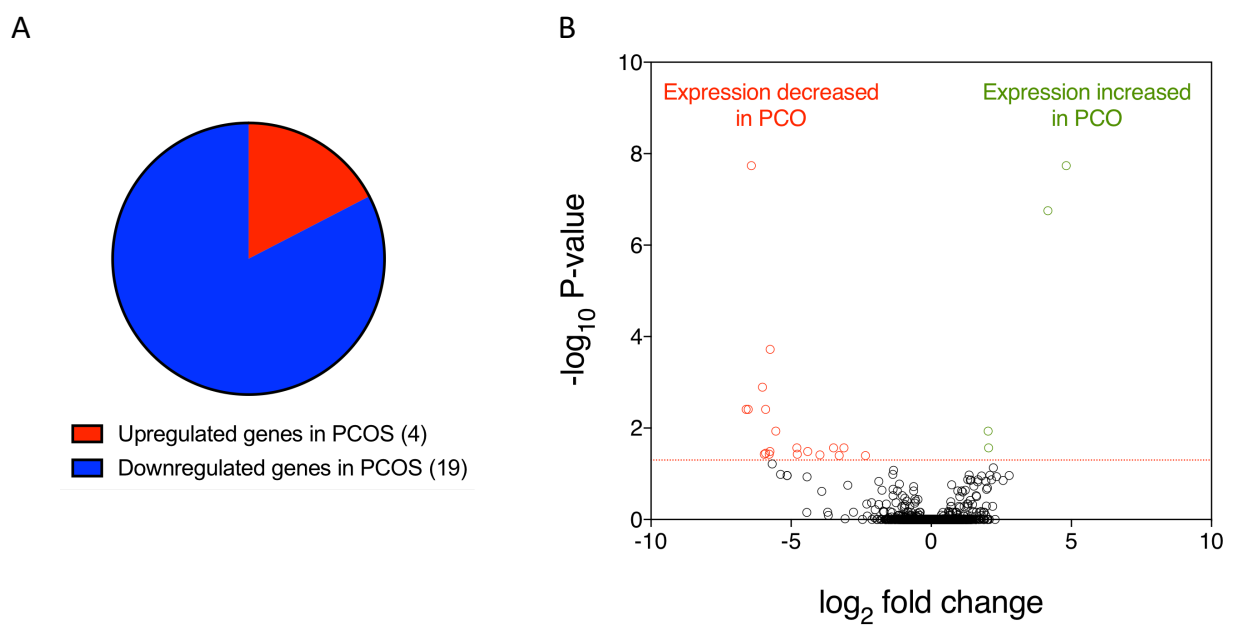


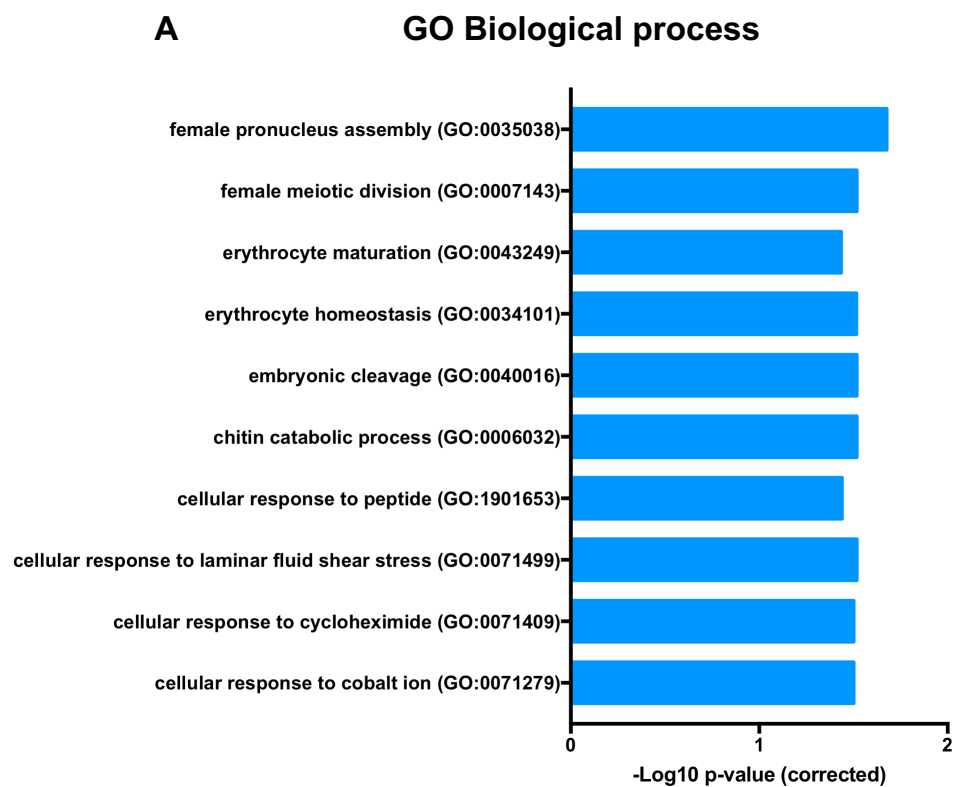
Figure 5.6 PCO transcriptional changes in GCs. **A** Pie chart indicating proportion of genes downregulated or upregulated by PCO **B** Volcano plot showing statistical significance ($-\log_{10} P$ value) plotted against $\log_2$ fold change for PCO vs control. Plot indicates significantly upregulated genes in green and downregulated genes in red.

5.2.6.2 Pathway enrichment and Gene Ontology analysis

Pathway and Gene Ontology (GO) analyses were used to understand the functions of the genes that were differentially expressed. InnateDB was used to complete both analyses (www.innatedb.com) (Breuer et al., 2013). Both KEGG (Kyoto Encyclopaedia of Genes and Genomes), (Moriya et al., 2007) and Reactome databases were used for pathway analysis

(Fabregat et al., 2018). After correcting for multiple comparisons there were no significant pathways found to be altered in PCO compared to control samples.

GO analysis assesses if there are specific biological categories that are enriched among the differentially expressed genes. It does this by comparing the frequency of individual genes that have altered expression with a reference list (the human genome). It searches within 3 categories; biological processes, cellular components or molecular functions. 44 GOs were found to be enriched in PCO; 33 in biologic processes, 8 in cellular component and 3 in molecular function. In the GO biologic process category the most enriched genes were in female pronucleus assembly, female meiotic division and embryonic cleavage (Figure 5.7A). In the GO cellular component the most enriched genes were in egg coat, perivitelline space and female pronucleus assembly and female germ cell nucleus (Figure 5.7B). In the GO molecular function category the most enriched genes were in oxidoreductase activity, chitinase activity and ATP binding (Figure 5.7C).



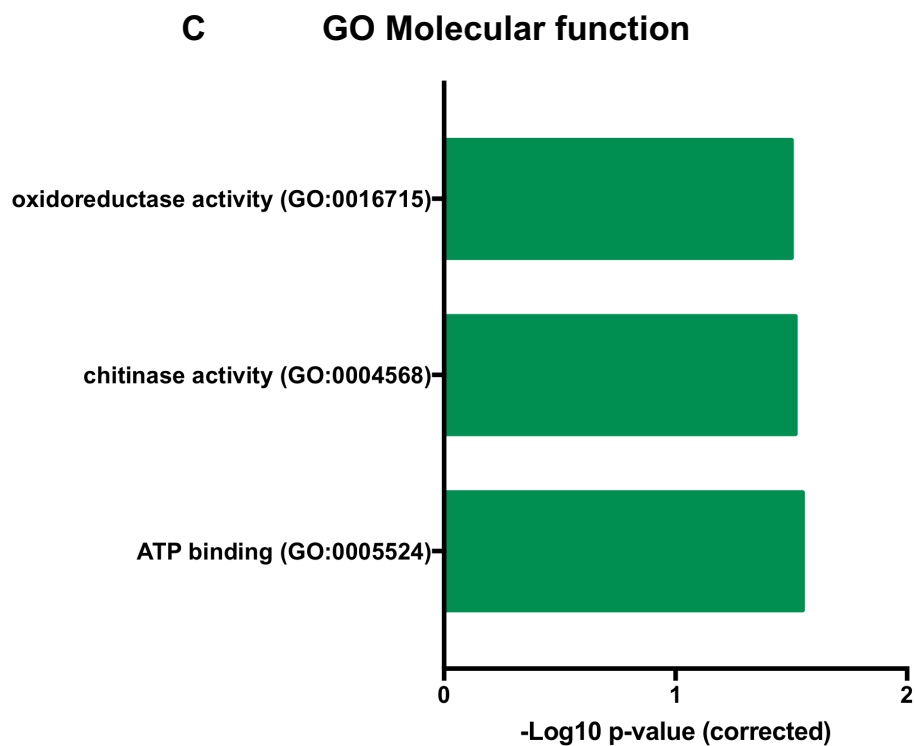
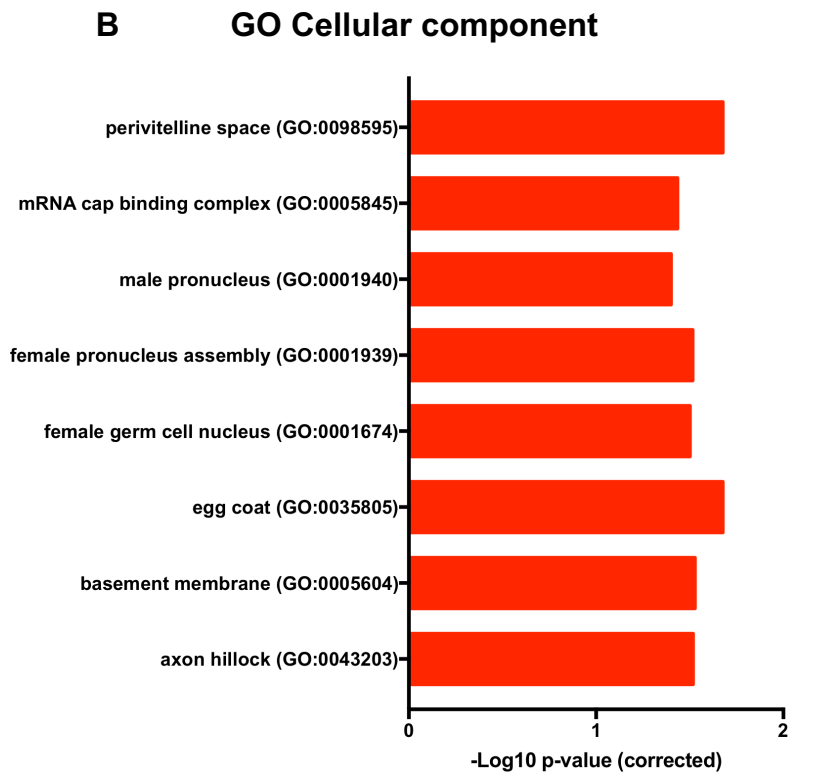


Figure 5.7 Gene Ontology (GO) of significantly overrepresented genes that are differentially in PCO. GO categories- A Biological process, B Cellular component and C Molecular function categories.

5.2.6.3 STRING protein-protein interaction network analysis

To gain a global view of the interactions occurring between the differentially expressed genes I used the STRING database to visualise protein-protein interactions (Figure 5.8). Network analysis clusters the proteins according to their biological function. There were 4 pseudogenes which do not encode proteins so were not included in the analysis. While there was no dense clustering between proteins, there were several proteins with shared biological functions, which were mainly shared with ZAR1 (zygote arrest 1) (Figure 5.8). ZAR1 encodes a protein that is thought to function in the initiation of embryogenesis and regulation of meiosis in female germ cells (Uzbekova et al., 2006, Wu et al., 2003b, Wu et al., 2003a).

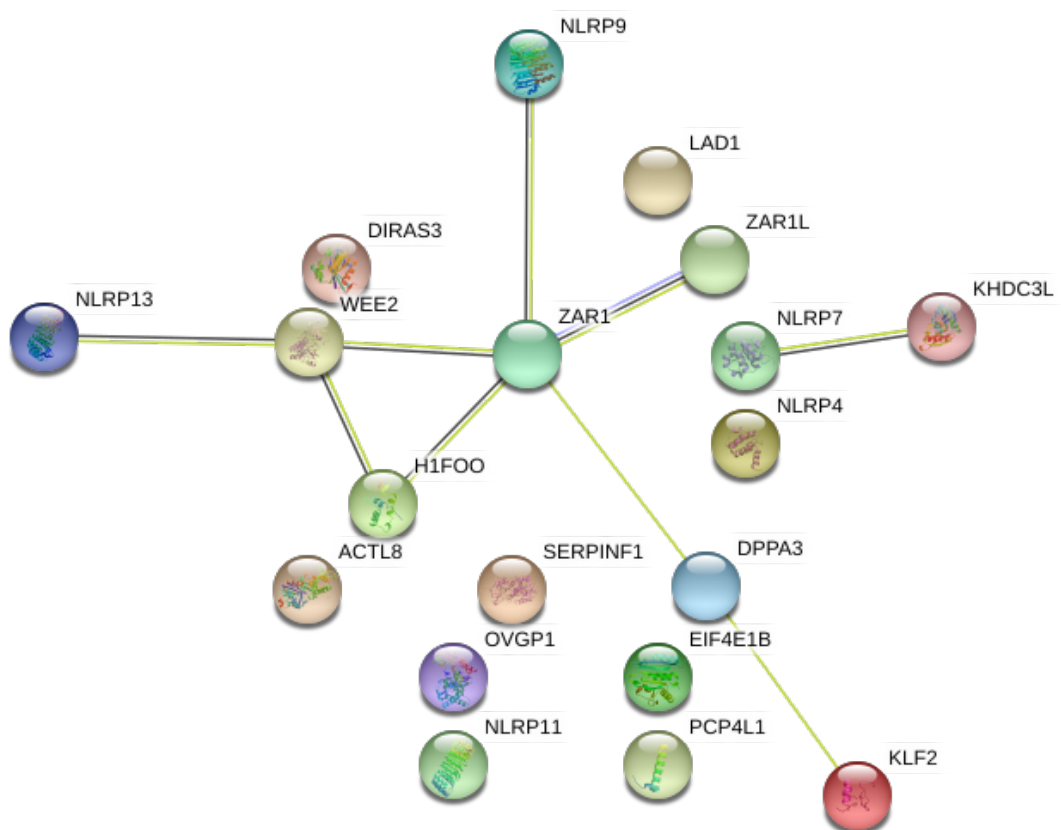


Figure 5.8 STRING protein-protein interaction network analysis demonstrating shared biological function between genes which are differentially expressed in PCO GCs. Filled nodes are those where some of the 3D structure is known. Empty nodes are those where the proteins have an unknown 3D structure. There is a small cluster of proteins linked through ZAR1. Most proteins shown are not known to have any shared biological function.

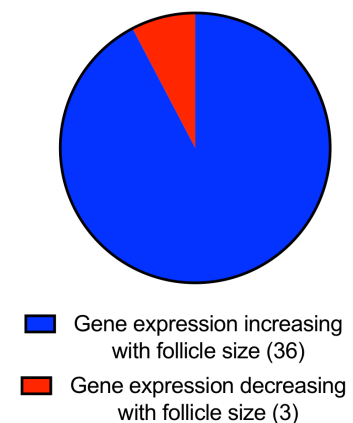
5.2.6.4 The effect of follicle size on gene expression

I also looked at the effect of follicle size on gene expression. For this, both control and PCO samples were combined to provide significant power for analysis. They were also analysed separately, however the data were insufficiently powered to provide significant changes. Changes in gene expression per 1 millimetre (mm) increase in follicle size were calculated. 39 genes changed expression with follicle size, and all but 3 of these were increased with increasing follicle size (Figure 5.9). The top 10 differentially expressed genes are given in Table 5.5.

Table 5.5 Top 10 genes differentially expressed with each 1mm increase in follicle size

Gene	Gene ID	Fold change	FDR p-value	LogCPM
Cytochrome P450, family 19, subfamily A, polypeptide 1	CYP19A1	1.62	3.45E-05	7.8
H19, imprinted maternally expressed transcript (non-protein coding)	H19	1.55	4.75E-05	11.2
Inhibin, alpha	INHA	1.55	0.0001	7.1
Cerebral cavernous malformation 2-like	CCM2L	1.59	0.0002	3.5
Cytochrome P450, family 11, subfamily A, polypeptide 1	CYP11A1	1.49	0.0012	6.4
Hydroxy-delta-5-steroid dehydrogenase, 3 beta- and steroid delta-isomerase 2	HSD3B2	1.43	0.0017	5.9
ATPase, Ca++ transporting, plasma membrane 4	ATP2B4	1.32	0.002	6.2
GRAM domain containing 1B	GRAMD1B	1.25	0.004	5.3
Rhomboid domain containing 2	RHBDD2	1.2	0.004	6.2
Inhibin, beta A	INHBA	1.4	0.0044	7.1

A



B

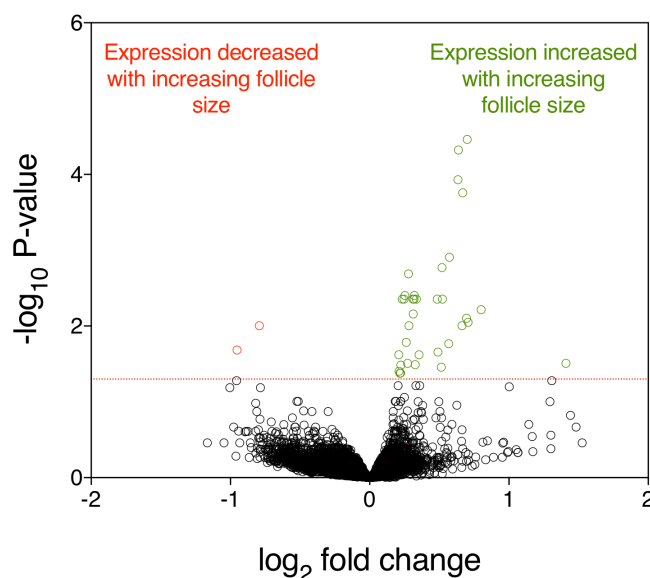


Figure 5.9 Transcriptional changes with increase in follicle size per millimetre in GCs. **A** Pie chart indicating proportion of genes downregulated or upregulated with each 1mm increase in follicle size. **B** Volcano plot showing statistical significance ($-\log_{10} [P \text{ value}]$) plotted against $\log_2$ fold change for gene expression changes with change in follicle size. Plot indicates significantly upregulated genes with increasing follicle size in green and downregulated genes with increasing follicle size in red.

5.2.6.5 Pathway enrichment and GO analysis

Again, InnateDB was used to complete pathway and GO analyses. The combined KEGG and Reactome pathway analysis revealed 15 enriched pathways. 11 of these 15 pathways were involved in steroid and lipid biosynthesis/metabolism and peptide and glycoprotein hormone biosynthesis/metabolism (Figure 5.10).

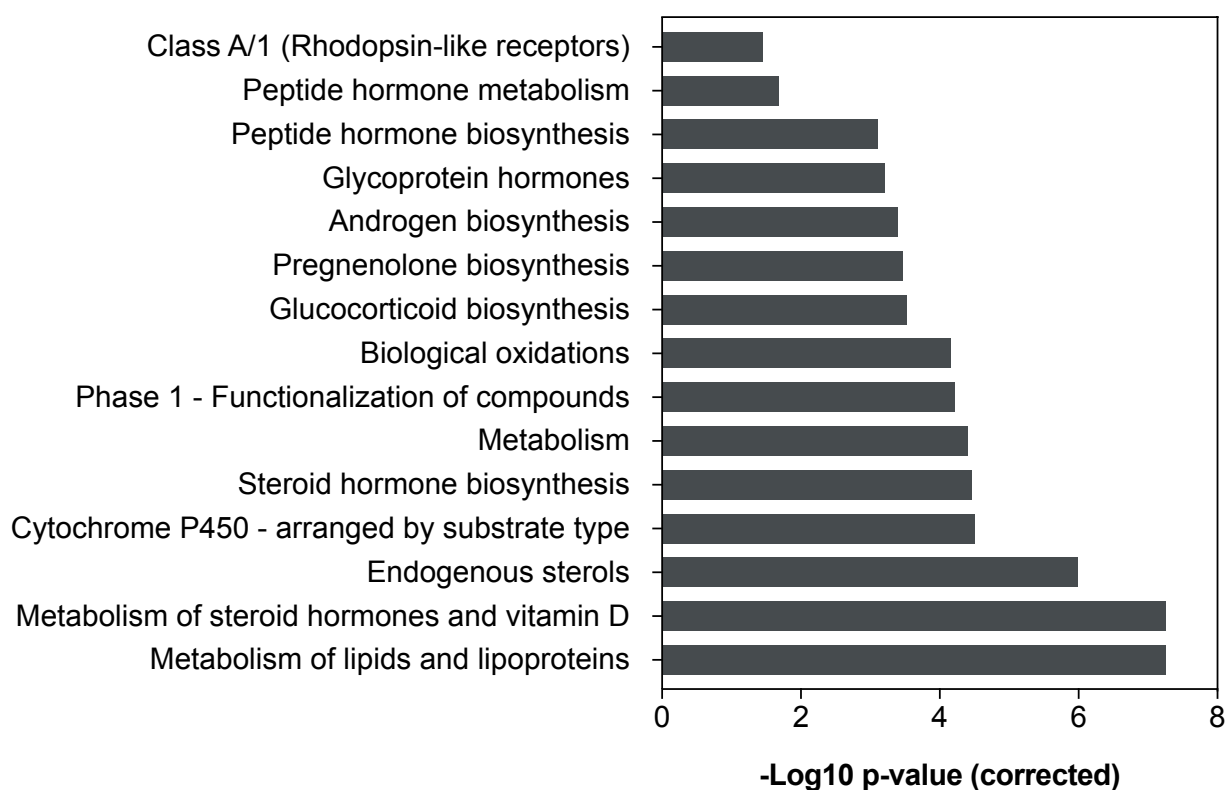
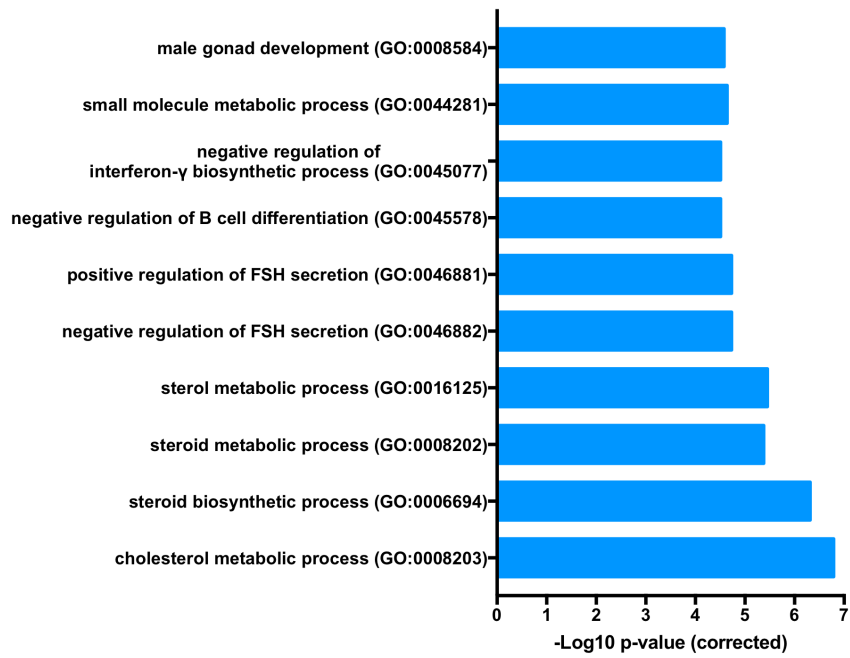
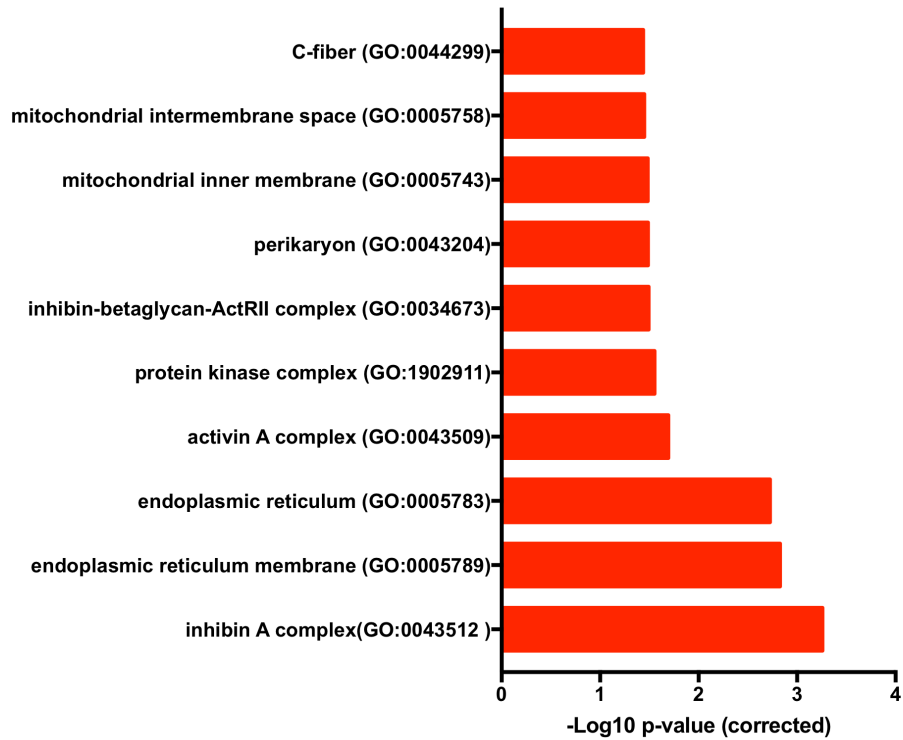


Figure 5.10 Combined KEGG and Reactome pathway analysis demonstrating pathways that are significantly enriched with each mm increase in follicle size.

Significant enrichment in 172 GOs were found, of which the 10 most significant in each category is given in Figure 5.11. The greatest changes in the GO biological process analysis were in cholesterol and steroid biosynthesis and metabolism, as well as regulation of FSH secretion (*INHBA*, *INHBB*) (Figure 5.11A). Changes in the GO cellular components were related to activin and inhibin complexes and endoplasmic reticulum, amongst others (Figure 5.11). GO molecular function enrichment was related to Haem and iron binding, receptor and GPCR binding, and hormone activity (Figure 5.11C).

A**GO Biological process****B****GO Cellular component**

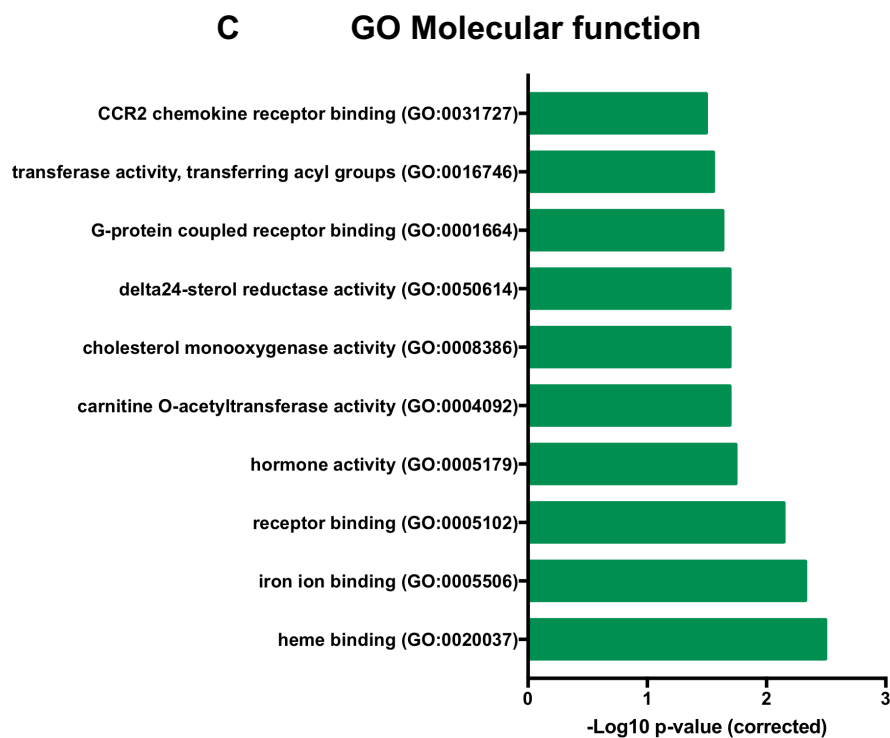


Figure 5.11 Gene Ontology (GO) of overrepresented differentially expressed genes with increasing follicle size. GO categories- A. Biological process, B. Cellular component and C. Molecular function categories.

5.2.6.6 STRING protein-protein interaction network analysis

Network analysis revealed distinct clusters of proteins according to their shared biological function. The largest cluster (Figure 5.12) shows a visually dense set of interconnected nodes that represent the interactions between 8 proteins. These proteins are all involved in steroidogenesis and ovarian gonadotropin function.

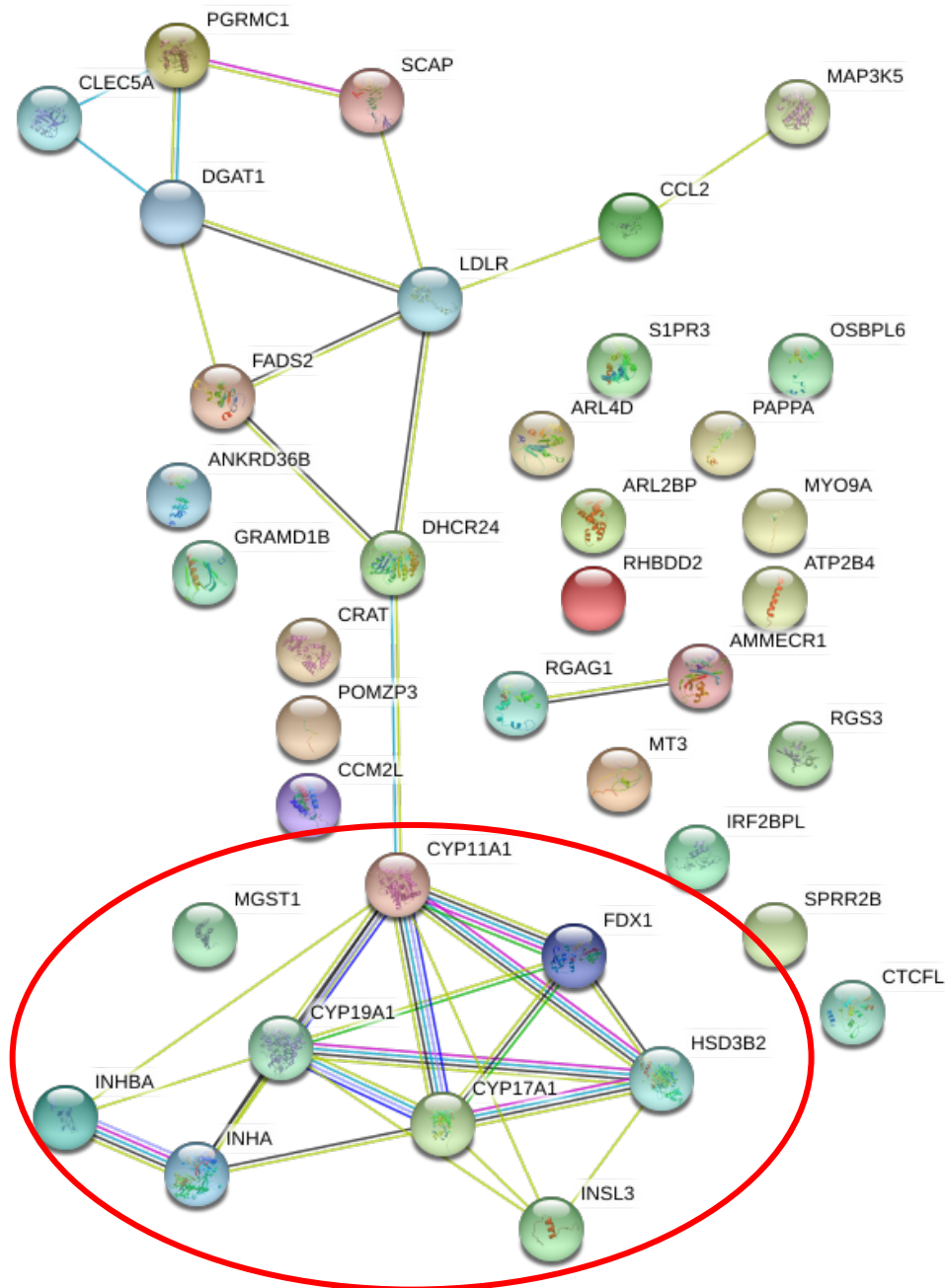


Figure 5.12 STRING protein-protein interaction network analysis demonstrating shared biological function between genes which have changing expression per mm increase in follicle size. Filled nodes are those where some of the 3D structure is known. Empty nodes are those where the proteins have an unknown 3D structure. The largest cluster (highlight in red) shows a visually dense set of interconnected nodes that represent the interactions between 8 genes. These genes predominantly control ovarian steroidogenesis.

5.3 Discussion

To my knowledge, this is the first systematic comparison of gene expression, using targeted RT-qPCR and RNA seq, in GCs of unstimulated hSAFs between normal and polycystic ovaries.

5.3.1 Targeted mRNA analysis

I first examined expression of key genes involved in growth and steroidogenesis with a particular emphasis on those factors that our group, and others, have previously implicated in the mechanism of follicle arrest and anovulation in PCOS (Mason et al., 1994, Willis et al., 1998). I found evidence of aberrant expression of gonadotropin receptors, *AR* and steroidogenic enzymes.

In GCs from unstimulated hSAFs, *LHCGR* was expressed at low levels (compared to GLCs) in samples from follicles of all sizes, from both control and PCO tissue (Figure 5.2), replicating a previous study of gene expression in follicles of normal women (Jeppesen et al., 2012a). Receptor expression did not correlate with follicle size. There was no overall increase in *LHCGR* seen in PCOS, however there was a wider distribution of expression in PCO follicles, with some expressing up to 20-fold higher than the median expression in GCs from normal ovaries. The lower overall level of *LHCGR* expression (in contrast to that of *FSHR*) in GCs from hSAFs is consistent with the observation that LHR is only functional once the antral follicle has reached a diameter of around 8-10 mm (and that in the dominant follicle alone) during the second half of the follicular phase of a normal cycle. This is supported by the lack of LH responsive steroid production in GCs until the follicle reaches a diameter of about 10mm (Hillier, 1994, Willis et al., 1998). However a significant proportion of hSAFs from women with PCOS show steroidogenic response to LH *in vitro* (Willis et al., 1998), a pattern that may echo the expression of *LHCGR* in PCO follicles in this study, with those that with higher expression prematurely producing progesterone. While I cannot assume changes in gene expression equates to altered protein expression and function, both *STAR* and *HSD3B2* (Figure 5.3), which were higher in PCO GCs, again suggesting augmented. GC steroidogenic activity and especially production of progesterone and 17-OH-progesterone. *StAR* is important in delivering cholesterol to the mitochondria as a substrate for CYP11A1, the first enzymatic step in steroidogenesis, but,

interestingly, expression of *CYP11A1* was significantly lower in GCs of PCO follicles despite the amplified progesterone response to LH observed in PCOS (Rice et al., 2005). *CYP11A1* is the key enzyme in metabolism of cholesterol to pregnenolone (and thereafter *HSD3B2* catalyses conversion to progesterone) but it is not unusual for there to be a discordance between gene and protein expression and/or function, which may be compensatory.

The underlying cause of hyperresponsiveness to LH in PCOS is not known. Insulin has been shown to augment not only basal production of oestradiol and progesterone, but also LH-stimulated steroid accumulation in GC cultures (Willis et al., 1996). Insulin receptor expression was not increased in PCO follicles in this study but insulin induction of *LHCGR* in GCs may have a part to play in women who are characteristically hyperinsulinaemic. High circulating LH levels and elevated LH pulse amplitude and frequency are typical biochemical features of PCOS (Balen, 1993) and may also contribute to premature LH responsiveness in GCs. Why it occurs in some but not all hSAFs also remains unclear. There has been one previous study in GCs from hSAFs which found a significantly higher expression of *LHCGR* in PCOS and, interestingly, this was a feature of most follicles analysed in that study (Jakimiuk et al., 2001). I also demonstrated a trend towards higher *LHCGR* expression in GLCs in PCOS, which has been discussed previously in Chapter 4.

There was a significantly lower expression of *FSHR* in GCs from hSAFs in PCO compared to control. *FSHR* is critical for FSH mediated follicle growth and development. The opposite might have been expected, as it has been shown in mouse and primate ovaries that androgens induce *FSHR* (Vendola et al., 1998, Laird et al., 2017), and given that there is higher production of oestradiol from PCOS GCs *in vitro* (Willis et al., 1998). However, in a study of rat ovaries (Orisaka et al., 2013) sustained follicle stimulation by LH decreases *FSHR* mRNA levels, *FSHR* signalling and inhibits FSH-induced follicular growth, so it is possible that inappropriate *LHCGR* activation inhibits *FSHR* expression in human follicles. In addition, I show in Chapter 6 (Figure 6.4) that LH treatment *in vitro* downregulates *FSHR*, therefore, reduced *FSHR* may be as a result of premature LH activity. Significantly, expression of *CYP19A1*, encoding P450 aromatase, was upregulated, which is consistent with increased production of oestradiol by GCs in response to FSH *in vitro*

(Mason et al., 1994) and does not support the notion that aromatase activity is reduced in SAFs from polycystic ovaries, as has been suggested, for example in the context of AMH action (Dewailly et al., 2016).

The reduced *FSHR* is also related to the significantly lower *AR* expression found in GCs from hSAFs in PCO. *AR* expression correlated strongly with *FSHR* (Figure 5.2E), as has been shown previously (Kristensen et al., 2017, Nielsen et al., 2011), suggesting a close interrelationship. Androgen treatment *in vitro* in certain models enhances *FSHR* expression, although the mechanism through which this happens is unknown (Laird et al., 2017). It is unlikely that the *AR* directly regulates *FSHR* as an androgen response element has not been identified on the *FSHR* gene promoter. A more likely explanation is that downregulation of both *FSHR* and *AR* is the result of enhanced LH activity. I show in Chapter 6 that *AR* expression is under the control of LH (Figure 6.5). LH treatment *in vitro* reduces *AR* expression. It is possible that the early responsiveness to LH seen in PCOS, likely as a result of some follicles expressing aberrantly high LHCGR and LH activity, inhibits *AR* expression, which in turn reduces *FSHR* expression. Reduced *AR* expression may also reflect increased exposure to androgen since our group have shown that, in mouse follicles, treatment with testosterone or dihydrotestosterone results in suppression of *Ar* in GCs (Laird et al., 2017). This combination of enhanced LHCGR activity, together with increased androgen action (acting by increasing intrafollicular cyclic AMP) may underpin the follicular arrest seen in PCOS (Franks et al., 2008). The action of LH on *AR* may also explain why *AR* falls dramatically in mature follicles after the LH surge. This pattern of expression manifests cardinal features of terminal differentiation of GCs or “premature luteinisation”.

It is significant that there were no differences between PCO and controls in gene expression of either AMH or its receptor. This suggests that the raised circulating AMH, consistently observed in PCOS, is a result of a higher number of antral follicles, rather than increased production of AMH per follicle. Indeed, Jeppesen and colleagues found a highly significant association between GC *AMH* mRNA expression and AMH protein level in the corresponding FF in a large number of individual antral follicles from normal human ovaries (Jeppesen et al., 2012b). They showed that *AMH* correlates with *AMHR2*, which I also found. Furthermore, *AMHR2* was also not significantly

different between control and PCO GCs in our study. These findings, together with evidence of increased, rather than diminished aromatase activity, support the contention that follicle arrest does not necessarily mean impaired steroidogenesis in these follicles.

Inhibins A and B have been shown to be lower in follicular fluid (FF) of size matched follicles in PCOS (Welt et al., 2005). Gene expression in GCs in this study showed the opposite, with higher *INHBA* in PCO and no significant difference in *INHBB*. This discordance has been seen previously in mature follicles (Fujiwara et al., 2000) and may reflect the temporal differences between GC gene transcription and protein translation and storage in FF. Follistatin expression in GC in this study was not different in PCOS, which matches the unchanged *FST* gene expression previously observed (Welt et al., 2005). The significance of increased *INHBB* expression in PCO GCs remains unclear.

This study also highlights GC dynamics and changes in expression between those from small antral follicles and large, luteinised follicles. GCs from large luteinised follicles have dramatically higher expression of genes involved in progesterone synthesis (*LHCGR*, *STAR*, *HSD3B2*, *CYP11A1*) and lower expression of genes involved in growth and oestradiol synthesis (*FSHR*, *CYP19A1*, *AMH*, *AMHR2*, *FST*) when compared to GCs from hSAFs.

The major strength of this study is the use of large numbers of unstimulated hSAFs from ovarian material that is normally very difficult to obtain. These GCs have not been exposed to exogenous gonadotropins as part of an ovarian stimulation process. This is, to my knowledge, the largest and most comprehensive study of GCs from hSAFs in PCOS and provides valuable insight into the pathogenesis of follicle arrest in PCOS.

The samples from hSAFs were obtained from women with clear morphological evidence of polycystic ovaries but an obvious limitation of the study is that I was not able to determine whether there were clinical features of polycystic ovary *syndrome*. Nevertheless, the clear

differences that I have found in gene expression between normal and PCO GCs remain valid and, if anything, may underestimate differences between normal follicles and those from women with PCOS. Another potential limitation of this data is the relatively small range of follicle size and the lack of data from follicles of greater than 8mm. However, it is the follicles that fall within this range that undergo arrest in women with PCOS and in which our lab has previously shown significant differences between normal and PCOS subjects in gonadotropin responsiveness (Mason et al., 1994, Willis et al., 1998). I did not see any obvious correlation between gene expression and follicle size, as has been shown in some previous studies, but that is likely to be because the majority of samples were from follicles between 4-7 mm whereas previously reported changes with follicle size were most pronounced when comparing results in small with large antral follicles (Kristensen et al., 2017).

In summary, GCs from women with PCO display an altered profile of gene expression compared to women with regular cycles, with increased *LHCGR* in a subpopulation of follicles, reduced *CYP11A1*, *FSHR* and *AR*, increased *STAR* and *CYP19A1*, increased *INHBB*. There were, however, no differences between PCO and controls in *INHBA*, *FST*, *AMH* or *AMH* receptor. Overall this picture is consistent with “premature luteinisation” in PCOS and is summarised in Figure 5.13. This contributes to the growing body of knowledge around the complex abnormal follicle dynamics in PCOS.

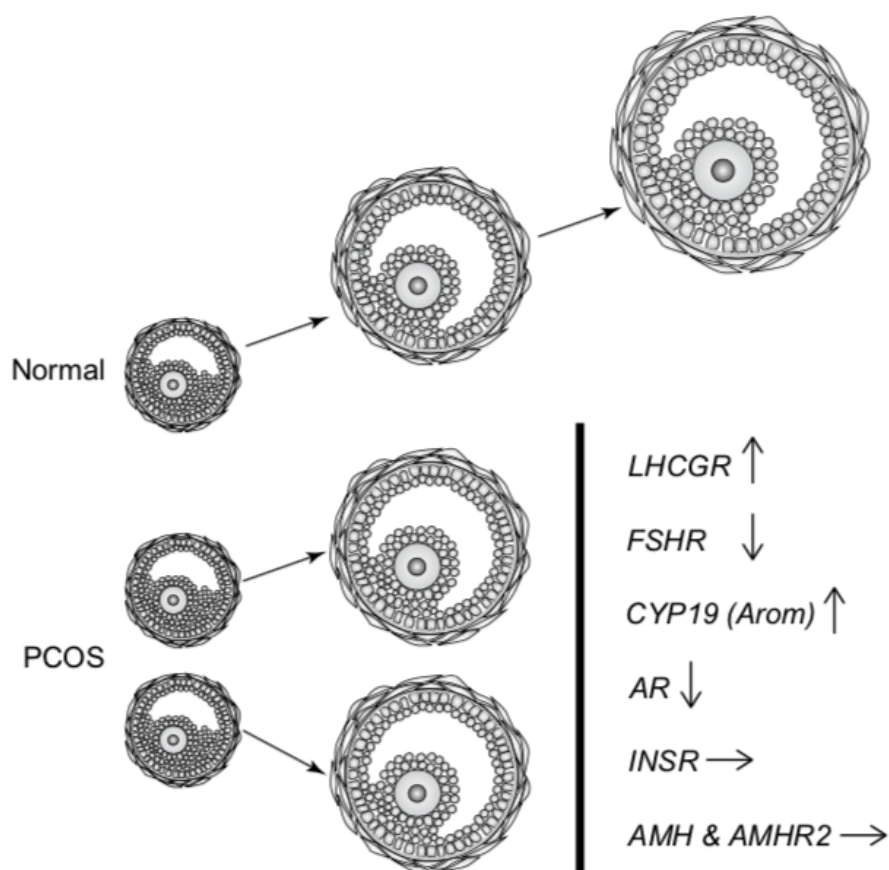


Figure 5.13 Summary of the key findings that we postulate are causally related to the mechanism of follicle arrest in PCOS. Expression of LHCGR is increased in a significant subpopulation of granulosa cells (GCs) in small antral follicles (SAFs) from PCOS. Increased LH activity may account for downregulation of both FSHR and AR but increased aromatase (CYP19A1) expression is consistent with the notion that steroidogenesis is exaggerated, or at least conserved, in arrested follicles (Franks et al., 2008). Hyperinsulinemia may contribute to follicle arrest but expression of insulin receptor (INSR) is unchanged. Expression of both AMH and AMHR is similar in normal and PCO follicles and the role of AMH in arrested follicle growth in PCOS remains uncertain. (Figure courtesy of Kate Hardy).

5.3.2 RNA sequencing

RNA sequencing was used to assess the transcriptional profile of GCs from control and PCO women. It was also used to profile changes in GC expression with increasing follicle size. To the

best of my knowledge this is the first-time transcriptomic analysis has been used to study these cells.

Surprisingly a very small number of transcripts were differentially expressed in PCOS. A number of these genes were also pseudogenes, which do not encode proteins. This is vastly different to our group's RNA sequencing project in GLCs, which showed that over 450 genes were differentially regulated in GLCs from women with and without PCOS (Lerner et al, unpublished). This disparity is likely to be for a number of reasons. The exogenous medications and IVF protocols may alter gene expression. Also, many of the genes found to be differentially regulated in the GLC project were related to cholesterol metabolism and steroidogenesis, so it may be that these genes are activated/regulated by LH activity. Or it may just be that biologically the GCs peri-ovulation differ more in PCOS than those from hSAFs.

While pathway analysis did not reveal any specific enriched pathways, GO analysis provided some insight into the biological processes these enriched genes are involved in. Interestingly female meiotic division is enriched in PCO GCs. The ovaries of women with PCOS show an increased density of preantral and antral follicles, including primordial and primary follicles (Webber et al., 2003). Perhaps this is associated with differences in regulation or activation of meiosis. Basement membrane genes were also highlighted and altered function of this structure has been previously implicated in the aetiology of PCOS. Alterations have been observed in the production and distribution of important basement membrane components type IV collagen, TIMP-3 and TIMP-4 (Oksjoki et al., 2004). Ovaries from a PCO mouse model have also been shown to have increased blood flow and enhanced selectivity of molecular permeation through blood follicle barriers (Zhou et al., 2008). Based on the above data, these may be in part due to differential gene regulation in GCs.

SERPINF1 was one of 4 genes upregulated in PCO. Also known as pigment derived endothelial factor (PEDF), it is an inhibitor of angiogenesis that negates VEGF activity (Chuderland et al., 2014). It has been shown previously to be higher in the serum of women with PCOS and to be associated with insulin resistance (Yang et al., 2011). PCOS is associated with impaired angiogenesis and excessive vascular formation (Amin et al., 2003). Ovarian hyperstimulation

syndrome, which women with PCOS are more likely to develop, is also associated with impaired angiogenesis and reduced PEDF (Chuderland et al., 2013).

Embryos from women with hyperandrogenic PCOS in one study showed slower breakdown and development from fertilisation to the 8 cell stage (Wissing et al., 2014). Embryonic cleavage was enriched in PCO GCs. However it is difficult to know the relevance of this in GCs from small follicles, which do not play a role directly in embryonic cleavage.

Further analysis on this data is currently ongoing in conjunction with Oxford Genomics Centre. We are doing an expression quantitative trait loci (eQTL) analysis (Nica and Dermitzakis, 2013). This analysis involves a direct association test between markers of genetic variation with gene expression levels typically measured in tens or hundreds of individuals. This has previously been done comparing GWAS data in PCOS with serum, fat and skin biopsies from women with and without PCOS (Pau et al., 2017). They found associations between certain genotypes and inflammation and variations in gonadotropins and gene expression in skin and fat. The vast majority of GWAS risk loci fall in non-coding regions of the genome. This may be because the GWAS risk loci alter the individual's disease risk via their effect on gene expression in different tissues. Analyses such as eQTL help to determine which genes are affected in which tissues. Another related analysis which we are completing on this data is an eCAVIAR analysis (eQTL and GWAS Causal Variant Identification in Associated Regions). This model allows for allelic heterogeneity, where multiple variants can be causal in a single locus, a phenomenon that is widespread in eQTL data (Hormozdiari et al., 2016).

Some of the fold changes in the PCO GCs were quite dramatic, with over 90 fold downregulated (*H1FOO*). However many of these genes also had a low expression suggesting expression of these genes is low. It is hard to speculate on the biological relevance of changes in genes which are of very low expression. Future work could examine protein expression, although this would be difficult given the small quantity of material when GCs are examined from individual SAFs, and the effect of SiRNA knockdown of certain genes (for example *H1FOO*, which may have a role in initiation of oocyte growth (Tanaka et al., 2005) on follicle development in normal and PCO

mouse ovary cultures. Some of these genes, especially those which align with GWAS data, and those which are also known to be altered in serum (such a SERPINF1/PEDF), could potentially be used as biomarkers of PCOS.

The transcriptomic analysis of GC changes with increasing follicle size revealed a group of genes that predominantly increased in expression with each 1mm increase in follicle size. STRING and GO analysis revealed interaction and enrichment in genes responsible for steroid and cholesterol biosynthesis and metabolism, regulation of FSH secretion and peptide hormone biosynthesis and metabolism and GPCRs and receptor binding. This demonstrates clearly that follicles are enhancing their steroidogenic capacity and receptor activity as they increase in size, preparing for ovulation and luteinisation. Endoplasmic reticulum (ER) and ER membrane were also enriched. ER proteins and stress are thought to be essential for ovarian follicle development and are implicated in follicle atresia (Sun et al., 2018, Huang et al., 2017). These data also support the view that ER has a role in follicle development.

The significant gene changes in RT-qPCR were not significantly different using RNA sequencing. This is not surprising as RNA sequencing detects thousands of transcripts and determines significance of these through correcting for multiple comparisons, as opposed to targeting and detecting a known sequence. The main difference between using RNA sequencing and RT-qPCR in general relate to discovery power and scalability, with both offering highly sensitive and reliable variant detection. It is difficult to compare and link the results of the two techniques, as they were done on mainly different samples, with different biological procedures and different statistical analyses.

5.3.3 Conclusions

Using targeted RT-qPCR I have highlighted the key genes underlying 'prematurely luteinised' follicles in PCO, with reduced *CYP11A1*, *FSHR* and *AR*, increased *STAR* and *CYP19A1*, increased *INHBB*, and no differences in *INHBA*, *FST*, *AMH* or *AMH* receptor. LHCGR expression was not different but there was a wider variation in expression in the PCO samples, with some having dramatically higher expression

RNA sequencing of PCO GCs revealed that very few genes were differentially expressed, and these were mainly downregulated and had low levels of expression. The genes that were significantly different using RT-qPCR were not significantly different using RNA sequencing, likely due to differences in the samples used, as well as differences in the techniques themselves and the statistical analysis. The role of most of the genes differentially expressed in the RNA sequencing in the ovary is unclear. Future work could involve measuring protein expression and to assess the impact of these on follicle growth using a knockdown mouse model. Future work currently ongoing will compare this data to known GWAS data. This could ultimately lead to identifying a potential biomarker of PCOS.

The differences between using RNA sequencing and RT-qPCR in general relate to discovery power.

RNA sequencing of GCs from follicles of different sizes has highlighted the changing role of the follicle as it grows, with increasing expression of genes controlling regulation of gonadotropins and steroidogenesis.

Chapter 6

The effects of *in vitro* DHT treatment on gonadotropin receptor function and steroidogenesis in human granulosa lutein cells

6.1 Introduction

Androgens play a critical role in the normal development and functioning of the female reproductive system. They act as a precursor for oestradiol, but they also have direct roles, via the androgen receptor (AR). Androgens are synthesised by theca cells, and subsequently diffuse into granulosa cells where they are aromatised to oestrogens (Hillier et al., 1994, Ghayee and Auchus, 2007; Burger et al, 2002). The AR mediates its effects by binding to genomic androgen response elements (ARE), which influence transcription of AR target genes, and via membrane-initiated non-genomic signalling. AR expression is highest in preantral/antral follicle and declines as the follicle matures (Figure 5.2D) (Hillier et al., 1997, Suzuki et al., 1994). The role of androgens in pre-antral/antral follicle development has been studied, however androgen action around the time of ovulation is unclear. Excess androgen is a key defining feature of PCOS and may affect ovarian function throughout the cycle.

Work from our lab (Laird et al., 2017) found that *in vitro* treatment of mouse follicles and granulosa cells with dihydrotestosterone (DHT) increased *Fshr*, *Star*, and decreased *Cyp11a1*. Similarly, in primate granulosa cells treatment *in vitro* with androgen augments *Fshr* (Weil et al., 1999, Sen and Hammes, 2010). *In vitro* treatment of rat pre-antral granulosa cells with testosterone stimulated progesterone production (Hillier et al., 1977). It also potentiated steroidogenic enzyme induction and augmented FSH-dependent cAMP accumulation in culture media (Hillier and de Zwart, 1982). The presence of androstenedione also augments FSH-dependent E₂ secretion in bovine granulosa cells (Hamel et al., 2005). So altogether, this points to androgen augmenting the expression and function of FSHR.

Androgens may also affect the LHR. We know that granulosa cells from certain small antral follicles from women with PCOS produce progesterone in response to LH and produce excess oestradiol in response to FSH compared to cells from women who do not have PCOS (Mason et al., 1994, Willis et al., 1998). It is not known exactly what mediates this augmentation, however AR expression positively correlates with both *LHCGR* and *FSHR* in human GCs (Figure 5.2)(Jeppesen et al., 2012a, Kristensen et al., 2017).

I hypothesised based on the evidence above that *in vitro* treatment with androgens may affect gonadotropin receptor function in GLCs. DHT was used for this *in vitro* treatment. DHT is formed from testosterone via the enzyme 5 alpha reductase (see Figure 1.2). It is the most potent agonist of the androgen receptor and unlike testosterone, does not aromatise to oestrogen.

6.1.1 Specific aims

The aims were to address the following questions:

1. Does DHT treatment *in vitro* affect LH and FSH receptor signalling in GLCs?
2. Does DHT treatment *in vitro* affect gene expression of gonadotropin receptors and steroid enzymes?
3. Does DHT affect LH and FSH induced steroidogenesis *in vitro* in GLCs?

6.2 Methods and Results

6.2.1 DHT augments LH induced cAMP accumulation but does not affect FSH action

In Chapter 4 I demonstrated that cAMP levels in response to LH were augmented in PCOS GLCs (Figure 4.1). While this may be due to increased LHR expression, and increased expression of receptor trafficking enzymes, I hypothesised that it may also be due to a direct androgen effect. To test this, after 3 days in culture GLCs from control women were pre-treated with DHT 10nM for 24 hours prior to treatment with LH or FSH. This dose was used as it has been demonstrated by our group to augment granulosa cell gene expression and function in both human and mouse models (Lerner et al., 2019, Laird et al., 2017). I tested this dose and also found it significantly affected gene expression and signalling assays (discussed below), therefore further doses were not used.

Untreated cells were incubated with ethanol as a vehicle control. DHT alone did not stimulate cAMP production. DHT pre-treatment augmented the cAMP response to LH (Figure 6.1A). 77% (17 of 22) samples showed this increase in cAMP in response to LH with DHT pre-treatment. DHT pre-treatment did not affect the cAMP response to FSH (Figure 6.1B).

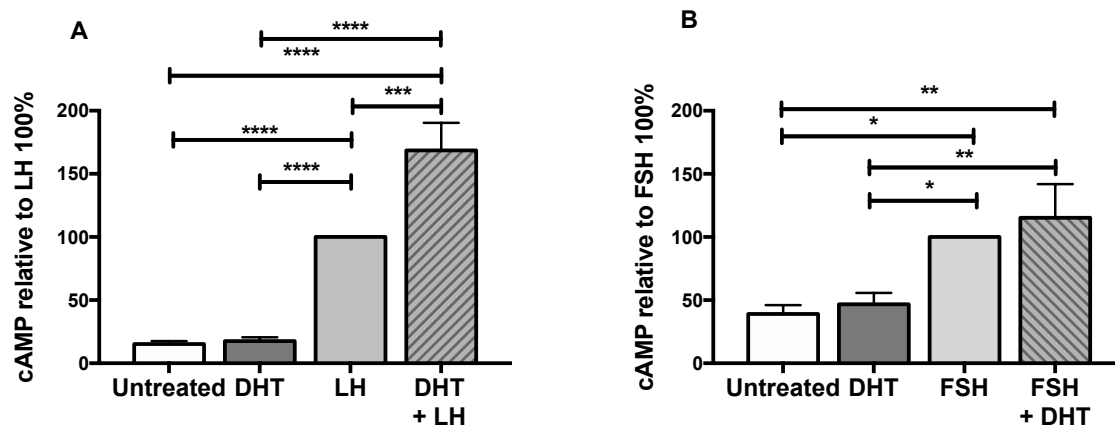


Figure 6.1 The impact of in vitro treatment with DHT on cAMP production in response to LH and FSH in GLCs. After 3 days in culture GLCs were treated with DHT 10nM or ethanol (vehicle control) for 24 hours. They were then treated with LH or FSH 10nM for 5 mins and cAMP assay completed. Data is normalised to the LH response (LH 100%). A DHT alone did not stimulate cAMP production. LH stimulated cAMP production ($p < 0.0001$). Pre-treatment with DHT augmented the response to LH ($p < 0.001$), ($n = 22$). B DHT alone did not stimulate cAMP production. FSH stimulated cAMP production ($p < 0.05$). DHT pre-treatment did not affect cAMP production in response to FSH ($n = 13$ mean $\pm$ SEM, One-way ANOVA used for both).

6.2.2 DHT augments pERK 1/2 signal intensity after LH treatment but does not affect FSH

In order to measure signalling further downstream of Gs/cAMP/PKA pathway, I measured pERK production in response to FSH and LH, with or without pre-treatment with DHT. The ERK pathway can also be activated by other G proteins and arrestins. Pre-treatment with DHT augmented the pERK 1/2 signal intensity generated in response to LH at western blot. 78% (11 of 14) samples showed this increase in pERK in response to LH with DHT pre-treatment. DHT did not affect the pERK 1/2 response to FSH.

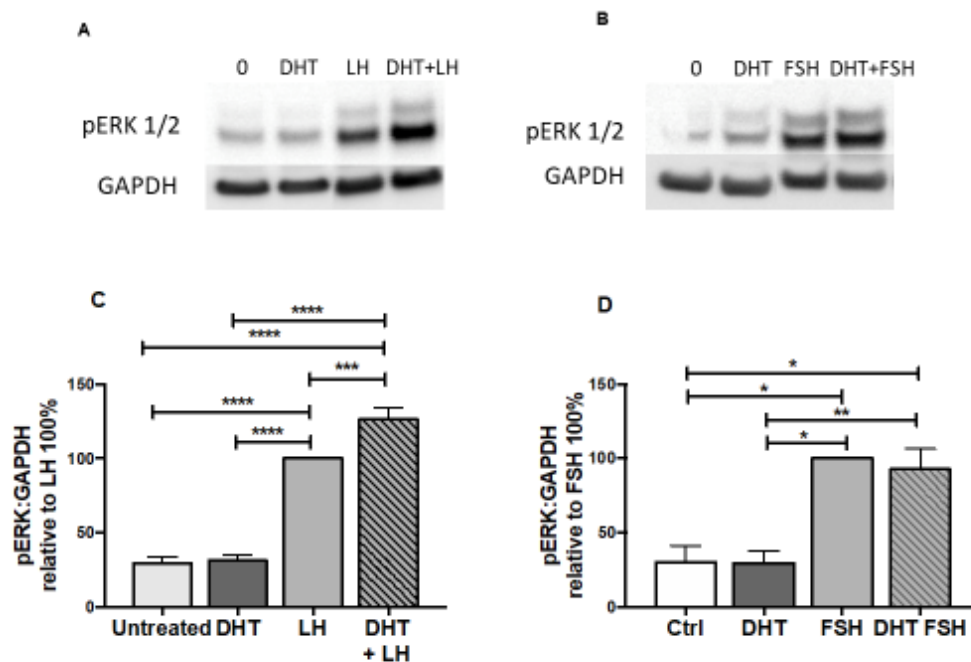


Figure 6.2 The impact of *in vitro* treatment with DHT on pERK 1/2 generation in response to LH and FSH in GLCs. After 3 days in culture GLCs were treated with 10nM DHT or ethanol (vehicle control) for 24 hours. They were then treated with LH or FSH 10nM for 5 minutes. Western blot was completed and probed for pERK 1/2. Data is normalised to the LH response (LH 100%). **A** Representative western blot demonstrating the effects of LH and DHT treatment on pERK 1/2 generation **B** Representative western blot demonstrating the effects of FSH and DHT treatment on pERK 1/2 generation **C** DHT treatment for 24 hours did not stimulate an increase in pERK. LH stimulated an increase in pERK ($p < 0.0001$). DHT pre-treatment augmented the LH induced increase in pERK ($p < 0.001$). One-way ANOVA, $n = 14$. **D** DHT treatment for 24 hours did not stimulate an increase in pERK. FSH stimulated an increase in pERK ($p < 0.05$). DHT pre-treatment did not affect the FSH induced increase in pERK, ($n = 8$, mean $\pm$ SEM, Kruskal Wallis test).

6.2.3 DHT augments LH induced IP-1 accumulation

After 3 days in culture GLCs were incubated with DHT 100nM or ethanol (vehicle control). After 24 hours, cells were incubated with LH 100nM for 2 hours and then IP-1 accumulation assay

carried out. Higher ligand concentrations were used as they are required to stimulate Gq signalling (Breen et al., 2013). DHT alone did not result in any significant change in IP-1 compared to vehicle control (Figure 6.3). LH treatment significantly increased IP-1. The combination of DHT and LH did not significantly further increased IP-1 accumulation compared to LH alone. Some samples (6 of 9) did show and increase with DHT and LH compared to LH alone, however as some samples (3 of 9) did not show any further increase in IP-1 with the combination of DHT and LH, the overall average increase was 8% and was not significant.

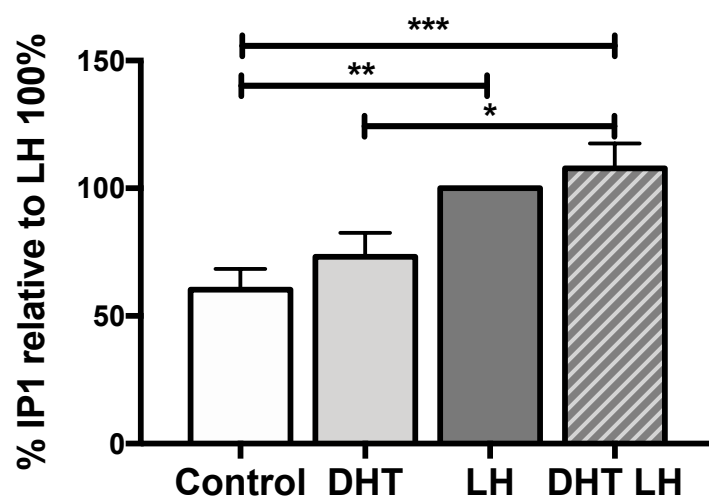


Figure 6.3 IP-1 accumulation (normalised to protein content) in GLCs treated with ethanol or DHT 100nM and then treated with LH 100nM for 2 hours. Data is normalised to the LH response (LH 100%). GLCs were treated with ethanol or DHT 100nM for 24 hours and then LH 100nM for 2 hours, (n=9). DHT alone did not increase IP-1. LH significantly increased IP-1 ($p<0.01$). DHT + LH further increased IP-1 accumulation by a further 8% ($p<0.001$). One-way ANOVA.

6.2.4 Effect of DHT and gonadotropins on receptor gene expression

In order to assess if the effects of DHT on gonadotropin receptor signalling were correlated with changes in gene expression of receptors, I measured mRNA levels of *LHCGR*, *FSHR* and *AR* in GLCs after treatment with DHT, with or without LH and FSH. LH and FSH treatment *in vitro* both upregulated *LHCGR* (Figure 6.4A). Unlike animal models, there was no significant increase in *FSHR* expression with DHT treatment alone (Figure 6.4B). LH and FSH treatment both

downregulated *FSHR* expression (Figure 6.4B). The addition of DHT in addition to LH and FSH did not affect expression of *LHCGR* or *FSHR* (Figure 6.4).

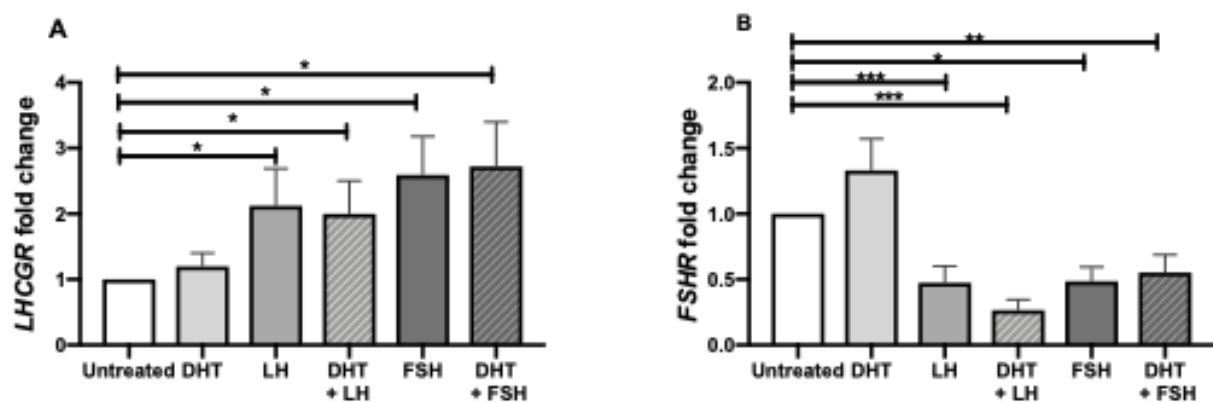


Figure 6.4 LHCGR and FSHR gene expression in vitro in GLCs treated with DHT, LH or FSH. GLC were cultured for 3 days and then treated with DHT 10nM or ethanol (vehicle control) for 24 hours. GLCs were then treated with DHT/ethanol, and LH or FSH 10nM for a further 24 hours. Cells were then lysed for RNA extraction, cDNA synthesis and RT-qPCR. Results are represented as fold change relative to the vehicle control (untreated normalised to 1). **A** DHT treatment alone did not affect LHCGR. LH and DHT + LH upregulated LHCGR to a similar degree ($p < 0.05$). FSH and FSH + DHT treatment upregulated LHCGR to a similar degree ($p < 0.05$). **B** DHT treatment alone did not affect FSHR, although there was a trend towards an increase in expression. LH and DHT + LH treatment downregulated FSHR ($p < 0.001$). FSH and FSH + DHT treatment also downregulated FSHR ($P < 0.05$, $p < 0.01$ respectively). $N = 10$, Kruskal Wallis test, mean $\pm$ SEM.

DHT treatment *in vitro* increased AR. LH downregulated AR expression, as did the combination of DHT and LH to a similar degree. FSH, both alone and combined with DHT, did not affect AR.

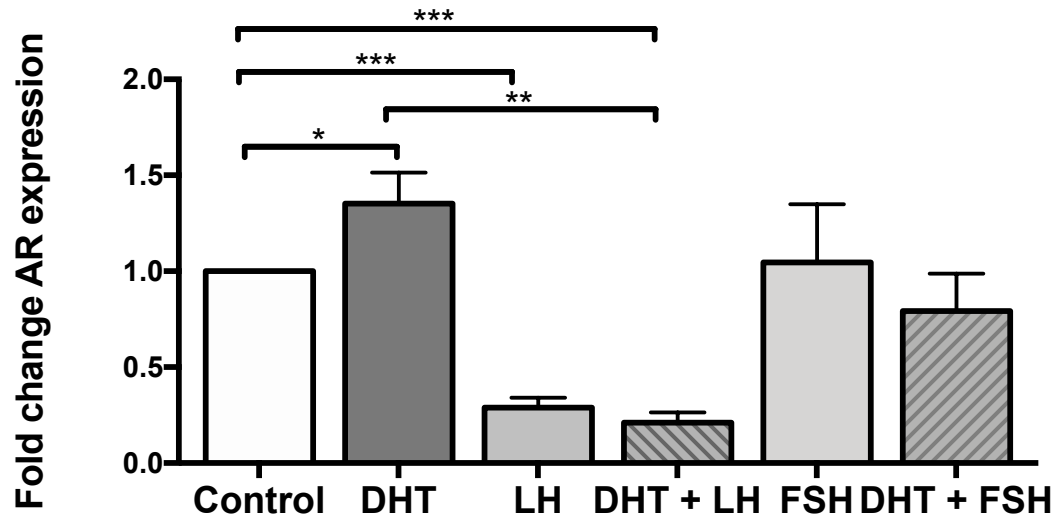


Figure 6.5 Androgen receptor (AR) gene expression in vitro in GLC treated with DHT, LH or FSH. (Work completed by Celia Simpson under my supervision). GLC were cultured for 3 days and then treated with DHT 10nM or ethanol (vehicle control) for 24 hours. GLCs were then treated with DHT/ethanol, and LH or FSH 10nM for a further 24 hours. Cells were then lysed for RNA extraction, cDNA synthesis and RT-qPCR. Results are represented as fold change relative to the vehicle control (untreated normalised to 1). DHT treatment increased AR expression ($p < 0.05$). LH and DHT+LH treatment reduced AR expression ($P < 0.001$). FSH and DHT+FSH treatment did not affect AR. (One-way ANOVA. $N=9$, mean $\pm$ SEM).

6.2.5 DHT treatment *in vitro* does not affect steroid gene expression

DHT treatment *in vitro* in GLCs appears to augment gonadotropin receptor signalling, which may be related to changes in receptor expression. To study if DHT induced changes in steroid enzyme gene expression, I treated GLCs with DHT for 24 hours, and then a further 24 hours with or without LH or FSH. DHT treatment alone did not affect gene expression. LH and FSH increased *STAR* and *CYP11A1* expression (Figure 6.6 A+B) and did not affect *HSD3B2* or *CYP19A1* expression. The combination of DHT and LH did not significantly change gene expression compared to LH alone, when compared with LH alone. FSH treatment increased *STAR*, *CYP11A1*, *HSD3B2* and *CYP19A1* expression (Figure 6.6). The combination of DHT and FSH did not change expression significantly compared to FSH alone.

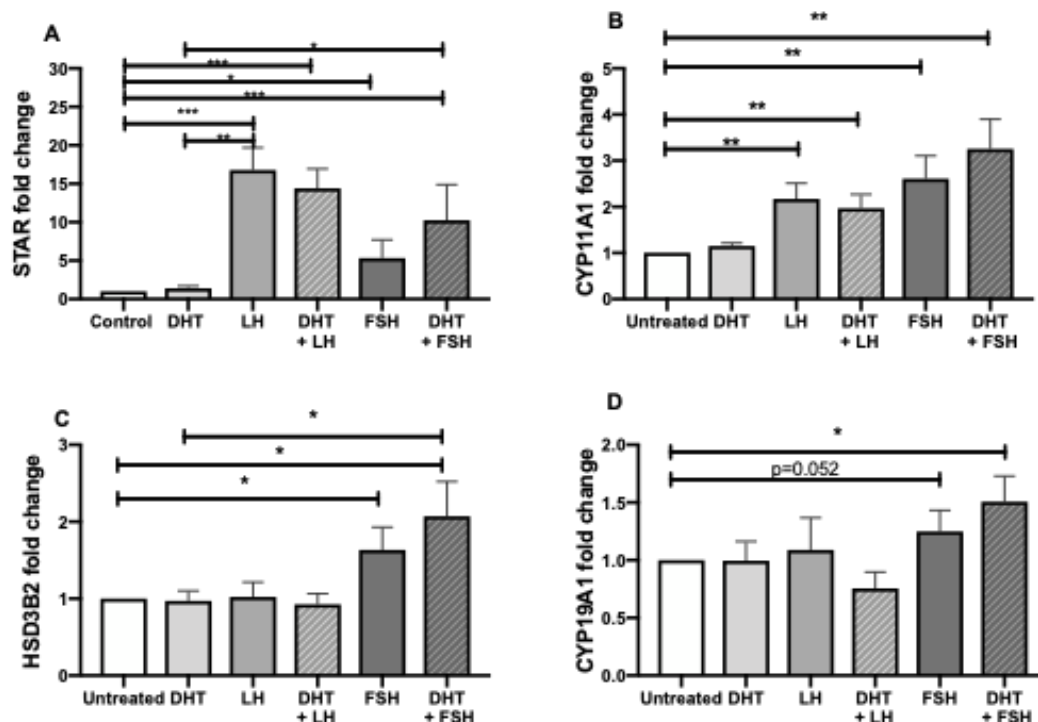


Figure 6.6 Steroid enzyme gene expression in vitro in GLCs treated with DHT, LH or FSH. GLC were cultured for 3 days and then treated with DHT 10nM or ethanol (vehicle control) for 24 hours. GLCs were then treated with DHT/ethanol, and LH or FSH 10nM for a further 24 hours. Cells were then lysed for RNA extraction, cDNA synthesis and RT-qPCR. Results are represented as fold change relative to the vehicle control (untreated normalised to 1). **A** STAR gene expression is not affected by DHT treatment alone. LH treatment increased STAR ($p < 0.001$). LH + DHT treatment also increased STAR ($p < 0.001$). There was no difference in STAR expression between samples treated with LH or treated with DHT + LH. FSH increased STAR ($p < 0.05$), as did DHT + FSH ($p < 0.001$). **B** CYP11A1 gene expression is not affected by DHT treatment alone. LH treatment increased CYP11A1 ($p < 0.01$). LH + DHT treatment also increased CYP11A1 ($p < 0.01$). There was no difference in CYP11A1 expression between samples treated with LH or treated with DHT + LH. FSH increased CYP11A1 ($p < 0.01$), as did DHT + FSH ($p < 0.01$). **C** HSD3B2 gene expression is not affected by DHT, LH or DHT + LH treatment. FSH increased HSD3B2 ($p < 0.05$), as did DHT + FSH ($p < 0.05$). **D** HSD3B2 gene expression is not affected by DHT, LH or DHT + LH treatment. FSH increased HSD3B2 ($p < 0.05$), as did DHT + FSH ($p < 0.05$). (N=10, mean $\pm$ SEM, Kruskal Wallis test).

6.2.6 The effect of DHT on progesterone production in GLCs

Given the effect of DHT on LH induced signalling, I measured its downstream impact on progesterone secretion into culture media. The AR antagonist flutamide was also used, to assess if any androgen related changes are reversed by adding an anti-androgen. After 3 days in culture GLCs were treated with ethanol (vehicle control), DHT 10 nM or flutamide 20 μ M. They were then treated for a further 24 hours with or without LH. The dose of flutamide is based on previous work in our lab which demonstrated these as the minimum effective dose in stimulating change in steroidogenesis and gene expression in GCs (Laird et al 2017, (Thomson, 2017)). DHT alone did not stimulate progesterone production (Figure 6.7). Flutamide alone also did not stimulate progesterone production. LH alone stimulated progesterone production. There was no further significant increase in progesterone production when DHT and LH were used in combination. While there was a trend towards a reduction in progesterone with flutamide use, it did not completely reverse the additive effect of DHT on LH-induced progesterone production.

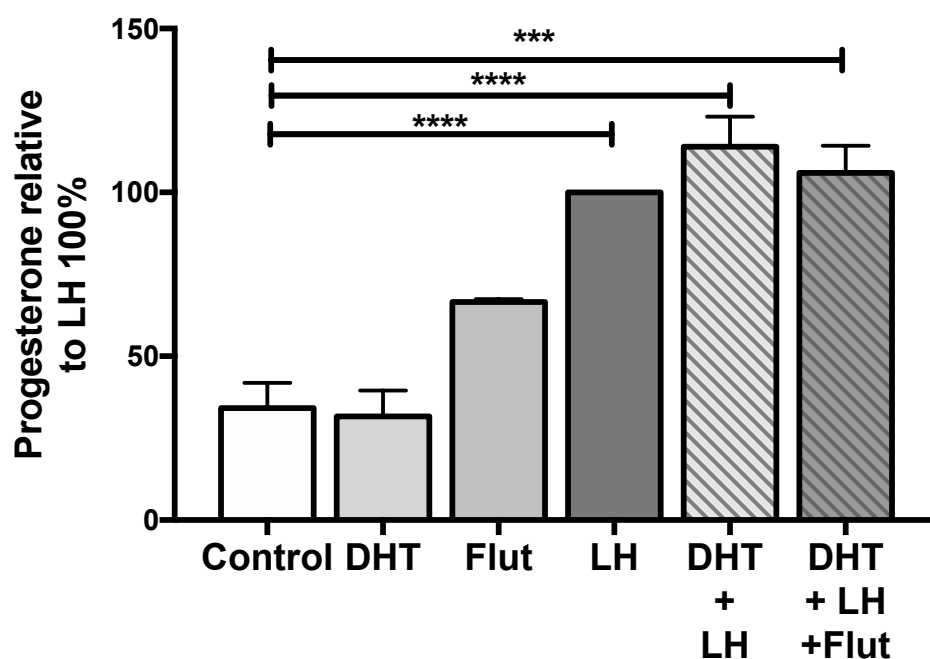


Figure 6.7 Progesterone production in conditioned media after treatment with ethanol, DHT, +/- LH, +/- Flutamide. Data is normalised to LH (100%). GLCs were treated in vitro with ethanol (vehicle control), DHT 10nM, flutamide 20 μ M, LH 10nM, DHT 10nM + LH 10nM, or DHT 10nM +

LH 10nM + flutamide 20μM. n=9. Culture media was collected, and progesterone assay completed, using the Abbott Architect iSR200 analyser using a micro-particle enhanced immunometric assay, read by chemiluminescence in the Hammersmith hospital pathology lab. LH treatment increased progesterone production ($p<0.0001$), as did DHT + LH ($p<0.0001$) and DHT + LH + flutamide ($p<0.001$). Flutamide treatment alone did not significantly increase progesterone production. The combination of DHT + LH stimulated 11% more progesterone production than LH alone ($p=0.1$, ns). One-way ANOVA, mean $\pm$ SEM.

6.3 Discussion

The role of androgens in pre-antral/antral follicle development has been studied, however direct androgen action peri-ovulation is unclear. I therefore studied the effect of androgen on gonadotropin receptor signalling and function in GLCs. Unlike in studies of GCs using animal models (Laird et al., 2017, Hamel et al., 2005), DHT did not stimulate a significant increase in *FSHR* or steroidogenic enzymes expression. It did however appear to augment LH induced LHR signalling.

I found that incubating GLCs with DHT augmented the cAMP response to LH and the generation of pErk 1/2 in response to LH. These changes did not appear to be related to an increase in *LHCGR* expression, but DHT treatment did increase the expression of AR. LHR and AR expression are known to be strongly positively correlated in human GCs (Kristensen et al., 2017), and AR expression is downregulated by LH treatment *in vitro* (Figure 6.5), so there appears to be a strong relationship between these two key receptors. This suppression of AR by LH likely explains the decline in AR seen in more mature follicles, after the LH surge (Hillier et al., 1997, Suzuki et al., 1994).

DHT did not significantly affect IP-1 accumulation in response to LH in GLCs. Androgens may therefore exert a bias effect on Gs signalling over Gq. This parallels what I saw in PCOS GLCs in Chapter 4, where cAMP/Gs signalling was augmented but IP-1 accumulation was not. This suggests that androgen exposure may have a direct role in explaining the augmented LH

signalling in PCOS. It would be interesting to see if DHT treatment in vitro augments expression of trafficking proteins GIPC and β -arrestin, which had a higher expression in PCOS GLCs. It would also be interesting to see the effect of supraphysiologic doses of DHT on LHR signalling, to more closely mimic the follicular hyperandrogenism seen in PCOS.

While the exact mechanism of DHT augmentation of LH signalling is unclear, one hypothesis is that it may be through interaction between AR and GPCR Family C group 6 Member A (GPRC6A). The importance of interactions between physiologically relevant GPCRs acting as steroid extranuclear receptors, such as the androgen regulated GPRC6A, is becoming increasingly recognised. Overexpression of GPRC6A in HEK293 cells stimulates signalling in response to androgen in cells lacking the androgen receptor (Pi et al., 2010). Androgen receptor signalling is attenuated in prostate cancer cells after SiRNA knockdown of GPRC6A (Pi et al., 2010). Future work could measure the expression of GPRC6A in PCOS GLCs and the effect of knocking out GPRC6A on LHR signalling.

Non-genomic steroid hormone signalling has also been hypothesised to involve variants of steroid receptors localised to the plasma membrane and membrane proteins with steroid-binding domains. This mechanism of signalling is fast and does not depend on transcription. Nuclear receptors have been found at the plasma membrane, from where they can alter signal transduction (Wilkenfeld et al., 2018). The AR associates with the plasma membrane at caveolae lipid rafts through interactions with proteins caveolin-1 and Src, from where it can activate G proteins and alter signalling pathways (Deng et al., 2017, Yu et al., 2012). Interestingly Src activation in response to LH is augmented in PCOS GLCs (Figure 4.8), so Src may be facilitating androgen receptor localisation to the cell membrane and androgen mediated augmented signalling. Activation of membrane associated AR triggers matrix metalloproteinase-mediated transactivation of the epidermal growth factor receptor (EGFR), which in turn leads to MAPK and Akt signalling (Sen et al., 2014). EGFR activation by LH is also augmented in PCOS GLCs (Figure 4.8). Future work could examine the effects of short-term, non-genomic, androgen treatment on LHR signalling. Future work could also examine AR and EGFR localisation and effect on LHR functioning and trafficking, using a cell line where tagged receptor can be transfected.

DHT treatment did not have an effect on FSHR signalling, despite a trend towards an increase in *FSHR* with DHT treatment, unlike what has been demonstrated in animal models. In rat granulosa cells, DHT caused a reduction in ERK phosphorylation in response to FSH, which led to reduced cyclin D2 mRNA expression and cell cycle arrest (Kayampilly and Menon, 2004). There is a strong positive correlation between *AR* and *FSHR* in GCs from hSAF (Figure 5.2). Both decline as the follicle matures, and the predominant role of FSHR is in small antral follicles, so GCs from these smaller follicles would likely be a better model for studying interactions between AR and FSHR. In addition, it is likely that the main effects of androgen on FSHR are related to aromatisation and the oestrogen effect, not a direct androgen effect as was measured here.

It is not possible however, to rule out an oestrogen effect as the cause of DHT induced LHR signal augmentation. DHT, while predominantly non-aromatisable, can be reduced into 3 β -diol, an androgen metabolite with oestrogen like effects (Handa et al., 2008, Picciarelli-Lima et al., 2006). However, oestrogen effects on LHR have been shown to be mediated through augmentation of FSH induction of LHCGR (Ikeda et al., 2008). DHT did not affect the FSH induced increase in LHCGR, suggesting it is unlikely there was a predominant oestrogen effect (Figure 6.4A).

DHT treatment did not alter gene expression of steroid enzymes and gonadotropin receptors. This differs from animal models, as *in-vitro* androgen treatment in mouse ovaries has been shown to augment FSH-induced follicle growth, increased antrum formation, increased *Star* expression and reduced *Cyp11a1* expression (Laird et al., 2017). Androgen effects were reversed by the AR antagonist flutamide. The presence of androstenedione also augments FSH-dependent E₂ secretion in bovine granulosa cells (Hamel et al., 2005). Future work could measure oestradiol output into conditioned media in GCs treated with FSH with or without DHT. This would ideally be done in GCs from hSAFs, as they produce oestradiol alone under normal conditions, and GLCs predominantly produce progesterone, not oestradiol. Theca cells, where androgens are produced and LHR is active, would also be a good model for studying LHR signalling in PCOS and in cells incubated with androgen.

Studies have examined the effects of DHT on gene expression in multiple tissues, using micro-array and RNA-sequencing. There appears to be a diverse response to DHT in different tissues, with little overlap of genes that are androgen regulated. One study used micro-array in mouse ovary, found 294 DHT regulated genes, and gene ontology analysis found these were involved in cell signalling and G protein signalling, amongst others (Otto-Duessel et al., 2012).

There was a trend towards higher progesterone production in GLCs treated with DHT and LH in some but not all samples (Figure 6.7). This is likely related to the augmented effect of DHT on LHR signalling, which may be genomically and non-genomically mediated. The anti-androgen flutamide did not completely inhibit this effect. This was because flutamide may exert an effect on LHR and progesterone production, perhaps as a partial agonist (Nguyen et al., 2007), although the mechanism of this is unclear. Secondly flutamide does not prevent GPRC6A mediated activation (Pi et al., 2010), which again adds to the argument for the key role this receptor plays in augmenting DHT effect on LHR function.

It is interesting that DHT augmented LHR signalling in samples from most but not all women. It is not clear why but could theoretically be related to variability of expression of androgen receptor, GPRC6A or EGFR. It may also be related to varying patterns of plasma membrane expression of nuclear receptors and the relationship to G proteins.

Future work could also examine if there are further differences in DHT effect on LHR signalling in PCOS GLCs. GLCs from women with PCOS have been shown to have two alternatively spliced variants of the androgen receptor (Wang et al., 2015), and these can alter the expression of genes related to androgen metabolism and folliculogenesis. Their effect on receptor signalling is unknown.

6.4 Conclusions

Overall it appears that around the time of ovulation, androgens are not only precursors for oestradiol synthesis, they directly contribute to reprogramming LHR signalling and function in GLCs. This is similar to what was seen in PCOS GLCs in chapter 4. The exact mechanism underlying this remains unclear, but may be related to both the genomic and non-genomic actions of AR.

Chapter 7

The in-vivo and in-vitro effects of kisspeptin on granulosa lutein cell function and the potential applications to reproductive medicine

7.1 Introduction

7.1.1 Ovarian kisspeptin action

Kisspeptin and its receptor are present in tissues throughout the body (Lee et al., 1996, Ohtaki et al., 2001), including the human and animal ovary (Cejudo Roman et al., 2012, Gaytan et al., 2009). In the ovary kisspeptin may play a direct role in the control of ovarian function; including steroidogenesis, follicle development, oocyte maturation and ovulation. However, this has not been studied in humans. In chicken GCs, the gene expression of CYP11A1, 3 β -HSD and STAR was increased after *in-vitro* treatment with kp-10 (Xiao et al., 2011). In rat luteal cells, treatment with kp-10 alone and with hCG increased expression levels of StAR, CYP11A and 3 β -HSD (Peng et al., 2013). Another study (Laoharatchatathanin et al., 2015) showed whole rat ovary expression of kisspeptin was increased after stimulation with hCG.

Kisspeptin signals via the kisspeptin receptor (KISS1R), which is a G α q/11-coupled GPCR. When kisspeptin binds to KISS1R, Gq/11 activates a series of second messengers, which ultimately leads to intracellular Ca²⁺ release (Stafford et al., 2002, Jiang et al., 2005, Kotani et al., 2001) and activation of the MEK-ERK (MAPK) pathway, which is necessary for LH-induced progesterone synthesis (Woods and Johnson, 2007, Jamaluddin et al., 1994, Jiang et al., 2005, Yuan and Connor, 1997). Reduced MAPK signalling is associated with granulosa cell survival, apoptosis and follicle atresia (Yang et al., 2017, Shiota et al., 2003). Activated KISS1R also recruits β -arrestin 1 & 2 to the cell membrane, which also modulates phosphorylated ERK 1/2 activation (Pampillo et al., 2009, Szereszewski et al., 2010, Goertzen et al., 2016). Kisspeptin has also been shown to stimulate phosphorylation of PI3K/AKT in a tissue specific manner (Peng et al., 2013, Ringel et al., 2002, Navenot et al., 2009). Kisspeptin receptor activity in human GLCs has never been explored.

7.1.2 Kisspeptin in reproductive endocrinology

The most dangerous complication of assisted reproductive technology (ART) is ovarian hyperstimulation syndrome (OHSS), which can be life-threatening (Golan et al., 1989, Whelan

and Vlahos, 2000) and can occur in up to 25% of IVF cycles. (HFEA, 2014, Toftager et al., 2016). Human chorionic gonadotropin (hCG) has traditionally been used to trigger oocyte maturation but its use is complicated by OHSS (Delvigne and Rozenberg, 2002a, Delvigne and Rozenberg, 2002b, Elchalal and Schenker, 1997). Gonadotropin releasing hormone agonists (GnRHa) have a shorter duration of action than hCG and a lower risk of inducing OHSS (Babayof et al., 2006, Corbett et al., 2014, Melo et al., 2009). However, GnRHa are often used as second line agents due to concerns regarding implantation rates (Griesinger et al., 2006, Youssef et al., 2014). Kisspeptin-54 (kp-54) has been shown to trigger oocyte maturation with no clinically significant OHSS (Abbara et al., 2015).

Several studies have compared alterations in gene expression, gonadotropin levels, vascular endothelial markers and growth factor profiles in serum, follicular fluid and GLCs between hCG and GnRHa triggered IVF protocols (Babayof et al., 2006, Borgbo et al., 2013, Cerrillo et al., 2011, Cerrillo et al., 2009)), however these have not been examined with kisspeptin use.

KISS1, Neurokinin B and its receptor have also been shown to be downregulated in GLC PCOS women with PCOS (Blasco et al., 2019), however another study showed *KISS1* and *KISS1R* were overexpressed in PCOS GLC ((Hu et al., 2018)).

7.1.2 Specific aims

The aims were to address the following questions:

- What are the *in vivo* effects of one or two doses of kp-54 on gene expression markers of ovarian function/steroidogenesis and OHSS in GLCs, in comparison to traditional triggers of oocyte maturation (hCG, GnRHa)?
- What, if any, are the *in vitro* effects of kisspeptin-10 (kp-10) in human GLCs?
- Are there differences in *KISS1* and *KISS1R* expression in both GLCs and in GCs from hSAFs in women with and without PCOS?

7.2 Methods and Results

7.2.1 In-vivo use of kp-54 as a maturation trigger results in an altered profile of expression of genes critical to GLC function when compared to hCG or GnRHa (Owens et al., 2018)

7.2.1.1 Study participants

12 women received hCG, 12 received GnRHa and 24 received kp-54 to trigger oocyte maturation. In the kp-54 group, 12 received one injection of kp-54 (9.6nmol/kg) and 12 received two injections of kp-54 at a 10hr interval (9.6nmol/kg x2). Women who received kp-54 to trigger oocyte maturation were significantly younger than those who received hCG or GnRHa (29 vs 33 years, $p = 0.001$) (Table I). Mean body mass index was similar in the three groups. Baseline AFC was lower in women who were triggered with hCG than with GnRHa or kp-54, as half of this group did not have PCO/PCOS ($p < 0.001$). AFC was not significantly different between the GnRHa and kp-54 groups. The mean dose of recombinant FSH received as part of IVF stimulation protocol was higher in women without PCOS who received hCG compared to those who had PCOS or had a GnRHa ($p = 0.01$) or kp-54 ($p = 0.002$) trigger. All the women in the GnRHa and kp-54 groups had PCO (or had multiple ovarian follicles without increased stroma). Half of the women in the hCG group had PCO/PCOS and half had IVF for male factor infertility, tubal problems or unexplained infertility.

Table 7.1 Baseline Characteristics (Owens et al., 2018)

	hCG trigger N=12	GnRH agonist trigger N=12	Kisspeptin-54 trigger N=24	ANOVA p-value
Mean Age (years) ($\pm$ SD)	33.62 $\pm$ 4.9	33.4 $\pm$ 3.8	29.5 $\pm$ 2.8	<0.001
Body Mass index (kg/m ²) ($\pm$ SD)	24.5 $\pm$ 3.7	23.8 $\pm$ 3.5	25.3 $\pm$ 3.3	ns

Dose of FSH (iU) (mean $\pm$ SD)	PCO 1804 $\pm$ 511 Non-PCO 3403 $\pm$ 1301	1785 $\pm$ 836	1607 $\pm$ 605	<0.001
Antral follicle count (mean $\pm$ SD)	14 $\pm$ 8	34 $\pm$ 12	41 $\pm$ 17	<0.0001
Ovarian morphology	50% PCO/PCOS 50% normal ovaries	PCO/PCOS	PCO/PCOS	

7.2.1.2 Serum LH and FSH levels following administration of kp-54 differ from GnRHa and hCG.

Serum gonadotropin measurements were recorded as part of the clinical trial data. Baseline (pre-treatment) serum concentrations of FSH and LH were all within the normal, premenopausal range. Serum LH and FSH peaked 4 hours after administration of kp-54. The mean baseline LH was 2.9 ± 3.8 IU/ml and the mean peak was 55 ± 30 IU/ml. The mean baseline FSH was 7.9 ± 2.9 IU/ml and peak was 10.3 ± 3.2 IU/ml. LH and FSH return to baseline levels within 24 hours. GnRHa (data adapted from (Fauser et al., 2002) result in a peak rise of LH at 4 hours of over 130 IU/ml and FSH of over 40 IU/ml and return to baseline within 36 hours. hCG does not affect FSH and causes a sustained rise in serum LH of over 100 IU/ml which lasts for up to 7 days ((Fauser et al., 2002).

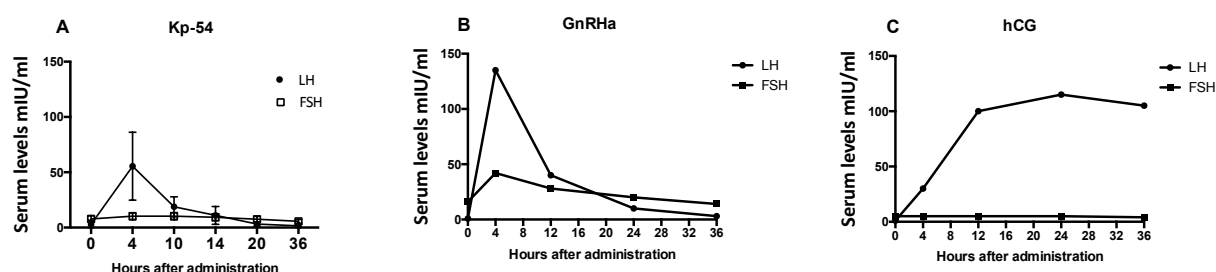


Figure 7.1 Mean $\pm$ SEM change in serum FSH and LH levels from 0 to 36 h after kp-54 administration (Owens et al., 2018), compared to GnRHa and hCG (adapted from Fauser et al, 2002). **A** LH peaks at 4 hours after administration of Kp-54 and returns to baseline within 14

hours. There is a small increase in FSH (<50% rise from baseline) and returns to baseline within 20 hours. **B** LH peaks at 4 hours after administration of GnRHa and returns to baseline within 24 hours. FSH peaks at 4 hours after administration of GnRHa and returns to baseline within 36 hours. The rise in LH and FSH are higher than after administration of Kp-54. **C** LH peaks at 24 hours after administration of hCG and remains elevated after 36 hours. There is no change in FSH.

7.2.1.3 Gene expression of gonadotropin but not kisspeptin receptors are higher in GLCs from women who received kp-54.

GLCs from women who had received kp-54 had a 14-fold ($p < 0.001$) and 8-fold ($p < 0.01$) higher expression of *FSHR* gene than GLCs from women who had received hCG or GnRHa, respectively (Fig. 8.2A). *FSHR* correlated strongly with baseline antral follicle count ($p < 0.0001$) (Figure 7.2B). It is known that fresh GLCs from women with PCO express more *FSHR* (2 fold, see Figure 4.13), however this does not explain the 8-fold higher *FSHR* expression in the kp-54 group as both GnRHa and kp-54 group all had PCO and AFC was similar. *FSHR* did not differ significantly between those who had one or two kisspeptin injections (Figure 7.2C).

GLCs from women who had received kp-54 had a 2-fold (ns) and 2.5-fold ($p < 0.05$) higher expression of *LHCGR* than GLCs from women who had received hCG or GnRHa, respectively (Figure 7.2D). *LHCGR* expression was significantly higher in women who received two injections of kp-54 10 hrs apart when compared with women who had received only one ($p < 0.05$) (Figure 7.2E). There was no direct correlation between *LHCGR* and baseline AFC or rise in serum LH (Figure 7.2 F, G).

Given that the women in the kp-54 group were younger, age was correlated with changes in serum LH or FSH, and expression of *LHCGR* and *FSHR*. There was no correlation between age and change in serum gonadotropins and gonadotropin receptor expression. Results were also analysed with the control women from the hCG group excluded, which did not change any of the above results, again to address the confounding issue on PCO.

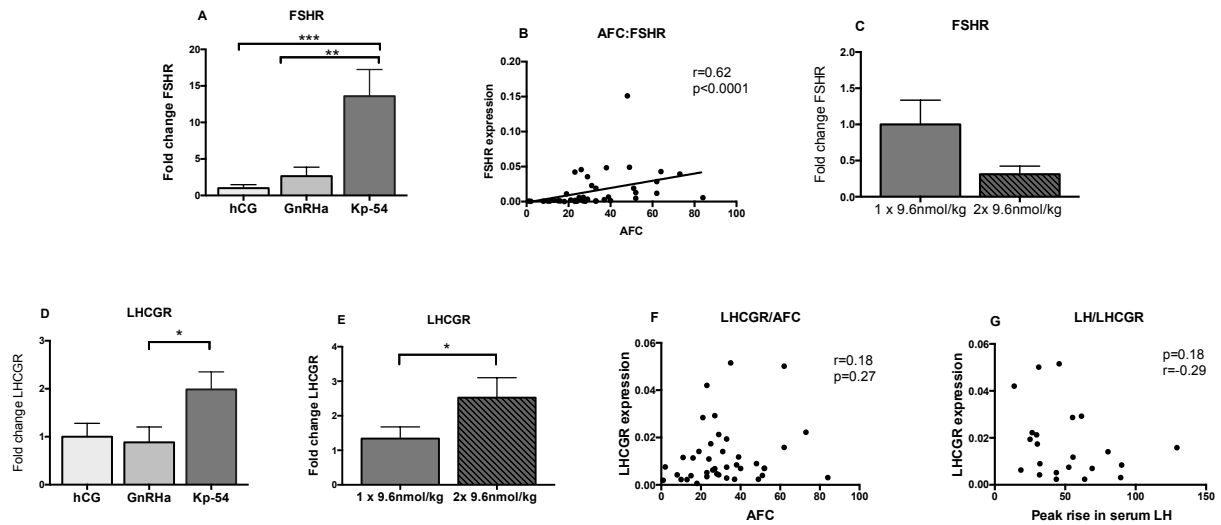


Figure 7.2 Gene expression of gonadotropin receptors in GLCs after oocyte maturation with hCG, GnRHa or kp-54, and correlation of gene expression with antral follicle count.

(Owens et al., 2018) **A** Follicle stimulating hormone receptor (FSHR) gene expression is higher in GLCs from women who received kp-54 as maturation trigger versus hCG ($P \leq 0.001$) or GnRHa ($P \leq 0.01$), mean $\pm$ SEM. **B** FSH correlates with baseline AFC $r=0.62$, $p<0.0001$. **C** There is no significant difference in FSHR in GLCs from women who had received one versus two kisspeptin injections, mean $\pm$ SEM. **D** Luteinizing hormone receptor gene expression (LHCGR) is higher in GLCs from women who received kp-54 as maturation trigger than GnRHa ($P < 0.05$) but is not higher than hCG, mean $\pm$ SEM. **E** LHCGR gene expression is higher in GLCs from women who received two kp-54 injections to trigger maturation (2×9.6 nmol/kg 10 h apart, $n = 12$) than women who received one injection (9.6 nmol/kg), $P = 0.038$, mean $\pm$ SEM. **F** LHCGR does not correlate with baseline AFC. **G** LHCGR does not correlate with rise in serum LH. Statistical tests- Kruskal Wallis, Mann Whitney and Spearman correlations.

KISS1R was expressed at low levels, and was similar in GLCs from women who received hCG, GnRHa or kp-54 (Figure 7.3A). There was no significant difference in KISS1R in GLC between those who had received one or two injections of kisspeptin (Figure 7.3B).

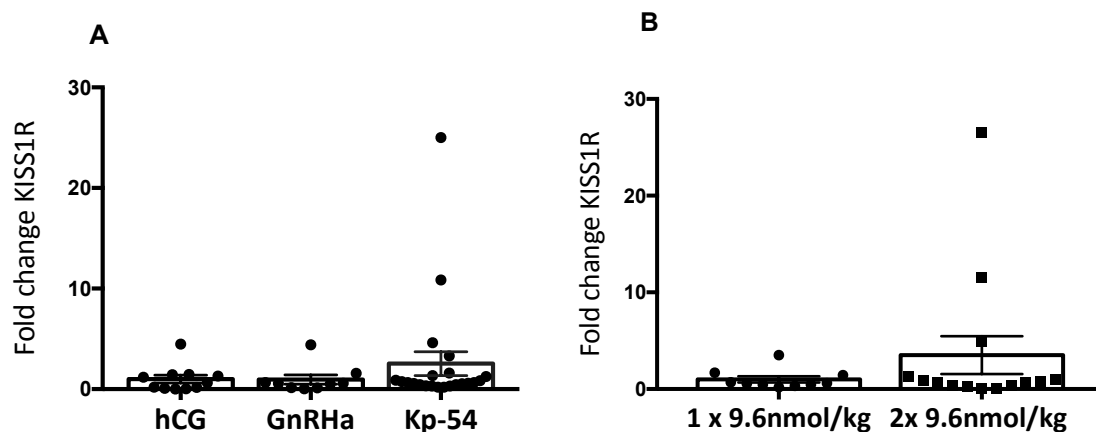


Figure 7.3 Kisspeptin receptor (KISS1R) gene expression in GLCs after oocyte maturation with hCG, GnRHa or kp-54. (Owens et al., 2018) **A** KISS1R was similar in GLCs from women who received hCG, GnRHa or kp-54 to trigger oocyte maturation. (Kruskal Wallis test) **B** KISS1R was similar in GLCs from women who had received one or two doses of kp-54. (Mann Whitney test, $n=12$ in each group, values are mean $\pm$ SEM).

7.2.1.4 Gene expression of ovarian steroidogenic enzymes, inhibin A and oestrogen receptors are higher in GLCs from women who received kp-54.

CYP19A1 (aromatase) expression was 3.6-fold ($p < 0.05$) and 4.5-fold ($p < 0.05$) higher in GLCs from women who had received kp-54 than hCG or GnRHa, respectively (Figure 7.4A). *CYP19A1* did not correlate with AFC (Figure 7.4B) and was not changed in those who had received two versus one injection of kp-54 (Figure 7.4C). Expression of *STAR* was 3.4-fold higher ($p < 0.01$) and 1.8-fold higher ($p < 0.05$) in women who had received kp-54 than PCO women in hCG group and GnRHa group, respectively (Figure 7.4D). *STAR* did not correlate with baseline AFC (Figure 7.4E). *STAR* expression was higher in those who had received two injections of kp-54 to trigger oocyte maturation than those who had received one ($p = 0.04$) (Figure 7.4F). 3β -Hydroxysteroid dehydrogenase 2 (*HSD3B2*) expression was 7.5- ($p < 0.01$) and 2.5-fold higher ($p < 0.05$) in kisspeptin group than those who had received hCG (PCO only) and GnRHa, respectively (Figure 7.4G). *HSD3B2* did not correlate with AFC (Figure 7.4H) and was not changed in those who had

received two versus one injection of kp-54 (Figure 7.4I). *CYP11A1* expression was similar amongst the three groups (Figure 7.4J).

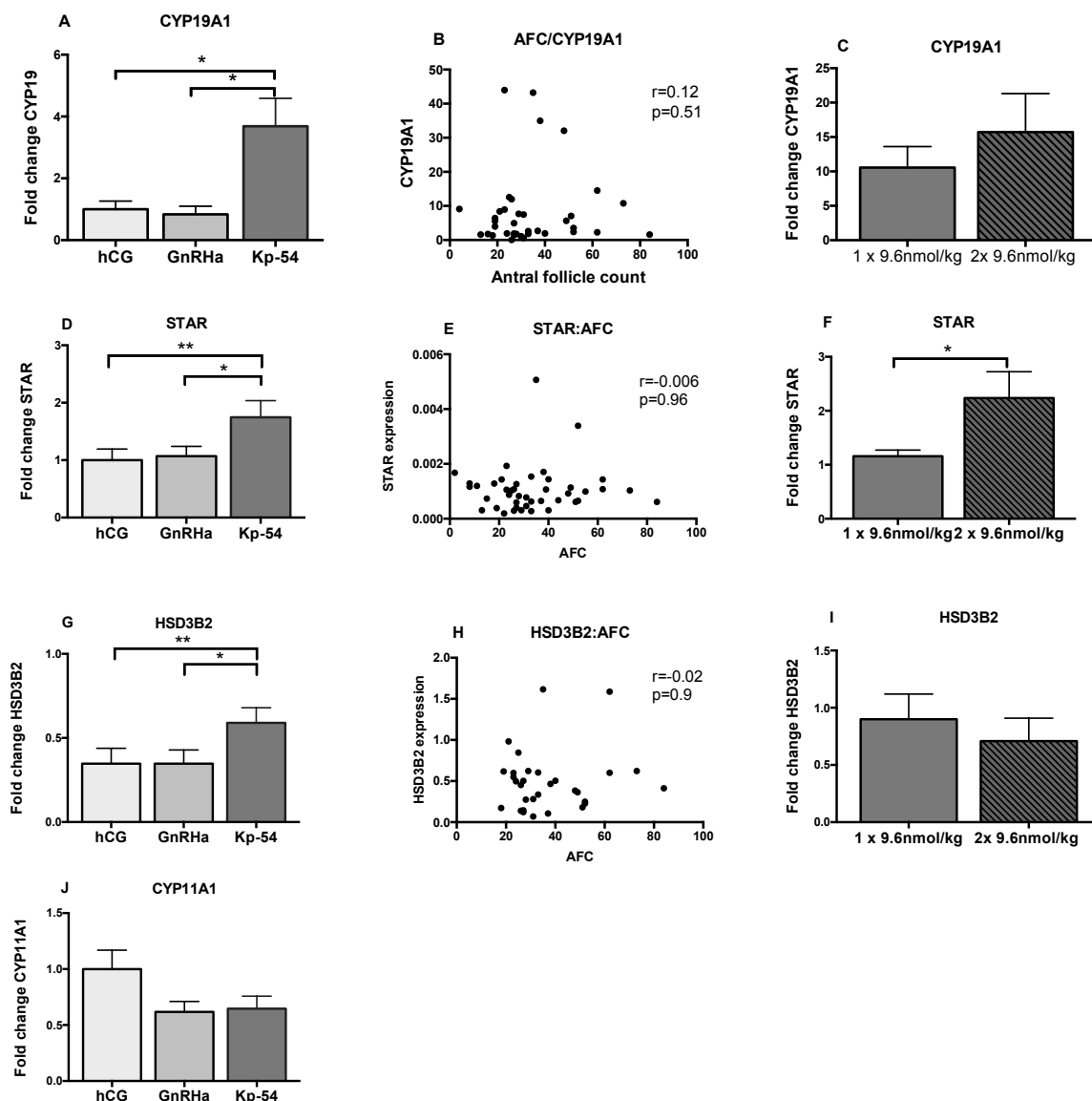


Figure 7.4 Gene expression of genes involved in ovarian steroidogenesis in GLCs after oocyte maturation with hCG, GnRHa or kp-54. (Owens et al., 2018) **A** Aromatase (*CYP19A1*) gene expression is higher in GLCs from women who received kisspeptin as maturation trigger than hCG ($P \leq 0.05$) or GnRHa ($P \leq 0.05$). **B** Aromatase gene expression does not correlate with baseline antral follicle count, $r = 0.12$, $P = 0.514$. **C** There is no significant difference in *CYP19A1* in GLCs from women who had received one versus two kisspeptin injections. **D** Steroidogenic acute regulatory protein (*STAR*) gene expression is higher in GLCs from polycystic ovary (PCO) women who received hCG or kisspeptin as maturation trigger than GnRHa ($P < 0.05$). **E** *STAR* does not

correlate with baseline AFC. **F** STAR gene expression is higher in women who received two kisspeptin injections than women who received one ($P = 0.04$). **G** 3 β -Hydroxysteroid dehydrogenase 2 (HSD3B2) gene expression is higher in GLCs from PCO women who received kisspeptin as maturation trigger than hCG ($P < 0.01$) or GnRHa ($P < 0.05$). **H** HSD3B2 does not correlate with AFC. **I** There is no significant difference in HSD3B2 in GLCs from women who had received one versus two kisspeptin injections. **J** CYP11A1 gene expression is the same in GLCs from women who received kisspeptin as maturation trigger than hCG or GnRHa. Statistical tests- Kruskal Wallis, Mann Whitney and Spearman correlations. Values are mean $\pm$ SEM.

The oestrogen receptors *ESR1* and *ESR2* both showed 3-fold higher expression in cells from kp-54 treated than GnRHa treated women (Figure 7.5). *INHBA* gene expression was 2.5-fold higher from GLCs from women who had received kp-54 than hCG or GnRHa ($P < 0.01$) (Figure 7.6). *INHBA* gene expression did not relate to pre-treatment AFC. *INHBB* expression was similar amongst the three groups (Figure 7.6).

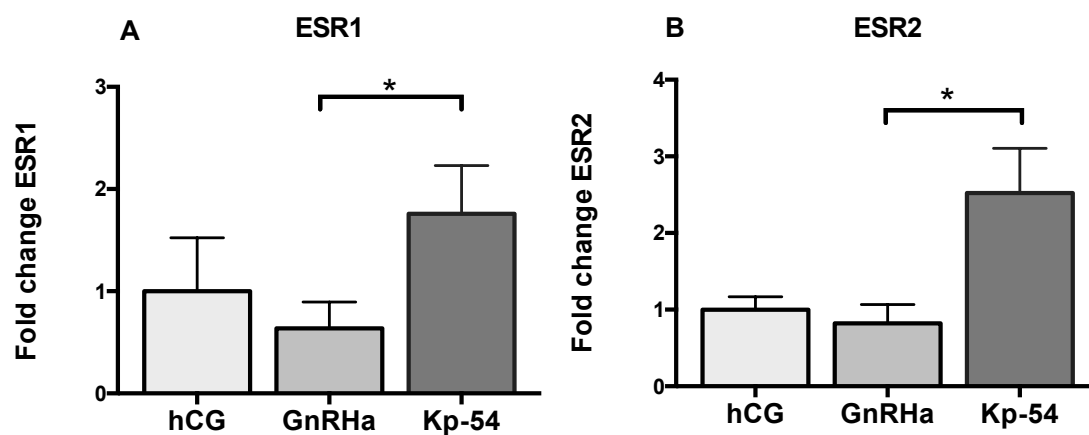


Figure 7.5 Oestrogen receptor 1 and 2 (*ESR1*, *ESR2*) gene expression in GLCs after oocyte maturation with hCG, GnRHa or kp-54. (Owens et al., 2018) Gene expression of *ESR1* and *ESR2* is higher in GLCs from women who received kisspeptin as maturation trigger than GnRHa ($P < 0.05$) but is not higher than hCG. (Kruskal Wallis test, mean $\pm$ SEM).

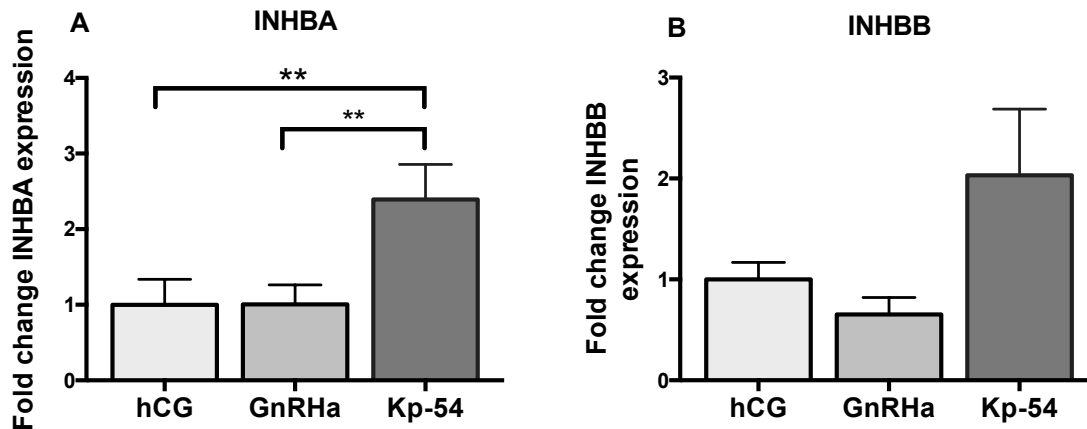


Figure 7.6 Inhibin A (INHBA) and inhibin B (INHBB) gene expression in GLCs after oocyte maturation with hCG, GnRHa or kp-54. (Owens et al., 2018), hCG group ($n = 12$), GnRHa (GnRHa) ($n = 12$) or kp-54 ($n = 24$), Values are mean $\pm$ SEM. **A** INHBA gene expression is higher in GLCs from women who received kp-54 as maturation trigger than hCG ($P \leq 0.01$) or GnRHa ($P \leq 0.01$). **B** INHBB gene expression was not significantly higher in GLCs from women who received hCG, GnRHa or kp5 as maturation trigger. Kruskal Wallis test, mean $\pm$ SEM.

7.2.1.1 Gene expression of markers of Ovarian Hyperstimulation Syndrome

There was no significant difference, amongst the three maturation triggers, between the gene expression of genes that are involved in vascular permeability and integrity; *VEGFA*, *SERPINF1* and *CHD5*, nor in expression of growth factors amphiregulin and epiregulin, which have been previously shown to be potential markers of OHSS (Figure 7.7).

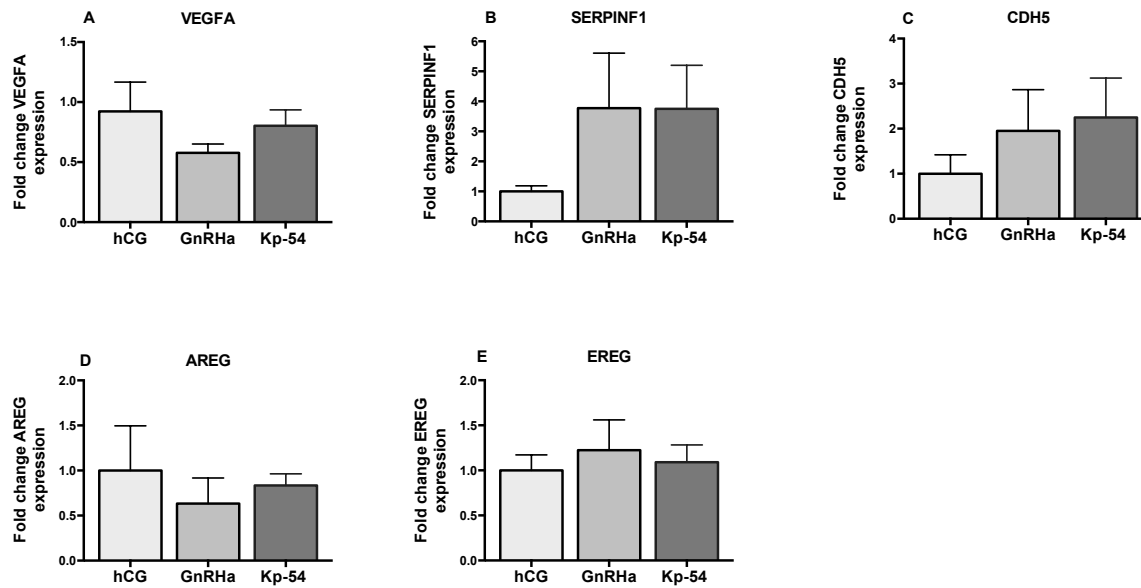


Figure 7.7 Gene expression of potential markers of ovarian hyperstimulation in GLCs after oocyte maturation with hCG, GnRHa or kp-54. (Owens et al., 2018) hCG group (n = 12), GnRHa (GnRHa) (n = 12) or Kp-54 (Kiss) (n = 24), Values are mean $\pm$ SEM. The hCG group were analysed with and without the control (non-polycystic ovary (PCO) women). Data are presented as the mean + SEM of individual gene expression values. There is no significant difference between gene expression in the three groups for any of the markers (A) vascular endothelial growth factor VEGFA, (B) Serpin Family F Member 1 (SERPINF1), (C) Cadherin-5 (CHD5), (D) amphiregulin (AREG) and (E) epiregulin (EREG). Kruskal Wallis test used for all.

7.2.2 *In vitro* actions of kisspeptin in GLCs.

In order to assess if the changes in gene expression seen in GLCs with *in vivo* use of kisspeptin were related either to the effect of the altered serum gonadotropin profiles seen after administering kisspeptin, or a direct effect of KISS1R activity within GLCs, GLCs and incubated them *in vitro* with kisspeptin. GLCs were cultured for 3 days, serum starved overnight and then treated *in vitro* with Kp-10, hCG or a combination of both.

7.2.2.1 Kisspeptin stimulates MAPK/ERK signalling but not pAKT in GLCs *in vitro*

Kp-10 activated pERK1/2 in most but not all patient samples. The pERK1/2 signal was sustained for 15 minutes (Figure 7.8D). Doses of 50nM and above generated a significant increase in pERK1/2 (Figure 7.8E). The response to kp-10 (mean fold increase in pERK1/2 was 1.48 ± 0.6) was much lower than that of hCG (mean fold increase in pERK1/2 5.7 ± 7.8) (Figure 7.8F). There was no additive effect when hCG was used in combination with Kp-10 (Figure 7.8F).

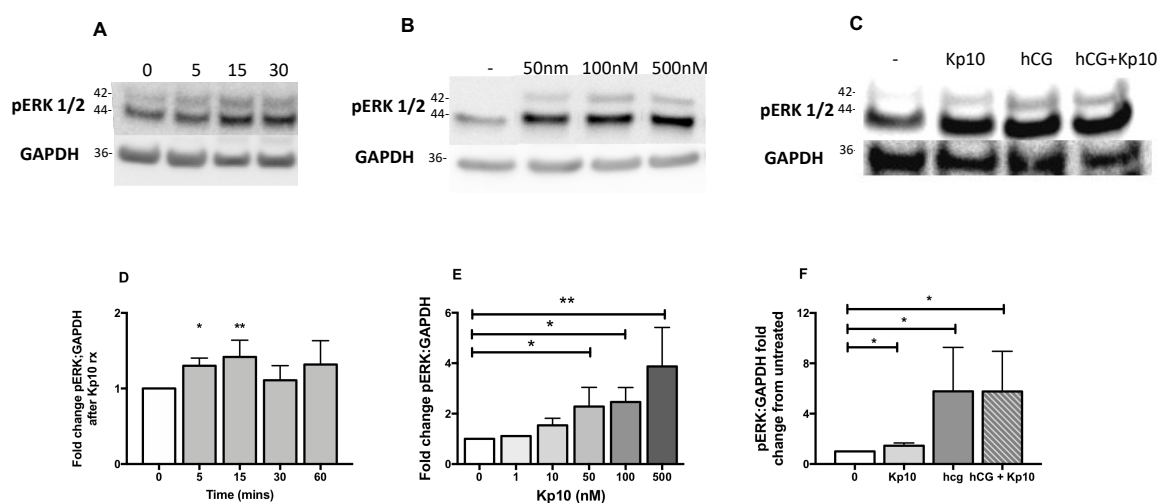


Figure 7.8 Kp-10 and gonadotropin dependent activation of MAPK signalling. GLCs were treated with Kp-10 of varying concentration, hCG or both for 5, 15, 30 and 60 minutes. Cells were then lysed, protein extracted and quantified. Total protein lysate was separated by gel electrophoresis and visualised through western blotting probed for phosphorylated ERK1/2 (pERK1/2) and phosphorylated AKT (pAKT). Signal intensity was calculated relative to GAPDH, which was used as an internal loading control. **A** Representative western blot showing an increase in pERK after *in vitro* treatment with Kp-10 100nM for 5, 15 and 30 mins. **B** Representative western blot showing an increase in pERK after *in vitro* treatment for 15 mins with Kp-10 50, 100 and 500nM. GAPDH is used as a loading control. **C** Representative western blot showing increase in pERK after *in vitro* treatment for 15 mins with Kp-10 100, hCG, Kp-10 100nM

+ hCG. **D** There was a sustained increase in pERK after *in vitro* treatment with Kp-10 100nM for 5, 15 mins ($n=5$). **E** Increase in pERK after *in vitro* treatment for 15 mins with 1nM (*ns*), 10nM (*ns*), 50nM ($p<0.05$), 100nM ($p<0.05$), 500nM Kp-10 ($p<0.01$). **F** There was an increase in pERK after treatment with Kp-10 100nM ($p<0.05$), hCG 10nM ($p<0.05$) and hCG+Kp-10 ($p<0.05$) for 15 mins (Kruskal Wallis, mean $\pm$ SEM).

There was no significant increase in pAKT signalling after incubation with kp-10, hCG or a combination of both (Figure 7.9).

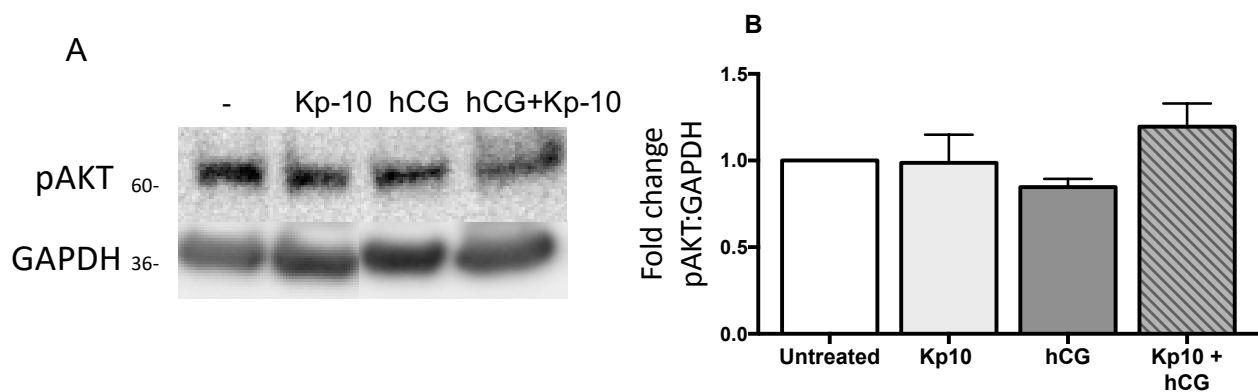


Figure 7.9 pAKT after treating with kisspeptin for 15 minutes. **A** Representative western blot showing no change in pAKT after treatment with Kp-10. **B** pAKT after *in vitro* treatment of GLCs for 15 mins with Kp-10 100nM, hCG 10nM or a hCG and Kp-10 ($n=5$). There is no change in pAKT after treatment with Kp-10 or hCG or both combined. Kruskal Wallis test, mean $\pm$ SEM.

7.2.2.2 $G\alpha_q/11$ signal activation *in vitro* in GLCs in response to kisspeptin

KISS1R is known to be $G\alpha_q$ coupled, and activation leads to an increase in intracellular calcium. To assess this in GLCs, calcium mobilisation was measured after administration of Kp-10 pre-incubated with the fluorescent calcium indicator dye Fluo4-AM. GLCs were imaged live via confocal microscopy and after administration of Kp-10. There was no response to Kp-10 100nM and 1000nM, $n=3$ (Figure 7.10 A). LH 100nM was used as a positive control, and led to a rapid and sustained rise in intracellular calcium mobilisation (Figure 7.10B).

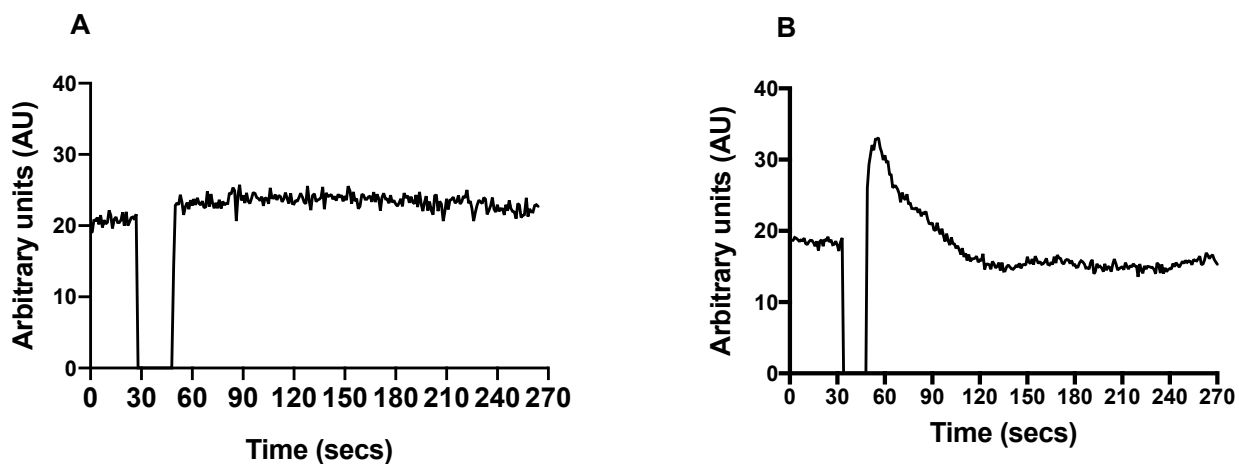


Figure 7.10 GLCs induce intracellular calcium in response to LH but not kp-10 *in vitro*. GLCs were cultured in glass dishes. On day 4 in culture they were incubated with fluorescent calcium inhibitor Fluo4-AM for 1 hr and imaged live via confocal microscopy for 30 seconds before Kp-10 or LH 100nM was added. **A** Fluorescent intensity plot from video A following Kp-10 stimulation, expressed in arbitrary units (AU). **B** Fluorescent intensity plot from video A following LH stimulation, expressed in arbitrary units (AU).

Intracellular accumulation of IP-1 was therefore used as an assay of an additional second messenger pathway of $G\alpha_q/11$. *In vitro* incubation with 50 or 100nM Kp-10 resulted in a significant accumulation of IP-1 (Figure 7.11). GLCs from some samples had an increase in IP1 after kisspeptin administration, where others did not, under the same experimental conditions.

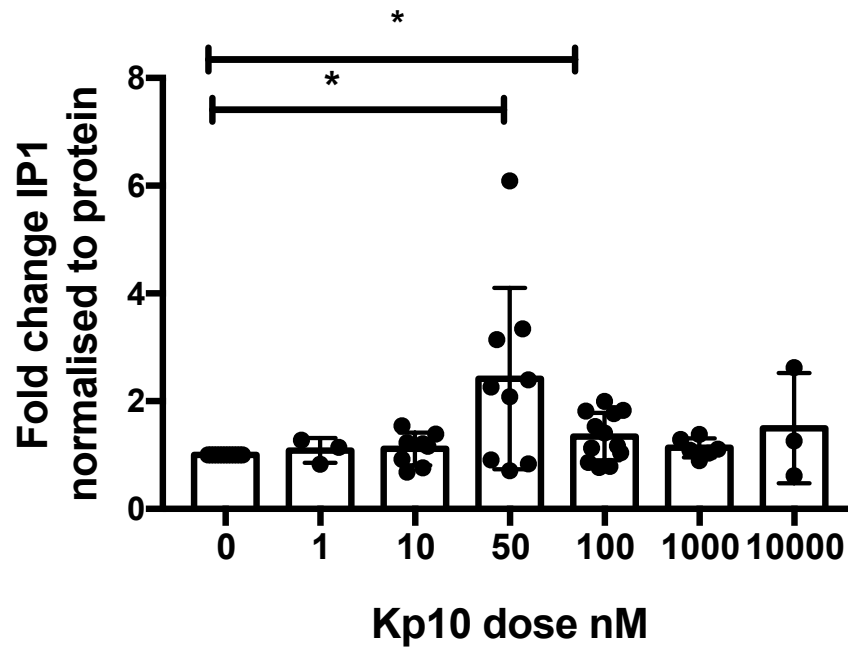


Figure 7. 11 IP1 assay after treatment with kp-10 for 1 hour in GLCs. *In vitro* treatment with Kp-10 50nm (n=9) and 100nm (n=12) resulted in a significant increase in IP1 compared to untreated GLCs ($p < 0.05$). Treatment with 1 (n=3), 10 (n=8), 50 (n=9), 1000 (n=6) and 10000nM (n=3) resulted in non-significant increases in IP1. Kruskal Wallis test, mean $\pm$ SEM.

7.2.2.3 *In vitro* treatment with kisspeptin does not alter gonadotropin and kisspeptin receptor expression in GLCs

GLCs were incubated with Kp-10 10nM-1uM, hCG 10nM or a combination of both for 24 hours to simulate the *in vivo* treatment. Cells were then lysed, RNA extracted and quantified, cDNA synthesised and RT-qPCR completed using optimised primer sets. Kp-10 did not alter expression of *FSHR*, *LHCGR* or *KISS1R*. hCG significantly upregulated *LHCGR* ($P < 0.01$) and downregulated *FSHR*. Combined treatment with Kp-10 and hCG did not have an additive effect beyond that of hCG alone (Figure 7.12).

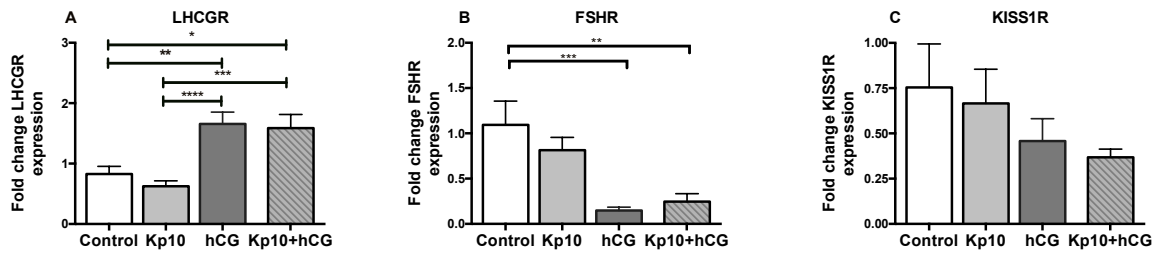


Figure 7.12 Gonadotropin and KISS1R expression in GLCs after in vitro treatment with Kp-10 100nM, hCG 10nM or a combination of hCG+Kp-10 for 24 hours, $n=13$, mean $\pm$ SEM. (Work completed by MSc student Georgina Poole under my supervision). **A In vitro treatment with hCG increases LHCGR ($p<0.01$). In vitro treatment with Kp-10 does not affect LHCGR, either alone or in addition to the effect of hCG. **B** In vitro treatment with hCG reduces FSHR ($p<0.001$). In vitro treatment with Kp-10 does not affect FSHR, either alone or in addition to the effect of hCG. **C** In vitro treatment with Kp-10, with or without hCG does not affect KISS1R. Kruskal Wallis test used for all.**

7.2.2.4 In vitro treatment with kisspeptin does not alter steroidogenic enzyme, oestrogen receptor or inhibin gene expression

In vitro treatment with Kp-10 did not alter expression of *CYP19A1*, *STAR*, *HSD3B2* or *CYP11A1*, either alone or in addition to the effect hCG. In vitro treatment with hCG increases *STAR* ($p<0.001$) and *CYP11A1* ($p<0.01$) but did not alter *CYP19A1* or *HSD3B2* (Figure 7.13).

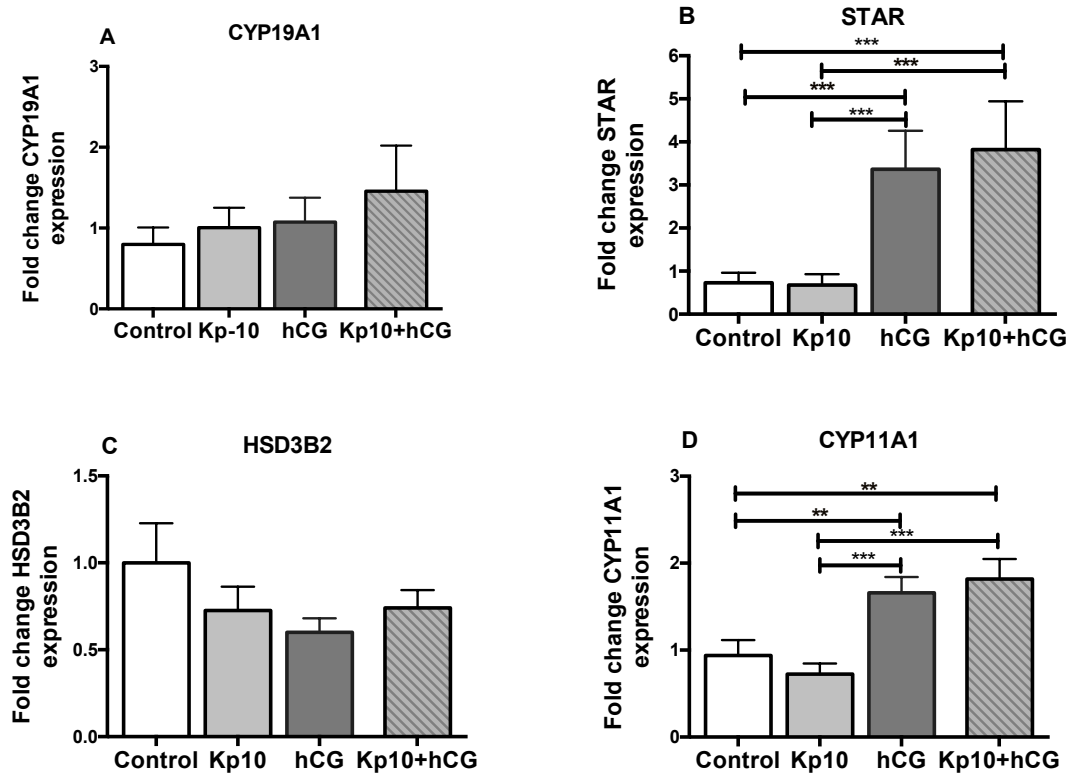


Figure 7.13 Steroid enzyme gene expression in GLCs after in vitro treatment with Kp-10 100nM, hCG 10nM or a combination of hCG+Kp-10 for 24 hours, $n=13$, mean $\pm$ SEM. (Work completed by MSc student Georgina Poole under my supervision). **A In vitro treatment with hCG and kp-10 did not affect aromatase expression (CYP19A1). **B** In vitro treatment with hCG increased STAR expression ($p<0.001$). Kp-10, either alone or in addition to hCG, did not affect STAR. **C** In vitro treatment with hCG and kp-10 did not affect HSD3B2 expression. **D** In vitro treatment with hCG increased CYP11A1 expression ($p<0.001$). Kp-10, either alone or in addition to hCG, did not affect CYP11A1. (Kruskal Wallis test).**

In vitro treatment with kp-10 does not alter expression of *ESR1* or *ESR2* (Figure 7.14), either alone or in addition to the effect hCG. In vitro treatment with hCG reduces *ESR1* ($p=0.06$) and *ESR2* ($p<0.05$) (Figure 7.14 A, B).

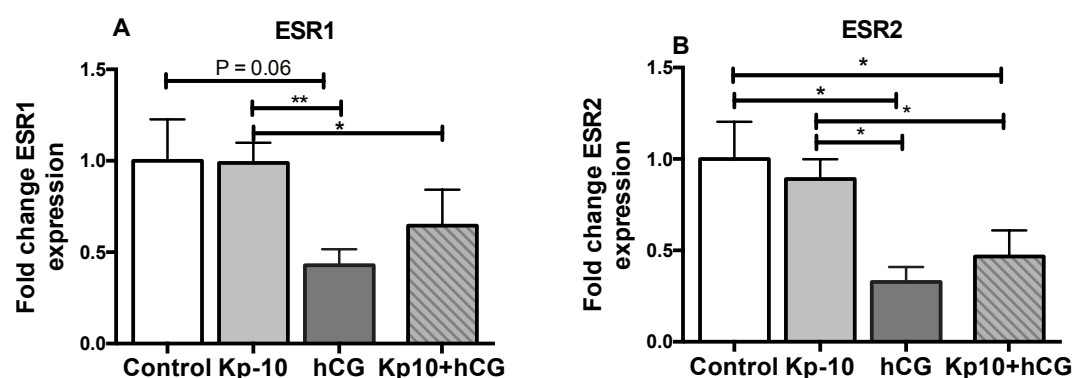


Figure 7.14 Oestrogen receptor 1 and 2 (ESR1, ESR2) expression in GLCs after in vitro treatment with Kp-10 100nM, hCG 10nM or a combination of hCG+Kp-10 for 24 hours, $n=13$, mean $\pm$ SEM. (Work completed by MSc student Georgina Poole under my supervision). In vitro treatment with hCG reduced expression of both ESR1 (A) and ESR2 (B). Kp-10, either alone or in addition to hCG, did not affect expression of ESR1 or ESR2. (Kruskal Wallis test).

In vitro treatment with kp-10 does not alter expression of *INHBA* or *INHBB*, either alone or in addition to the effect hCG (Figure 7.15). In vitro treatment with hCG reduces *INHBB* ($p<0.05$) (Figure 7.15B) but does not affect *INHBA* (Figure 7.15A).

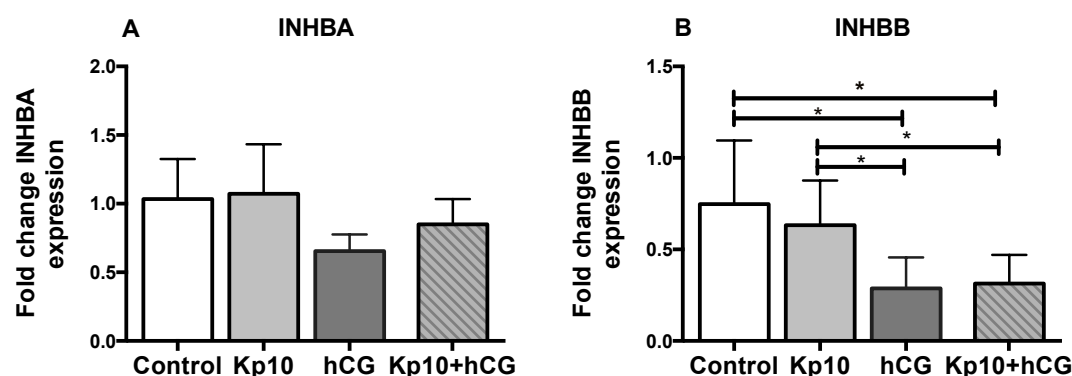


Figure 7.15 Inhibin expression in GLCs after in vitro treatment with Kp-10 100nM, hCG 10nM or a combination of hCG+Kp-10 for 24 hours, $n=13$, mean $\pm$ SEM. (Work completed by MSc student Georgina Poole under my supervision). A *INHBA* expression was not affected by in vitro treatment

with Kp-10 or hCG. **B** *In vitro* treatment with hCG reduced INHBB ($p<0.05$). Kp-10, either alone or in addition to hCG, did not affect INHBB. (Kruskal Wallis Test)

7.2.2.5 *In vitro* treatment with kisspeptin in most GLCs does not significantly increase progesterone production

GLCs were incubated *in vitro* Kp-10 and Kp-54 for 24 hours, alone and in combination with hCG. Media was then collected and progesterone in media was quantified using Abbott Architect iSR200 analyser, a micro-particle enhanced immunometric assay, read by chemiluminescence. *In vitro* treatment with kisspeptins did not result in a significant increase in progesterone, either alone or in addition to hCG (Figure 7.16A). hCG treatment resulted in an increase in progesterone output in to culture media ($p<0.05$) (Figure 7.16A). However, in GLCs from one patient (patient 654), incubation with kp-10 increased progesterone production by 30% and when co-treated with hCG increased progesterone production by an additional 33% (Figure 7.16B). *KISS1R* expression in GLC from this one patient was 34 times higher than that of the average of the other GLC samples (Figure 7.16C).

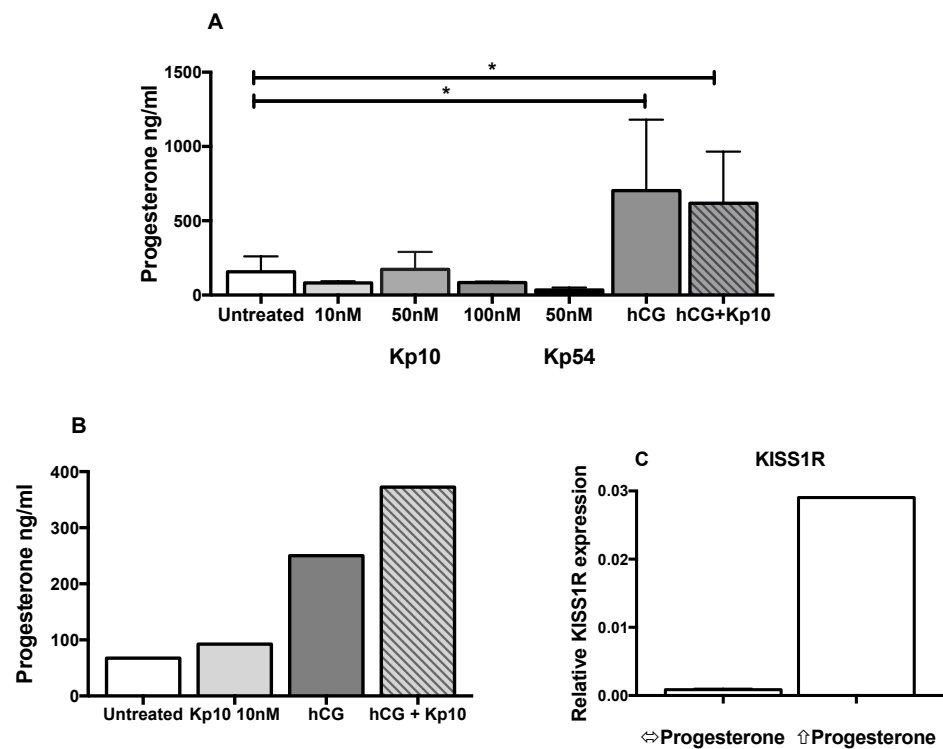


Figure 7.16 Progesterone concentration in conditioned culture media after treatment with Kp-10, Kp-54, hCG for 24 hours, n=6. **A** In vitro treatment with Kp-10 10, 50 and 100nM and Kp-54 50nM did not stimulate progesterone production, either alone or in combination with hCG. hCG stimulates progesterone production (Kruskal Wallis, $p < 0.05$, mean $\pm$ SEM) **B** In GLCs from one patient (patient 654), in vitro treatment with Kp-10 10nM increased progesterone production by 37% alone and in combination with hCG increased progesterone production by 49%. **C** KISS1R was 34 times higher in GLCs from patient 654 compared to the average from the other patients.

7.2.3 KISS1/KISS1R expression in granulosa cells from small antral follicle is higher than luteinised granulosa cells and does not differ in those with and without PCOS.

Two studies have examined *KISS1* and *KISS1R* in GLCs and shown conflicting results. Therefore, I examined gene expression in both GLCs and in GCs from hSAFs. *KISS1* and *KISS1R* are significantly higher in granulosa cells from small antral follicles (GCSAF) compared to GLCs ($p < 0.001$, $p < 0.0001$) (Figure 7.17). *KISS1* expression is negligible in GLCs (Ct values > 31). *KISS1* and *KISS1R* are not significantly different between control and PCOS GCSAF and GLCs (Figure 7.18).

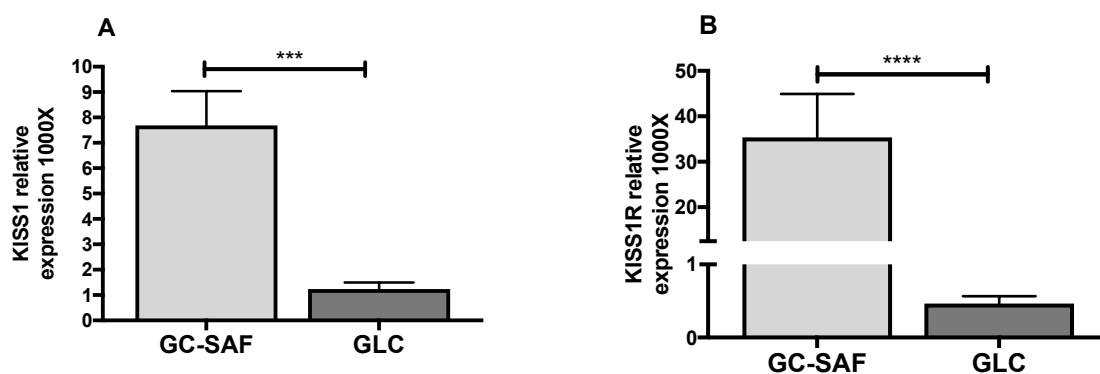


Figure 7.17 *KISS1* and *KISS1R* expression in unstimulated GCs from hSAFs (n=29) compared to GLCS (n=14), mean $\pm$ SEM. **A** Mean *KISS1* expression is 6-fold higher in GC SAF compared to GLC ($p < 0.001$). **B** Mean *KISS1R* is 76-fold higher in GC SAF compared to GLC ($p < 0.0001$). Mann Whitney test.

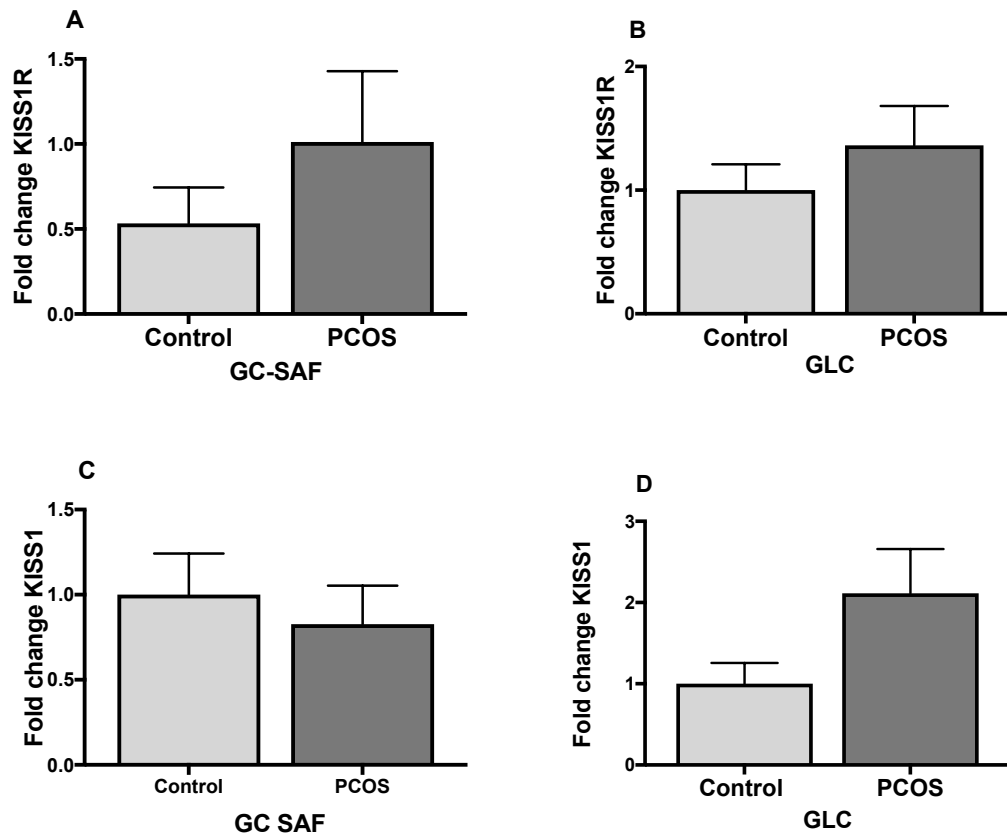


Figure 7.18 KISS1R and KISS1 expression in control and PCOS GCs from hSAFS (n=16 control, n=14 PCOS) and GLCs (n=8 control, n=6 PCOS). **A** There is no significant difference in KISS1R in PCOS GC SAF. **B** There is no significant difference in KISS1R in PCOS GLCs. **C** There is no significant difference in KISS1 in PCOS GC SAF. **D** There is no significant difference in KISS1 in PCOS GLCs. (Mann Whitney test, mean $\pm$ SEM).

7.3 Discussion

7.3.1 *In vivo* effects of kisspeptin (Owens et al., 2018)

To my knowledge this is the first study to examine the *in vivo* effects of kp-54 on human GLCs. As the therapeutic role for kisspeptin continues to grow, it is important that its mechanism of action and effects on target organs are comprehensively examined.

Kisspeptin, when given as a trigger for oocyte maturation in IVF, alters GLC gene expression considerably when compared to traditional triggers of maturation. It results in a higher expression of *FSHR* and *LHCGR* (Figure 7.2), and higher expression of genes involved in ovarian steroid production and action; steroidogenic acute regulatory protein, 3 β -hydroxysteroid dehydrogenase, aromatase, inhibin A, oestrogen receptors 1 and 2 (Figure 7.4). These changes occur in the absence of changes in the expression of *KISS1R* (Figure 7.3). These alterations in gene expression are not seen in GLCs treated *in vitro* with Kp-10 (Figure 7.12-7.15) and may be consistent with an indirect effect of kisspeptin-induced changes in gonadotropin secretion. GLCs from women who received kp-54 do not have changes in markers of ovarian hyperstimulation *VEGF*, *PEDF*, Cadherin 5, amphiregulin and epiregulin (Figure 7.7), despite these women having a high baseline risk of OHSS.

Kp-54 administration stimulates a rise in both LH and FSH but with a relatively greater effect on LH, a similar pattern to what has been shown previously (Figure 7.1) (Narayanaswamy et al., 2016). Doses of GnRHa used in current IVF treatment are likely to be at the high end of the dose–response curve, resulting in a higher amplitude of FSH and LH secretion than observed with kp-54 (Vuong et al., 2016). Kisspeptin stimulates GnRH neurons to secrete endogenous GnRH, whereas a GnRHa can also stimulate GnRH receptors, which are not contiguous with GnRH neurons resulting in higher amplitude of gonadotropin secretion at equimolar doses (Jayasena et al., 2015). The lower amplitude of gonadotropin secretion with kp-54, compared with GnRHa or hCG may explain the significantly higher expression of *FSHR* and *LHCGR* (Gonen et al., 1990, Fauser et al., 2002, Declerck et al., 2014). In fact, the changes in serum gonadotropins are closer to that of the peri-ovulatory changes in gonadotropins in the natural unstimulated cycle (Direito et al., 2013). FSH and LH have been shown to downregulate expression of their own receptors (Hirsh et al., 2005, Xu et al., 2011, Jeppesen et al., 2012a). However, one study (Rimon et al., 2004) showed that in luteinised granulosa cells, LH administration resulted in increased density of *LHCGR* transcripts, although these cells responded inefficiently to gonadotropin stimulation. The rise in *FSHR* expression is not related to dose of recombinant FSH administration during the stimulation phase of the IVF cycles, as women triggered with kp-54 or GnRHa received similar doses of recombinant FSH, and less than those who received hCG. It is also not explained by the fact that women in the kisspeptin group all had PCO, despite the strong correlation between AFC and *FSHR*, as the women in the GnRHa group also all had PCO and while *FSHR* is higher in PCO

GLC it is two-fold higher (see figure 3.3), not 8-14 fold, as seen with kisspeptin compared to GnRHa and hCG respectively (Catteau-Jonard et al., 2008).

The relatively high *FSHR* gene expression after kp-54 administration and subsequent augmentation of downstream signalling, could in turn increase expression of enzymes involved in progesterone synthesis (*STAR*, *HSD3B2*), androgen synthesis (*HSD3B2*) and aromatisation of androgens to oestrogens (*CYP19A1*). Oestradiol has been shown to upregulate expression of its own receptor and the increased production of oestradiol may be an explanation for the observed increase in *ESR 1* and *2* (Barton and Shapiro, 1988, Ing and Tornesi, 1997). Enhanced production and/or action may in turn contribute to upregulation of FSH and LH receptor expression (Dankbar et al., 1995). I also saw a higher expression of inhibin A in GLCs from kisspeptin treated women than hCG or GnRHAs. Inhibins are produced by granulosa cells and negatively feedback to inhibit FSH secretion by the pituitary. Inhibin A is the predominant form produced during the late follicular phase and it has been shown previously that inhibin A, in addition to its feedback on the pituitary, can act directly on the ovaries and can suppress *FSHR* gene expression in granulosa cells (Lu et al., 2009). It is possible that the upregulation of inhibin A may be a compensatory response to the higher *FSHR* expression.

There was no significant difference seen in markers of potential OHSS between the three groups, despite reports in the literature showing that triggering maturation with hCG results in an increased likelihood of OHSS, and studies showing alterations in these markers in hCG treated women (Cerrillo *et al.*, 2009, 2011; Haas *et al.*, 2014; Borgbo *et al.*, 2013). One possible explanation for this is that the hCG group in this study had lower baseline risk of OHSS with a lower baseline AFC and older age, and hence also did not have any evidence of changes in gene expression associated with OHSS. The kisspeptin trial specifically targeted women at high risk of OHSS. In addition, we know that no women in this study actually developed clinically significant OHSS. Recently it has been shown in a rat OHSS model that kisspeptin-10 prevented the increased vascular permeability of OHSS probably by the activation of KISS1R and the inhibition of VEGF (Zhai et al., 2017). Kisspeptin triggering could therefore additionally prevent OHSS through a direct ovarian action, thus explaining why these women at high risk of OHSS did not have higher VEGF expression. In addition, although identical quantities of RNA were used and gene expression was normalised to two housekeeper genes, the analysis does not account for

the potential impact of differing numbers of follicles aspirated from individual women and possible differences in gene expression amongst follicles from a single individual.

There are several limitations to this *in vivo* study. Nearly all the women had PCO. We have dealt with this confounder by completing the analyses with and without the women with PCOS, to interrogate this as a confounder and found no differences. As part of the selection process for inclusion in the kisspeptin trial women had to be 34 years of age or younger, as they were pre-selecting women at higher risk of OHSS. Younger women are at higher risk of OHSS and this may have cofounded the data relating to markers of ovarian hyperstimulation.

7.3.2 *In vitro* effects of kisspeptin

7.3.2.1 Kisspeptin receptor signalling in GLCs

When kisspeptin binds to KISS1R, the phosphorylated Gq/11 protein activates phospholipase C (PLC)- β . This leads to activation of phosphatidylinositol 4,5-bisphosphate (PIP₂), accumulation of inositol-(1,4,5)-triphosphate (IP₃), protein kinase C (PKC) activation, and intracellular Ca²⁺ release (Stafford et al., 2002, Jiang et al., 2005, Kotani et al., 2001). Studies in Chinese hamster ovary (CHO) KISS1R cell lines have shown that kisspeptin stimulation results in a biphasic increase in intracellular Ca²⁺, with a rapid acute increase followed by a more sustained second phase (Kuohung et al., 2010, Min et al., 2014). Activation of KISS1R by Kp-10 initiated calcium oscillations in GnRH neurons in the mouse nasal explants (Constantin et al., 2009). Calcium signalling in response to activation of KISS1R has not however been previously demonstrated in human ovarian tissue. While I was unable to directly measure calcium signalling after kisspeptin administration in GLCs, the IP₁ assay was used to indirectly demonstrate activation of this pathway.

PKC activation in general also activates the ERK/MAPK pathway, which appears to be necessary for LH-induced progesterone synthesis (Woods and Johnson, 2007, Jamaluddin et al., 1994, Jiang et al., 2005, Yuan and Connor, 1997). Therefore, the PLC/PKC/ERK pathway mediates progesterone synthesis in granulosa-luteal cells. Reduced MAPK signalling is associated with granulosa cell survival, apoptosis and follicle atresia (Yang et al., 2017, Shiota et al., 2003). In

addition kisspeptin or kisspeptin receptor mutant mice displayed increased follicular atresia (Colledge, 2009, Gaytan et al., 2014), suggesting the kisspeptin system may be involved in preventing cellular apoptosis. I demonstrated that kisspeptin treatment *in vitro* activates this ERK/MAPK pathway, although to a much lower extent than hCG.

Activated KISS1R also recruits β -arrestin 1 & 2 to the cell membrane, which also modulate phosphorylated ERK 1/2 and contributes to the sustained ERK signal profile evident in GLCs and other tissues (Pampillo et al., 2009, Szereszewski et al., 2010, Goertzen et al., 2016). This arrestin signalling has been demonstrated in mouse models where ablation of $G\alpha_q/11$ in GnRH neurons only partially inactivates gonadotropin secretion (Babwah et al., 2015). In addition kisspeptin dependent LH secretion is diminished in mice lacking β -arrestin-1 or β -arrestin-2 (Ahow et al., 2014). This may explain why patients bearing some types of $G\alpha_q/11$ -uncoupled KISS1R mutants display only partial gonadotropic deficiency (Tenenbaum-Rakover et al., 2007, de Roux et al., 2003). Future work could measure arrestin recruitment in GLCs.

In vitro treatment with kisspeptin in GLCs activated $G\alpha_q/11$ signalling pathways. The pERK signal intensity is modest and much lower than that following hCG administration. This is likely because of the low level of expression of KISS1R in these cells. Granulosa cells from human small antral follicles have significantly higher expression of KISS1R, and express KISS1, so future work should concentrate on the role of kisspeptin in these cells.

I used accumulation of IP1, a metabolite of IP3, as a marker of activation of calcium signalling. It was significantly higher after treatment with kisspeptin. Direct visualisation of calcium mobilisation after treatment with kisspeptin however, using a fluorescent calcium dye, did not demonstrate any calcium release. The increase in IP1 was modest, and is measured as accumulation over time, and therefore the low level calcium mobilisation may not have been visible as fluorescence at confocal microscopy. Also, given the variability of expression of Kiss1R, the 3 samples I tried may have all had relatively low kisspeptin receptor activity.

Kisspeptin has been shown to stimulate phosphorylation of PI3K/AKT in a tissue specific manner. This signalling pathway plays an essential part in granulosa cell apoptosis as well as primordial follicular survival, growth and transition to primary follicles (Zheng et al., 2012, Cecconi et al., 2012). Kisspeptin administration has been shown to activate PI3K/AKT in pre-optic neurons and thyroid cancer cell lines stably overexpressing KISS1R (Hanchate et al., 2012, Stathatos et al., 2005), but not in rat luteal cells or papillary thyroid cancer cells (Peng et al., 2013, Ringel et al., 2002). In fact in colorectal cancer cell lines stably over-expressing KISS1R pAKT was inhibited by k-10 administration (Chen et al., 2016). Similar to rat luteal cells, there was no activation of pAKT in GLCs *in vitro* after treatment with kisspeptin. There was also no activation of AKT after treatment with hCG. This mirrors a study by Casarini and colleagues, which showed that hCG treatment in GLCs *in vitro*, unlike LH, does not activate AKT (ref).

7.1.2.1 The role of kisspeptin receptor in GLCs.

It is known that hypothalamic kisspeptin affects ovarian activity indirectly via stimulating gonadotropin secretion, however both kisspeptin and KISS1R are expressed in the human ovary (Gaytan et al., 2009). *In vitro* treatment of cultured rat luteal cells with Kp-10 increased basal and hCG-induced progesterone levels but not oestradiol production, with concomitant increases in levels of key steroidogenic enzymes *STAR*, *CYP11A*, and *HSD3B2* and increased the phosphorylation of Erk1/2 mitogen-activated protein kinase (Gaytan et al., 2009). A study in pre-ovulatory chicken granulosa cells treated with kisspeptin-10 found an increase in progesterone secretion and steroidogenic gene expression (Xiao et al., 2011). These studies suggest a direct effect of kisspeptin-10 in reproduction.

Kisspeptin treatment *in vitro* in GLCs, unlike *in vivo*, did not cause or contribute to changes in gene expression of gonadotropin receptors, kisspeptin receptors or steroid enzymes or progesterone synthesis. Although there were signs of activation of intracellular signalling, this did not have any downstream effect, that I could measure. Interestingly GLCs from one patient had dramatically higher *KISS1R* and *in vitro* treatment with kisspeptin appeared to contribute to progesterone synthesis. While this is only one patient, perhaps kisspeptin is contributing directly

to steroidogenesis in GLCs from women how have a high endogenous expression of *KISS1R*. It also adds to the argument about examining kisspeptin action in GCs from smaller follicles, which have a dramatically higher expression of *KISS1* and *KISS1R*. It has been shown in animal models that kisspeptin may have a role in follicle maturation and follicle development (Saadeldin et al., 2012, Byri et al., 2017, Fernandois et al., 2016). Future studies could involve *in vitro* treatment of GCs from smaller follicles, although this tissue is difficult to obtain.

In addition, most of my *in vitro* studies were completed using Kp-10. Kp-54, which was used in the *in vivo* study, may result in differences in signal activation. *In vivo* Kp-54 is more potent with a longer half-life (d'Anglemont de Tassigny et al., 2017). Kp-10 has been used in all animal ovarian *in vitro* studies. However in binding and functional assays using CHO-K1 cells transfected with rat and human Kiss1R, it was found that kp-10 and 54 had the same affinity for the receptor and similar biological activity (Kotani et al., 2001). It is the C-terminal part which is common to all the kisspeptin peptides is responsible both for the binding and the activation of Kiss1R.

7.3.3 Proposed model of KISS1R signalling in GLCs

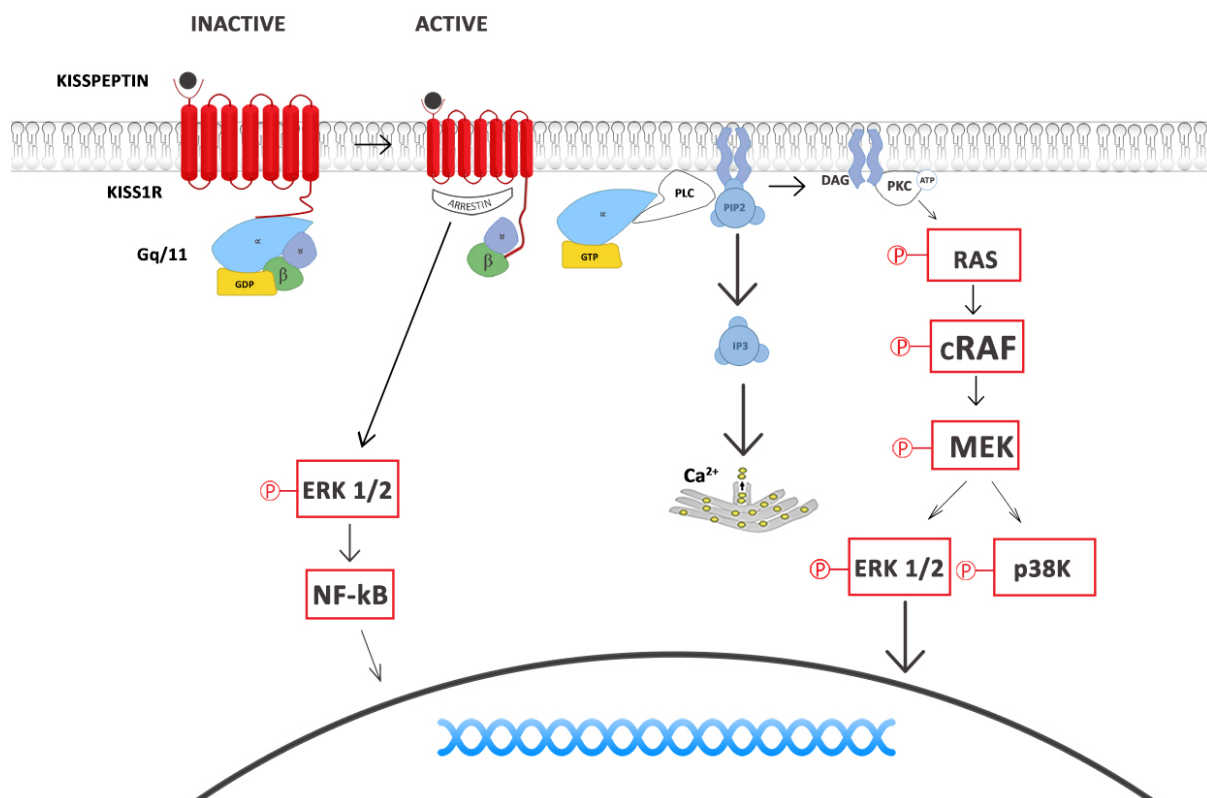


Figure 7.19 Kisspeptin receptor signalling in GLCs. Following ligand binding, the activated Kiss1 receptor switches on the cognate heterotrimeric ($\alpha\beta\gamma$) G protein by promoting the exchange for GDP for GTP in the α subunit. The G proteins detach from the receptor and cellular transduction commences. The phosphorylated $G\alpha_q/11$ protein activates phospholipase C (PLC)- β . This leads to activation of phosphatidylinositol 4,5-bisphosphate (PIP₂), accumulation of inositol-(1,4,5)-triphosphate (IP₃), protein kinase C (PKC) activation, and intracellular Ca²⁺ release. PKC activation also activates the ERK/MAPK pathway. The activated KISS1R also recruits β -arrestin 1 & 2 to the cell membrane, which also modulate phosphorylated ERK 1/2. This differs from, for example, KISS1R on GnRH KISS neurons which also activates PI3K/AKT signalling.

7.1.3 Ovarian kisspeptin in PCOS

Disordered gonadotropin secretion is a feature of PCOS, with LH hypersecretion and a relative deficiency of FSH. Given that kisspeptin controls GnRH secretion and predominantly stimulates LH release, it is plausible that kisspeptin may play a role in PCOS. It has been shown that in women with PCOS serum kisspeptin correlates positively with LH and leptin (Emekci Ozay et al., 2016). Some studies have shown elevated serum kisspeptin in women with PCOS and a negative correlation with FSH and a positive correlation with serum androgens (Gorkem et al., 2018), however other studies have found no elevation in serum kisspeptin in PCOS (Emekci Ozay et al., 2016).

Two studies have examined kisspeptin in the human ovary in PCOS, one showing lower expression of *KISS1* and *KISS1R* in PCOS mural and cumulus cells (Blasco et al., 2019) and the other showing higher expression of *KISS1* and *KISS1R* in PCOS GLCs, but no difference in *KISS1* in follicular fluid (Hu et al., 2018). I found no differential expression of either *KISS1* and *KISS1R* in PCOS in a large number of GCSAF, or in GLCs. Expression of both *KISS1* and *KISS1R* however are low in GLCs (*KISS1* negligible) and the relative impact of even significant changes in genes of such low expression is unclear, especially when no direct role for kisspeptin in these cells has been found.

7.3.4 Conclusions

Kp-54 *in vivo* augments gonadotropin receptor gene expression and steroidogenic enzymes, when compared with traditional oocyte maturation triggers hCG and GnRHa. The increases in serum gonadotropins are closer to that of the physiological menstrual cycle than other triggers and the expression of receptors and steroidogenic enzymes potentially creates an ovarian environment augmented for progesterone synthesis and luteal phase implantation. This mirrors what has been shown in animal models treated with kisspeptin (Gaytan et al., 2009, Xiao et al., 2011).

Overall it appears that changes in markers of ovarian steroidogenesis seen in those who had received kp-54 are related to the altered gonadotropin profile, as opposed to being related to direct ovarian stimulation by kisspeptin.

The kisspeptin receptor gene is expressed in GLCs and *in vitro* treatment activates two of the main Gq coupled receptors signalling pathways. However, this receptor activation does not result in progesterone production or augmentation of gonadotropin receptor and steroid enzyme expression *in vitro*. Future work should concentrate on cells with higher receptor expression, such as granulosa cells from smaller follicles or granulosa cell lines transfected with kisspeptin receptor.

Kisspeptin and kisspeptin receptor expression are not different in cells from women with or without PCOS. While kisspeptin may play a role in the aetiology of PCOS, ovarian kisspeptin action does not appear to play a part in this.

Chapter 8

General discussion and future perspectives

The overall goal of this thesis was to study gonadotropin receptor expression, signalling, trafficking and functioning in GLCs from women with and without PCOS. My main hypothesis, based on data from GWAS studies and *in vitro* data, was that LHR and FSHR function are altered in PCOS and this contributes to the aetiology of the condition.

To study this I have: (i) investigated gonadotropin receptor signalling pathways, receptor trafficking and downstream function in GLCs from women with and without PCOS; (ii) identified gene expression changes in GCs from small antral follicles from women with PCO; (iii) examined the effect of increasing follicle size on GC gene expression using RNA sequencing; (iv) examined the effect of androgen treatment on gonadotropin receptor signalling and function in GLCs and (v); established the direct and indirect effects of kisspeptin on gonadotropin receptor and GLC function.

My main findings are: (i) cAMP production in response to LH but not FSH is augmented in PCOS GLCs. This is associated with an increased expression of GIPC and β -arrestin 2, and no change in ERK or $G\alpha_q/11$ signalling. This augmentation results in a distinct profile of activation of downstream phosphoproteins, with no change in progesterone production *in vitro*. (ii) GCs from PCO SAFs displayed higher LHCGR expression in some follicles, higher *CYP19A1*, *STAR*, *INHBA* and lower *FSHR*, *AR*, *CYP11A1* expression. (iii) RNA sequencing of GCs revealed a small number of mainly downregulated genes in PCO; (iv) DHT treatment *in vitro* augments LHR but not FSHR $G\alpha_s$ signalling in GLCs; (v) Kisspeptin-54 as an oocyte maturation trigger results in a unique gene profile in GLCs compared with other maturation triggers; with higher expression of gonadotropin receptors and steroid enzymes, although markers of OHSS were not altered by kisspeptin administration; (vi) *In vitro* treatment in GLC with kisspeptin-10 activated ERK and $G\alpha_q/11$ signalling but did not alter KISS1R expression or steroidogenesis.

While I have shown that $G\alpha_s$ /cAMP signalling is augmented in PCOS GLCs in response to LH treatment *in vitro*, it remains unclear what the exact molecular mechanism underlying this is. I have postulated that it is related to augmented β -arrestin mediated receptor internalisation to the VEE, augmented trafficking away from the plasma membrane to the VEE via GIPC and

increased LHR expression (summarised in Figure 8.1). However it may also be related to APPL1, despite APPL1 gene expression being similar in PCOS and control GLCs. Knockdown of APPL1 enhances intracellular cAMP production in response to LH in HEK293 cells (Sposini et al., 2017). ERK signalling is not affected by APPL1 knockdown, again similar to PCOS GLCs. APPL1 is essential for LHR recycling from the VEE via a mechanism that involves LHR- dependent cAMP/PKA activation and APPL1 phosphorylation on S410 (Sposini et al., 2017). So future work to establish the mechanism underlying this enhanced LHR signal generation in PCOS GLCs could involve analysing the effect of APPL1 knockdown, and expression of APPL1 phosphorylated on S410, using phospho-specific antibodies. There may be a unique bias for $G\alpha_s$ over $G\alpha_q/11$ LHR signalling in PCOS GLCs, as there was higher cAMP but a trend towards lower IP1 accumulation in response to LH treatment. This could be further studied by assessing if increased $G\alpha_s$ -cAMP-PKA activity negatively regulates $G\alpha_q/11$ coupling and vice versa, which could be demonstrated by inhibiting PKA and then examining $G\alpha_q/11$ signalling and by inhibiting $G\alpha_q/11$ and then measuring cAMP levels in PCOS and control GLCs.

Other undiscovered endosomal proteins may also be involved in LHR trafficking, the expression and/or function of which may differ in PCOS. Novel techniques have been recently developed to track GPCRs and detect and measure proteins in close proximity at their native levels, for example using engineered ascorbic acid peroxidase (APEX) proximity labelling, which combines quantitative proteomics with a system of spatial references in order to capture the environment of a protein as it exists in situ (Paek et al., 2017, Lobingier et al., 2017). It also allows analysis of protein interaction networks that are dynamic. APEX fused to $\beta 2AR$ and the delta opioid receptor has been used to demonstrate agonist-responsive recruitment of β -arrestin-2, tagged with GFP (Lobingier et al., 2017).

This technique has also been used to measure the response of the angiotensin II type 1 receptor to balanced and biased agonists (Paek et al., 2017, Hung et al., 2016). However utilising this technique in GLCs would be difficult, as it involves transfection of receptor-peroxidase constructs, but trafficking pathways are conserved and use of heterologous cells lines are feasible and identified proteins could then be examined in GLCs.

The development of super resolution imaging technologies, such as PD-PALM (photoactivatable dyes- photoactivated localization microscopy), is enabling the visualisation and study of small intracellular compartments (Eichel et al., 2016, Jonas et al., 2015), thus making it theoretically possible to live track a GPCR from the plasma membrane, through to its engagement with trafficking machinery and movement to the endosomal compartment. This would allow me to visualise LHR internalisation and trafficking in order to highlight differences in PCOS. It would also allow comparison of the proportion of receptor trafficked to the VEE versus the EE, which may also differ in PCOS GLCs. I had intended to utilise this imaging technology to study LHR in GLCs, however it was not possible as I was unable to complete plasmid transfection in these cells. One option for studying this further is in granulosa or ovary cell lines, such as KGN or CHO cells, however response to gonadotropins is not necessarily the same (Thiruppathi et al., 2001) and receptor signalling may or may not be the same as primary cells. GCs from smaller follicles, which are not terminally differentiated and still divide *in vitro*, would likely to be easier to transfect and therefore tag the receptor and or trafficking proteins with a fluorescent label. While animal models have traditionally been used for studying PCOS, they are unlikely to be a good model for studying gonadotropin receptor trafficking and signalling. There are clear species-dependent variations in gonadotropin receptor function. Human and rodent LHR trafficking is distinct, as LHR inactivating mutations in women result in a mild phenotype of anovulatory infertility with normal pubertal maturation (Themmen and Huhtaniemi, 2000), whereas in mice the females are hypogonadal with defective folliculogenesis and anovulation (Zhang et al., 2001).

While I have demonstrated the altered LHR signalling pathways in GLCs from women with PCOS, what is less clear is the downstream effects of this. Progesterone output, the main role of LHR in GLCs, was the same in control and PCOS GLCs, however *in vitro* progesterone output into culture media may not reflect *in vivo* progesterone production in follicles through LHR action. I also studied FSHR signalling pathways, and while there were no differences in cAMP or pERK signalling assays after FSH treatment, there were differences in downstream protein phosphorylation. Future work on FSHR signalling in PCOS should evaluate it in GCs from smaller follicles, the main site of FSHR action and this will be done in the next phase of this project, through our collaboration with the University of Copenhagen. We have received MRC funding to

continue studying gonadotropin receptor action in the ovary, based on the data within this thesis.

I have established in GC from PCO women from SAFs that there is a distinct GC gene expression profile, with a subset of follicles expressing dramatically higher expression of LHCGR, with lower expression of AR, FSHR and CYP11A1, and higher expression of *CYP19A1*, *STAR* and *INHBA*. These are the follicles that, typically, then arrest and do not mature. The expression of *STAR* and *HSD3B2* are augmented in PCO follicles, which may explain and provide a possible molecular mechanism to explain why certain PCO follicles prematurely make progesterone and make more oestradiol than control follicles. It will be very interesting going forward to study these GCs from PCO follicles, by treating them with gonadotropins *in vitro* and to establish which FSHR signalling pathways are active and to study the trafficking of the receptor, using similar signalling assays as were used during my PhD. Given that GCs from certain PCOS follicles respond to LH, it will also be interesting to establish the signalling pathways involved in this premature LHR activity. This will be done as part of the next phase of this project.

The premature LHR activity may be related to excess androgen exposure. Therefore the next phase of this project will also involve theca cell culture, the site of ovarian androgen production. Theca cell hyper-responsiveness in PCOS has been established but the receptor signalling pathways underlying this have not been elucidated. APPL1 knockdown in bovine theca cells has been shown to enhance LH-stimulated androgen production (Comim et al., 2013a). This may be related to alterations in LHR recycling and increased cAMP, but little is known about LHR trafficking and signalling in theca cells.

The ultimate aim of all of this work is to exploit differences in ovarian LHR and FSHR function in PCOS as a drug target. One of the key issues in PCOS is follicle arrest and anovulation, which appears to be related to premature LHR activity in GCs from small antral follicles, which is associated with higher LHCGR expression. This premature LH activity may be related to hyperandrogenism or chronic stimulation from high circulating LH. Follicle arrest and anovulation

may also be related to reduced FSH secretion, which appears to reduce FSHR expression in GCs. Partial inhibition of LHR G α s signalling during or prior to puberty may play a future role in the management of PCOS. In recent years, there have been significant efforts to develop functionally selective biased ligands, that preferentially activate one GPCR signalling pathway over another. For example β -arrestin-biased ligands have been developed for certain receptors, such as for dopamine receptor 2, and these show promise in the treatment of psychosis, with fewer side effects (Park et al., 2016).

Allosteric small molecule compounds of the FSHR have been developed in recent years, in order to try and provide more targeted, stable treatments which can be administered orally. They consist of allosteric agonists, and positive and negative allosteric modulators (Anderson et al., 2018). Compounds targeting the LHR have also been identified, however most effort has gone into studying FSHR targeted compounds, as FSH is used daily in ovarian stimulation protocols for IVF. One of these FSH compounds, compound 5, a thiazolidenidione, was demonstrated to stimulate steroid production and increase ovulation in FSHR-transfected rat primary granulosa cells (Yanofsky et al., 2006). Another compound, Org 214444-0, a hexahydroquinoline, induced follicular growth in a rat ovulation assay by potentiating endogenous FSH activity (van Koppen et al., 2013). Unfortunately human testing on these FSH compounds has thus far failed to demonstrate an effect (Gerrits et al., 2016). It would be fascinating to develop and test small molecule allosteric modulators of LH which may 'correct' the ligand bias found in PCOS GLCs, and of FSH which may augment signalling to counteract the reduced FSHR found in PCO GCs (Nataraja et al., 2015).

I also carried out RNA sequencing of PCO GCs and surprisingly a very small number of transcripts (23) were differentially expressed in PCO. Most of these genes were downregulated (19) and their expression was low. A number of these genes were also pseudogenes, which do not encode proteins, and pathway analysis did not reveal any enriched pathways. However gene ontology analysis did reveal some interesting processes to be involved, such as meiotic division and basement membrane function. While the functional significance of these genes is uncertain, their downregulation was large, with some of the genes up to 90-fold downregulated (*H1FOO*),

so they may be useful as biomarkers of PCOS. It would be interesting to test serum levels of the proteins encoded by some of these genes, and see if they are detectable and are different in PCOS. MicroRNAs (MiRNAs) have been studied in serum (Sathyapalan et al., 2015) and follicular fluid (Xue et al., 2018) of women with PCOS. It would be interesting to study MiRNAs in GCs, and to compare them with those found in other tissues, which may add further strength to developing their role as biomarkers of the condition.

We will also learn more through further analysis which is ongoing on the sequencing results. eQTL analysis (Nica and Dermitzakis, 2013) comparing sequencing data with existing GWAS data is being carried out, as has been completed previously in PCOS with serum, fat and skin biopsies (Pau et al., 2017). The vast majority of GWAS risk loci fall in non-coding regions of the genome. This may be because the GWAS risk loci alter the individual's disease risk via their effect on gene expression in different tissues. Analyses such as eQTL help to determine which genes are affected in which tissues. Another related analysis which we are completing on this data is an eCAVIAR analysis (eQTL and GWAS Causal Variant Identification in Associated Regions). This model allows for allelic heterogeneity, where multiple variants can be causal in a single locus, a phenomenon that is widespread in eQTL data (Hormozdiari et al., 2016).

RNA sequencing of GCs from follicles of increasing sizes highlighted the genetic fingerprint of growing follicles, i.e. what genes increase in expression and the rate at which they increase as follicles mature. This analysis was completed in GCs from all follicles, both PCO and control, with the linear regression analysis controlling for PCO, and highlighted genes mainly involved in steroidogenesis and gonadotropin regulation. There was insufficient power to separately analyse PCO, but it would be fascinating in the future to use RNA seq on a larger subset of follicles to model differential gene expression in PCO and the exact follicle size at which changes start to emerge.

The limitations of studying primary GCS *in vitro* is their 2D nature and that their behaviour *in vitro* is likely different to that *in vivo*. Follicle culture systems have been developed in mice and

have been successful in completing growth *in vitro* from immature primordial stages as far as IVF of oocytes followed by embryo transfer and production of live offspring (Eppig and O'Brien, 1996). While this has not yet been completed in human tissue, there have been studies which have successfully cultured ovarian follicles and cortices, using a multi-step approach to support the changing requirements of the developing oocyte and GCs (McLaughlin et al., 2018, Telfer et al., 2008, Liebenthron et al., 2013). It would be fascinating to examine the growth of control and PCO follicles *in vitro* and the effect of premature LH and excess androgen exposure on follicle maturation, in order to functionally demonstrate the sequence of events underlying follicle arrest in PCOS.

An organoid platform has been developed for studying ovarian cancer (Kopper et al., 2019) and has a huge potential as a preclinical model of disease for research and drug testing. These ovarian cancer organoids retain the histological and genomic features of the lesion from which they were derived, and can be genetically modified. Different organoid lines have been formed for differing subtypes, facilitating study of heterogenous disease. Organoids can be generated from somatic cells, adult stem cells or pluripotent stem cells (Kopper et al., 2019). Development of a PCOS GC organoid model would facilitate studying this heterogenous condition, permitting study of gonadotropin and androgen receptor signalling and interactions, heterodimerisation and the effect of knockdown of genes found to be downregulated in RNA sequencing of GCs. It would also allow testing of small molecule allosteric modulators of LHR and FSHR. It could also be used to examine the functional effects of other GWAS targets, and to recreate the genetic signature from GWAS studies.

I also studied the effect of androgen and kisspeptin receptor on gonadotropin receptor and GLC function. Androgen receptor and androgen actions are critical to ovarian function, as well as being key to the pathogenesis of PCOS. The role of androgens in growing follicles are clear but its role around the time of ovulation is unclear. *In vitro* treatment with DHT augmented LHR $G\alpha_s$ and MAPK signalling in GLCs. Future work should examine the effects of supra-physiological doses of androgen on GLC function, more closely mimicking the hyperandrogenism of PCOS, and study AR signalling pathways in control and PCOS GLCs, including studying its localisation and

crosstalk with GPRC6A and EGFR. There are whole functional assays that monitor receptor activation by either nuclear translocation or receptor co-activator interactions that could be used to study this, and high affinity labelled ligands for nuclear hormone receptors have been developed (Wilson, 2009).

Kisspeptin receptor is expressed by GLC and *in vitro* treatment with kisspeptin-10 activated $G\alpha_q/11$ and ERK signalling. It did not however alter expression of its receptor or of gonadotropin receptors or steroid enzymes or alter progesterone production, which differs from studies in animal GCs, suggesting either that the action of the receptor is minimal or that it has alternate effects, unrelated to gonadotropin receptor action or steroidogenesis. Gene expression is much higher in GCs from SAFs so future work should concentrate on measuring signal activation and downstream effects *in vitro* in these cells.

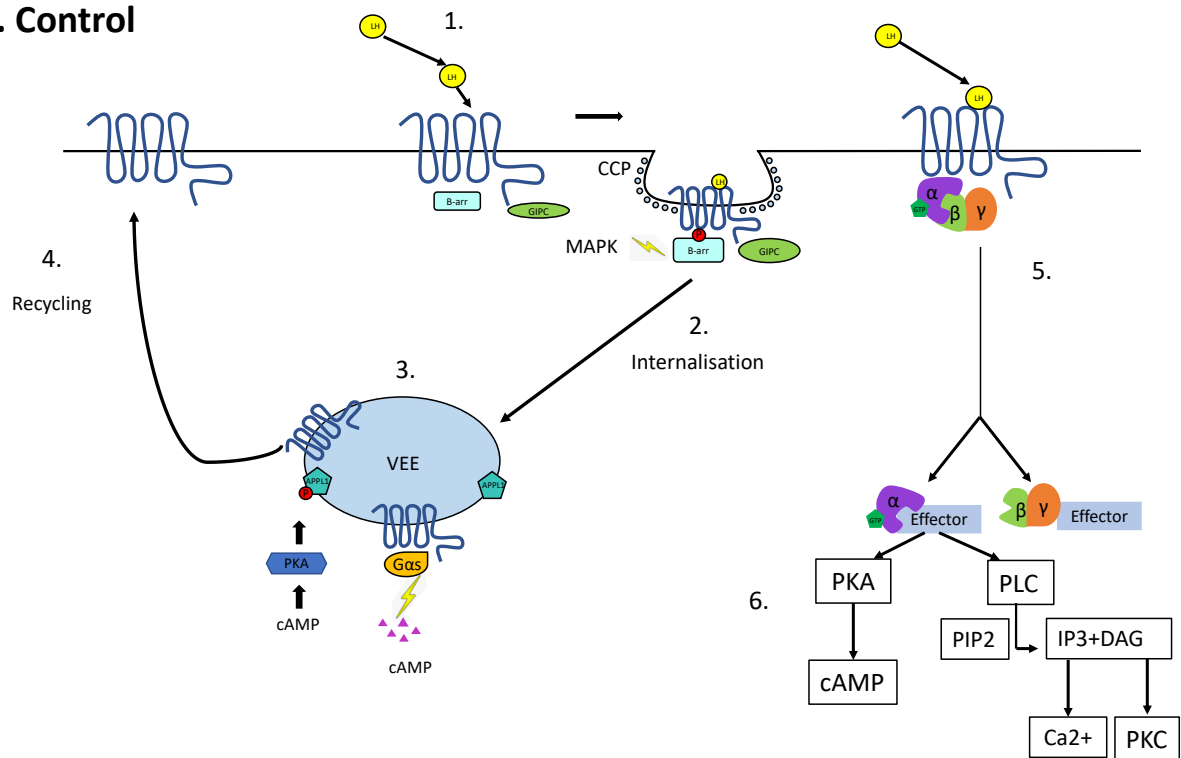
Kisspeptin receptor is also expressed in the oocyte in animal models (Tanyapanyachon et al., 2018) (Saadeldin et al., 2012, Saadeldin et al., 2018), and has been shown to enhance *in vitro* maturation and embryonic developmental competence in sheep and pig models (Saadeldin et al., 2018) (Byri et al., 2017). Future work should establish whether kisspeptin and its receptor are expressed in human oocytes, and if kisspeptin treatment contributes to *in vitro* maturation of human oocytes. This could be done in two ways; using the immature oocytes from ICSI that are not suitable for fertilisation, or using oocytes extracted from SAFs at the time of ovary cryopreservation. The first option is being launched as a study in Hammersmith hospital IVF unit this year, and I have been providing guidance on experimental protocol. The second option could be explored in the future through our collaboration with Professor Claus Yding Andersen.

Kisspeptin also has a therapeutic role in inducing oocyte maturation in IVF. I have demonstrated that using kisspeptin-54 as opposed to standard treatment with hCG or GnRHa creates a dramatically different serum gonadotropin profile and this results in a distinct GLC receptor and steroid gene expression profile. There were no differences in gene expression of markers of OHSS, likely because of the selection of high-risk patients for kisspeptin treatment. The next

phase of testing kisspeptin in the clinical setting in IVF will likely involve a randomised controlled trial of kisspeptin-54 versus the other triggers, to establish its efficacy and OHSS rate compared to standard treatment. It would be interesting as part of this study to measure serum and follicular fluid markers of OHSS such as VEGF, as well as to repeat gene expression in groups that have been matched, i.e. clinical parameters are the same.

In conclusion, there are distinct differences in signalling and trafficking of the LHR in GLCs from women from PCOS, with bias towards $G\alpha s$ signalling which may be driven by enhanced trafficking to the VEE, which results in altered downstream phosphoprotein regulation and LHR expression. A proposed model of LHR signalling in PCOS is demonstrated in Figure 8.1 below. Androgen treatment *in vitro* in GLCs augments LHR function but kisspeptin treatment does not appear to alter gonadotropin receptor function or steroidogenesis. GCs from small antral follicles in women with PCO display a distinct gene expression profile which may explain the premature luteinisation and follicle arrest seen in the condition.

A. Control



B. PCOS

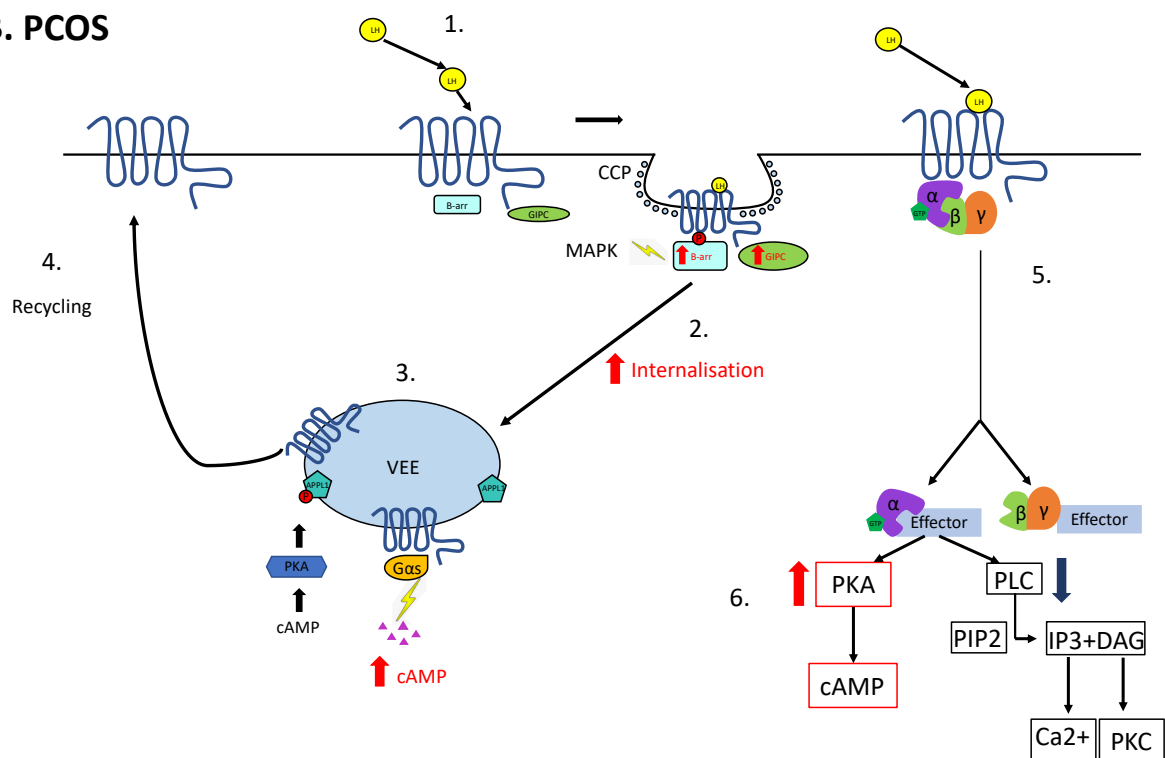


Figure 8.1 Proposed model of LHR trafficking and signalling in control (A) and PCOS (B) GLCs. **1.** LH binds to the receptor which undergoes a conformational change. **2.** The receptor is internalised after recruitment of arrestins via clathrin coated pits. In PCOS there is augmented β -arrestin expression which enhances receptor internalisation. The receptor is then trafficked to the VEE, which is mediated by GIPC. This trafficking is enhanced in PCOS as GIPC expression is augmented, and as a result of enhanced internalisation. **3.** Endosomal generated cAMP signalling is generated at the VEE and this is augmented in PCOS. APPL1 marks the VEE. **4.** APPL1 facilitates receptor recycling, after it is phosphorylated by PKA on Ser410. It is unclear if this is altered in PCOS **5.** After the activated receptor undergoes a conformational change and promotes the exchange of GDP for GTP for the $G\alpha$ subunit, after which the $G\alpha$ subunit dissociates from the $G\beta\gamma$ and from the receptor and both commence signal transduction through effectors. **6.** Signal transduction in $G_{\alpha s}$ and $G_{\alpha q}/11$ results in generation of second messengers. In PCOS there is a bias towards $G_{\alpha s}$ signalling over $G_{\alpha q}/11$, which results in increased cAMP production.

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Appendix

Appendix A

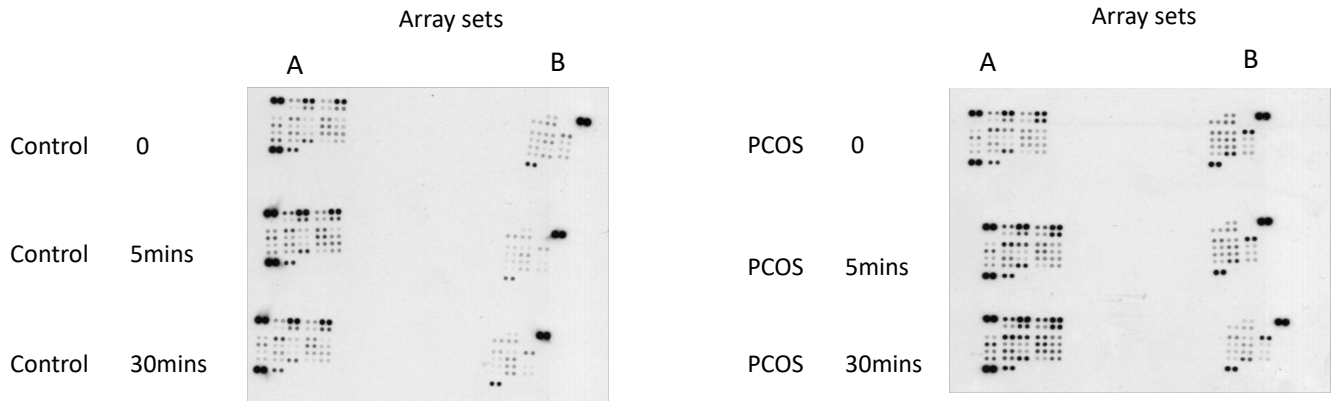


Figure A1.1 Phospho-Kinase array blots of control and PCOS GLCs treated with LH for 0, 5, 30 minutes. Pooled cell lysates were probed using the phospho-kinase array kit (RnD systems). Each array set contained 43 different phospo-antibodies in duplicate. Reference spots (upper and lower left hand corner and upper right hand of each set) were also included for normalisation. See www.rndsystems.com/products/proteome-profiler-human-phospho-kinase-array-kit_ary003b for a list of co-ordinates for each protein target.

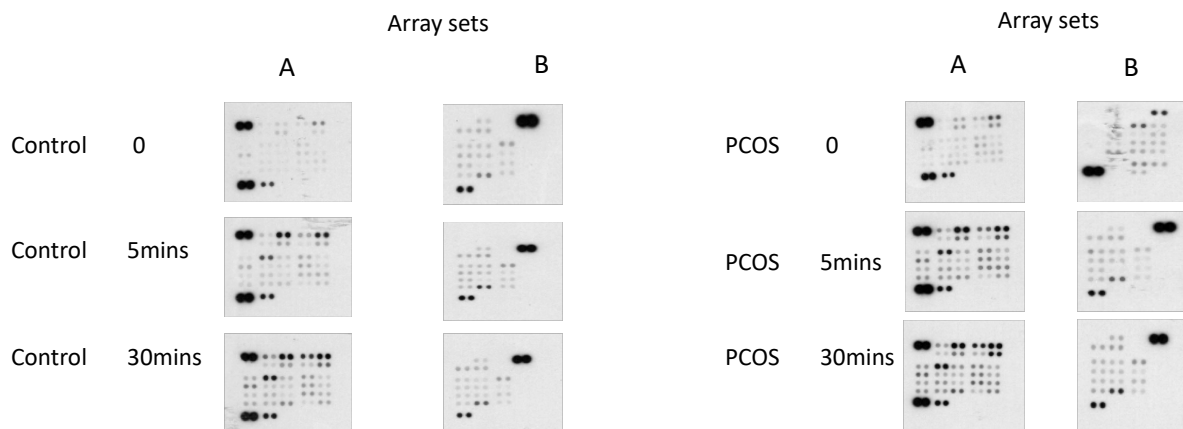


Figure A1.2 Phospho-Kinase array blots of control and PCOS GLCs treated with FSH for 0, 5, 30 minutes. Pooled cell lysates were probed using the phospho-kinase array kit (RnD systems). Each array set contained 43 different phospho-antibodies in duplicate. Reference spots (upper and lower left hand corner and upper right hand of each set) were also included for normalisation. See www.rndsystems.com/products/proteome-profiler-human-phospho-kinase-array-kit_ary003b for a list of co-ordinates for each protein target.

Publications and presentations

Publications (from which data is included in this thesis)

1. Owens LA, Kristensen SG, Lerner A, Christopoulos G, Lavery S, Hanyaloglu AC, Hardy K, Yding Andersen C, Franks S. Gene expression in granulosa cells from small antral follicles from women with or without polycystic ovary syndrome. Manuscript being revised for JCEM.
2. Lerner A*, Owens LA*, Coates M, Simpson C, Poole G, Velupillai J, Liyanage M, Christopoulos G, Lavery S, Hardy K, Franks S. Expression of genes controlling steroid metabolism and action in granulosa-lutein cells of women with polycystic ovaries. *Mol Cell Endocrinol*. 2019 Apr 15;486:47-54. doi: 10.1016/j.mce.2019.02.015. Epub 2019 Feb 22. *Joint first authors
3. Owens LA, Abbara A, Lerner A, O'floinn S, Christopoulos G, Khanjani S, Islam R, Hardy K, Hanyaloglu AC, Lavery SA, Dhillon WS, Franks S. The direct and indirect effects of kisspeptin-54 on granulosa lutein cell function. *Hum Reprod*. 2017 Dec 1;1-11. doi: 10.1093/humrep/dex357

Presentations

1. Differential regulation of genes relevant to reproductive function in human granulosa cells of small antral follicles from women with or without polycystic ovary syndrome. Endocrine Society meeting, 2019 New Orleans (poster)
2. The *in vivo* and *in vitro* effects of kisspeptin on human ovarian function, European Congress of Endocrinology, 2018 Barcelona (oral)
3. Alterations in gonadotropin receptors and signal activation in granulosa lutein cells from women with polycystic ovary syndrome, European Congress of Endocrinology, 2018 Barcelona (poster)
4. A study of gonadotropin receptor function in granulosa lutein cells from women with Polycystic Ovary Syndrome. British Pharmacological Society Cell Signalling meeting, Nottingham, April 2018 (poster)

5. The *in vivo* and *in vitro* effects of kisspeptin on human ovarian function, Irish Endocrine Society meeting 2018, Ireland (oral)
6. Insight into the molecular mechanisms underlying enhanced gonadotropin hormone receptor activity in polycystic ovarian syndrome Irish Endocrine Society meeting 2016, Ireland (oral)
7. Insight into the molecular mechanisms underlying enhanced gonadotropin hormone receptor activity in polycystic ovarian syndrome, British Endocrine society meeting 2016, Brighton.
8. Insight into the molecular mechanisms underlying enhanced gonadotropin hormone receptor activity in polycystic ovarian syndrome. Society of Reproduction and Fertility annual meeting 2016, Winchester