Investigation into the Expression and Localisation of c-kit and the Regulation of Kit Ligand Gene Expression in the Adult Human Ovary

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The most beautiful thing we can experience is the mysterious.
It is the source of all true art and science.

Albert Einstein

Question everything. Learn something. Answer nothing.

Euripides

I love fools' experiments. I am always making them.

Charles Darwin

This thesis is dedicated to my mum and dad.

Thank you for everything.

ABSTRACT

Folliculogenesis is a complex process that is central to the ovary's primary function, the production of healthy oocytes. One of the essential ligand/receptor pairs that mediates folliculogenesis is kit ligand (KITL), a granulosa-derived cytokine growth factor, and its receptor, c-kit. Since their discovery two decades ago, the KITL/c-kit system has been extensively studied in animal models, in particular the mouse, in which it has been demonstrated to be crucial for normal folliculogenesis and fertility. To date, little investigation into KITL and c-kit has been performed in the adult human ovary. Previously, this laboratory showed abnormally increased KITL protein levels in human polycystic ovaries (PCO) compared to non-PCO, suggesting that KITL may contribute to several PCO phenotypes according to the range of actions KITL has been shown to have in animal folliculogenesis. Thus, this thesis aimed to characterise KITL and c-kit expression and localisation in the adult human ovary, including polycystic ovaries, and examined regulation of KITL gene expression by endocrine and intraovarian factors.

To perform these studies, human ovarian tissues were obtained. These included granulosa cell subtypes cumulus and mural granulosa cells from women undergoing assisted reproductive technology treatment at infertility clinics, fresh ovarian cortex from the Royal Adelaide Hospital and archival paraffin-embedded human ovarian tissue from the Institute of Medical and Veterinary Sciences. The human granulosa tumour cell line, KGN, was also used as a model.

KITL and c-kit isoforms were demonstrated to be present in the human ovary throughout follicle development. KITL-2 was shown to be expressed primarily by granulosa cells representing preantral follicles, while KITL-1 was the predominant isoform expressed in preovulatory granulosa cells, suggesting that KITL-2 may play a greater role during early follicle development which then diminishes in preovulatory follicles with increased KITL-1 levels. Both c-kit mRNA isoforms were found to be present in human ovarian cortex. Examination of c-kit localisation throughout follicle development by immunohistochemistry revealed that all follicular cell types in preantral and antral follicles expressed c-kit protein. This may suggest that KITL has an unknown autocrine function in granulosa cells unique to the human ovary, as animals models have previously demonstrated c-kit staining to be confined to the theca layer and the oocyte. c-kit staining patterns were found to be different in PCO compared to non-PCO preantral and antral follicles, suggesting a potential involvement for c-kit in PCO pathology. Collectively these results suggest, as demonstrated in various animal models, that the KITL/c-kit system is present in the human ovary and may have some involvement in the mediation of human folliculogenesis.

Regulation of KITL gene expression was examined using KGN and cumulus cells. Based on previous studies, the candidate regulatory factors that were examined included androgen receptor (AR), endocrine factor follicle-stimulating hormone (FSH), theca-derived factor keratinocyte growth factor (KGF) and oocyte-secreted factors bone morphogenetic factor-15 (BMP-15) and growth differentiation factor-9 (GDF-9). Of these candidate factors, GDF-9 was found to directly decrease KITL gene expression in KGN

cells and cumulus cells via ALK 4/5/7 receptors. There was also some evidence for a slight reversal of the GDF-9 effect on KITL expression by the addition of the potent androgen 5α-dihydrotestosterone (DHT). The results of these studies indicated KITL gene expression is regulated by GDF-9 in human granulosa cells and are consistent with observations of negative regulation of KITL expression in mouse granulosa cells.

Evidence shown in this thesis suggests that the ratio of KITL isoforms in granulosa cells changes throughout human folliculogenesis. Follicular target cells for KITL signalling were found to include granulosa cells, theca cells and the oocyte, suggesting that the KITL/c-kit system may have potential roles throughout human folliculogenesis as demonstrated in animal models. Furthermore, this thesis has demonstrated that GDF-9 directly regulates KITL gene expression in human granulosa cells. From these results, this thesis proposes an *in vivo* model in which abnormally low levels of GDF-9, shown by a previous study to be characteristic of PCOS oocytes, results in increased KITL levels and this effect may be further exacerbated by the reversal of GDF-9 inhibition by excess androgen. This thesis has provided a greater understanding of the molecular mechanisms involved in human folliculogenesis which may be of use in future therapeutic strategies and diagnosis in assisted reproductive technology, and provide a basis for understanding human ovarian function and ovarian disease.

DECLARATION

I, Astrud R. R. Tuck, certify that this work contains no material which has been accepted for the award of any other degree or diploma in any university or other tertiary institution, and, to the best of my knowledge and belief, contains no material previously published or written by another person, except where due reference has been made in the text.

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Astrud R R Tuck

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Hickey TE, Tuck AR, Dodd T, Norman RJ, Tilley WD. Increased kit ligand in polycystic ovaries. Annual Meeting of the Endocrine Society, San Francisco, CA, USA, 2008.

Hickey TE, Tuck AR, Dodd T, Norman RJ, Tilley WD. Increased expression of an androgen receptor regulated gene, kit ligand, in polycystic ovaries. Annual Meeting of the Society for Reproductive Biology, Melbourne, VIC, 2008.

Tuck AR,Yang X, Tilley WD, Hickey TE. Kit ligand expression and regulation in human polycystic ovaries. Australian Society for Medical Research Annual Scientific Meeting of the SA Division, Adelaide, SA, 2009.

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Tuck AR, Robker RL, Tilley WD, Hickey TE. Kit ligand expression and regulation in human ovarian granulosa cells. University of Adelaide Faculty of Health Sciences Postgraduate Expo, Adelaide, SA, 2009.

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ABBREVIATIONS

3,4-DCI	3,4-dichloroisocoumarin
A	antrum
ALK	anaplastic lymphoma kinase
ANOVA	analysis of variance
AR	androgen receptor
ARE	androgen response element
ART	assisted reproductive technology
bFGF	basal fibroblast growth factor
BL	basal lamina
BMI	body mass index
BMP	bone morphogenetic factor
BMPR	bone morphogenetic factor receptor
bp	base pair
BSA	bovine serum albumin
cAMP	cyclic adenosine monophosphate
CA	California
CAlb	corpus albicans
CC	cumulus cells
cDNA	complementary DNA

CL	corpus luteum
CO_2	carbon dioxide
COC	cumulus-oocyte-complex
COOH	carboxyl group
DAB	3,3'-Diaminobenzidine
DBD	DNA binding domain
DCC	dextran coated charcoal
DCC-FBS	dextran coated charcoal-fetal bovine serum
DFP	diisopropylfluorophosphate
DHT	5α-dihydrotestosterone
DMSO	dimethyl sulphoxide
DNA	deoxyribonucleic acid
DNase 1	deoxyribonuclease 1
EDTA	ethylenediamine tetra-acetic acid
EGF	epidermal growth factor
ERK	extracellular signal-regulated kinase
EtOH	ethanol
FAI	free androgen index
FBS	fetal bovine serum
FSH	follicle stimulating hormone
FSHR	follicle stimulating hormone receptor
g	gram
GC	granulosa cells

GDF	growth differentiation factor
GnRH	gonadotrophin releasing hormone
h	hour
hCG	human chorionic gonadotrophin
HSP	heat shock protein
IGF-1	insulin-like growth factor-1
IgG	immunoglobulin
IU	international units
IVF	*in vitro* fertilisation
IVM	*in vitro* maturation
kb	kilo base
kD	kilo Dalton
KGF	keratinocyte growth factor
KITL	kit ligand
L	litre
LBD	ligand binding domain
LH	luteinising hormone
M	molar
mA	milliampere
MAP	mitogen activated protein
MAPK	mitogen activated protein kinase
mg	milligram
MGC	mural granulosa cells

min	minute
mL	millilitre
mM	millimolar
mRNA	messenger RNA
NaCL	sodium chloride
ng	nanogram
NH_2	amino group
nmol	nanomolar
NTD	amino-terminal domain
O	oocyte
OHF	hydroxyflutamide
PBS	phosphate-buffered saline
PCO	polycystic ovaries
PCOS	polycystic ovarian syndrome
PCR	polymerase chain reaction
PMA	phorbol 12-myristate 13-acetate
POF	premature ovarian failure
PTX3	pentraxin 3
qPCR	quantitative polymerase chain reaction
RIPA	radioimmunoprecipitation assay buffer
RNA	ribonucleic acid
RNase	ribonuclease
rpm	revolutions per minute

RT	reverse transcriptase
S	stroma
SA	South Australia
SCF	stem cell factor
SD	standard deviation
sec	second
SEM	standard error of the mean
SHBG	steroid hormone binding globulin
SMAD	mothers against decapentaplegic protein
StAR	steroidogenic acute regulatory protein
T	theca layer
TβR	transforming growth factor β receptor
TBS	tris buffered saline
TBST	tris buffered saline-tween 20
TGF	transforming growth factor
UK	United Kingdom
USA	United States of America
V	volt

Other:

°C	degrees Celsius
µg	microgram
µl	microlitre

µm	micron
µM	micromolar

CHAPTER 1

Introduction

Chapter 1: Introduction

1.1 OVERVIEW

A healthy, functioning ovary is essential for female fertility and reproductive success. Pregnancy is the most notable sign of fertility, which is dependent on the timely release of an oocyte, capable of being fertilised by sperm. Many women at some point in their reproductive lifespan choose to prevent pregnancy, often with the use of steroid hormone-based contraception. Infertility refers to the inability to conceive and produce offspring which can have many causes, several of which include ovarian dysfunction and disease. The ovary undergoes a highly complex and organised process known as folliculogenesis to produce a healthy, mature oocyte, and disruption or aberration in this process may result in infertility or sub-fertility.

Folliculogenesis is dependent on endocrine and intraovarian signalling pathways coordinating the timely proliferation and differentiation of somatic cells within the ovary, in conjunction with the growth and maturation of the oocyte. Much of what is currently known and understood about this complex process has been deduced from studies using animal models. Premenopausal human ovarian tissue is difficult to obtain and maintain in culture, thus animal models, in particular the mouse, have been widely utilised to study folliculogenesis. Mice reproduce quickly, are easy to maintain and their genetics is well understood and easy to manipulate experimentally. However, a disadvantage of many animal models is that they have markedly different ovarian cycles to humans, such as the

rapid production of several ovulated oocytes during a single cycle. While these models have been incredibly useful in gaining a significant insight into many aspects of folliculogenesis and its essential regulatory genes, it must be determined exactly how new knowledge gleaned from animals can be applied to the human ovary. Thus, in order to fully understand human fertility and its disorders, in addition to developing new therapeutic strategies to treat disease, more study into the molecular and cellular mechanisms of the human ovary remains vital.

This chapter gives an overview of mammalian folliculogenesis, including human, highlighting the important role of the various signalling pathways involved at each stage. In particular, the contribution of cytokine factor kit ligand and its receptor c-kit throughout follicular development is discussed, as well their possible involvement in the female infertility disorder, polycystic ovary syndrome.

1.2 FOLLICULOGENESIS

The ovary has two critical functions which are pivotal to female fecundity and reproductive success: the production of healthy, mature oocytes and the generation of sex steroid hormones which are essential for the development of secondary sex characteristics and regulation of the hypothalamic-pituitary-ovarian axis. A highly organised composition of germ cells and somatic cells comprise the ovary and give rise to the ovarian follicle, a structure that is central to the reproductive capacity of the ovary. The follicle contains a single germ cell, the oocyte, surrounded by somatic cells including granulosa and theca cells. These somatic cells provide an environment for the oocyte's nurture, survival and regulation to enable the oocyte to become competent and capable of being fertilised by sperm in the reproductive tract after ovulation. There is strong evidence that the oocyte directs follicle development, but follicular cells are interdependent and require complex autocrine, paracrine, juxtacrine and endocrine communication to ensure harmonious cell growth, timely steroidogenesis, oocyte maturation and ovulation. Thus, fertility is dependent on the coordination of regulatory signalling pathways, and unravelling the mechanisms behind this complex network of events is essential for understanding ovarian dysfunctions and disease in women.

Folliculogenesis begins during prenatal life with gonadal development. Primordial germ cells divide to give rise to approximately 7 million oocytes which form syncytia, or nests, of oocytes that subsequently break down to form individual primordial follicles. These

follicles consist of a single oocyte, arrested in prophase during the first stage of meiosis, surrounded by a single layer of flattened granulosa cells. These cells support oocyte growth and maturation by producing growth factors, nutrients, metabolic precursors and other small molecules much of which is mediated by direct bidirectional communication via intracellular membrane gap junctions (Anderson and Albertini 1976; Cecconi *et al.* 2004). Due to extensive apoptosis of oocytes prior to birth, only 2 million primordial follicles remain after birth, and this quiescent follicle pool serves as the oocyte reservoir throughout female reproductive life. These primordial follicles are located in the cortex, the most superficial layer of the ovary (Figure 1.1). Of this initial follicle pool, only 0.02% will ovulate, while the remaining follicles degenerate in a process called atresia.

Follicle activation is the process by which primordial follicles initiate growth and this continuous process begins long before the onset of puberty, independently of pituitary gonadotrophins (Fortune *et al.* 2000; Richards and Pangas 2010). Little is known about the signalling pathways that trigger follicle activation. Figure 1.2 shows examples of factors involved at each stage of follicle development. Oocyte-secreted factors, shown to be expressed by primordial follicles in mice, are believed to be involved in follicle activation and these include two members of the transforming growth factor-β (TGFβ) family: bone morphogenetic protein-15 (BMP-15) and growth differentiation factor-9 (GDF-9). These factors appear to enhance primordial follicle activation but are not essential, as follicles enter the growing pool regardless of their presence (Choi and Rajkovic 2006). Squamous granulosa cells also contribute to follicle activation and there is strong evidence for the

Figure 1.1. Schematic representation of human folliculogenesis. Primordial follicles, consisting of an oocyte surrounded by a single layer of flattened granulosa cells, are activated and growth of the oocyte and granulosa cells ensues. Cuboidal granulosa cells characterise a primary follicle, followed by proliferation of granulosa cells to form two or more layers around the oocyte, thus creating a secondary follicle. The fluid-filled antrum forms and stromal cells are recruited to proliferate and differentiate into the theca layer, which surrounds the rapidly proliferating granulosa cells of the antral follicle. Granulosa cells differentiate into cumulus cells, which immediately surround the oocyte, and mural granulosa cells, which surround the antrum and form the follicle wall. In response to the LH surge, the dominant antral follicle extrudes the cumulus-oocyte complex into the reproductive tract. The ovulated follicle then undergoes terminal differentiation to form the corpus luteum, and if pregnancy does not occur, it degrades into a fibrous tissue mass known as the corpus albicans. Original source of modified figure is unknown.

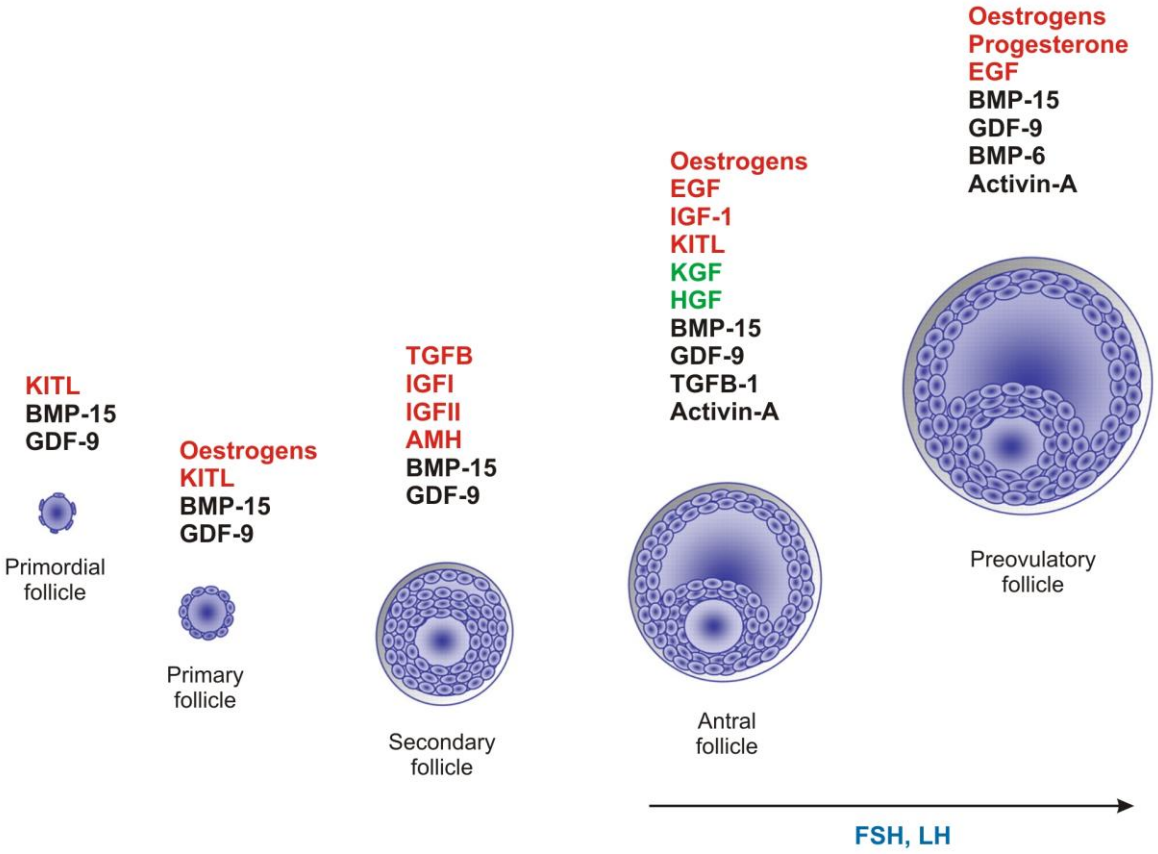


Figure 1.2. Examples of factors involved in each stage of follicle development. Factors in red are granulosa-derived, those in black are oocyte-secreted factors, those in green are theca-derived factors and those in blue are endocrine factors. Abbreviations: KITL, kit ligand; BMP-15, bone morphogenetic protein-15; GDF-9, growth differentiation factor-9; TGFβ, transforming growth factor-β; IGF1, insulin-like growth factor-1; IGFII, insulin-like growth factor-II; AMH, anti-Müllerian hormone; EGF, epidermal growth factor; KGF, keratinocyte growth factor; HGF, hepatocyte growth factor; TGFβ-1, transforming growth factor-β1; BMP-6, bone morphogenetic factor-6.

Modified from original figure (Hillier 2009).

requirement of kit ligand (KITL) in this process, a granulosa-cell secreted cytokine growth factor which signals via its receptor c-kit located on the oocyte (Yoshida *et al.* 1997; Parrott and Skinner 1999; Hutt *et al.* 2006). Upon activation, the layer of flattened granulosa cells transition to become cuboidal in shape and the oocyte begins to grow and mature, at which stage the follicle is now called a primary follicle.

Proliferation of granulosa cells in the primary follicle to form two or more layers constitutes a secondary follicle. This transition is regulated by GDF-9, BMP-15 and another granulosa-derived TGFβ family member, anti-Müllerian hormone (AMH) (McGrath *et al.* 1995; Elvin *et al.* 1999a; Elvin *et al.* 1999b; Otsuka *et al.* 2000; Yan *et al.* 2001; Durlinger *et al.* 2002). GDF-9 and BMP-15 are produced by oocytes throughout folliculogenesis until ovulation (Dong *et al.* 1996; Dube *et al.* 1998). Studies in mice have demonstrated the essential role of GDF-9 whereby follicular development ceases at the primary stage in its absence (McGrath *et al.* 1995; Elvin *et al.* 1999a; Elvin *et al.* 1999b). BMP-15 is important but not critical, as its absence negatively affects granulosa cell proliferation but does not halt follicle development in a null mouse model (Dong *et al.* 1996; Otsuka *et al.* 2000; Yan *et al.* 2001). Studies in sheep however, a mono-ovulatory species, have demonstrated the requirement of both GDF-9 and BMP-15 in normal follicular development and ovulation. Ewes are completely infertile if they possess a homozygous mutation in either gene, resulting in a lack of GDF-9 or BMP-15, or if they are fully-immunised against either factor (Otsuka *et al.* 2011). In addition to granulosa cell

growth, secondary follicles are characterised by the recruitment of ovarian stromal cells to form a primitive thecal layer outside the basement membrane that surrounds the granulosa cell layer, as well as continued oocyte growth which remains arrested at prophase. Cyclic follicle recruitment to the antral stage begins at puberty when sufficient levels of follicle-stimulating hormone (FSH), secreted from the pituitary, is in circulation and this phase of growth is completely gonadotrophin-dependent. Granulosa cells increase expression of FSH receptor and theca cells express luteinising hormone (LH) receptor. Antral follicles are characterised by the formation of the fluid-filled antrum and rapid proliferation of granulosa cells to form two differentiated cell types called mural granulosa cells, the outermost layer that surrounds the antrum, and cumulus cells, that surround the oocyte. In addition, the theca layer expands into two layers called the theca interna, capable of secreting steroid hormones including estrogen and androgens, and the theca externa which contains smooth muscle. Proliferation of granulosa cells, theca cells and formation of the antrum results in a three- to ten-fold increases in the overall volume of the follicle.

Antrum formation is driven by granulosa cell-secretion of IGF-1, believed to be upregulated by FSH (Baker *et al.* 1996; Itoh *et al.* 2002; Webb *et al.* 2004). Mutant mice lacking the β-chain of FSH do not develop an antrum, and expression patterns suggest that IGF-1 is the principal factor involved (Baker *et al.* 1996; Kumar *et al.* 1997). Additionally, blockade of the c-kit receptor inhibits antrum formation in the developing mouse, suggesting that its ligand (KITL) is also important in this process (Yoshida *et al.* 1997). There is evidence that theca formation is driven by the recruitment of stromal cells by

KITL, which then promotes differentiation and proliferation of theca cells as shown by bovine ovarian fragment culture and isolated ovarian stromal cells (Parrott and Skinner 1997; Parrott and Skinner 1998b). Bidirectional communication between the theca layer and granulosa cells, essential for antral follicle formation, is believed to be mediated by KITL and theca-secreted factors keratinocyte growth factor (KGF) and hepatocyte growth factor (HGF) (Parrott and Skinner 1998b). Isolated bovine theca and granulosa cell co-cultures were used to demonstrate a positive feedback loop between each cell type via these growth factors. In addition, LH receptor (LHR) agonist human chorionic gonadotrophin (hCG) was shown to stimulate expression of KITL, KGF and HGF in cultured, isolated bovine antral cells suggesting that these factors also play a role in mediating gonadotrophin action (Parrott and Skinner 1998b). The involvement of the oocyte in antrum formation is poorly understood, but it has been demonstrated to be essential in this process, as antrum formation does not occur in 3-dimensional cultures of mouse granulosa cells in the absence of an oocyte (Huang *et al.* 2006).

The LH surge acts as a trigger for a series of events leading to ovulation (Duggavathi and Murphy 2009). The fully grown oocyte undergoes meiotic resumption and nuclear maturation as indicated by germinal vesicle breakdown (GVBD) (Fan and Sun 2004). Completion of the first meiotic division and extrusion of the first polar body leads to meiosis II, where the oocyte again arrests in metaphase and remains in this state until fertilisation. The follicular wall undergoes focal proteolytic degradation and the cumulus-oocyte-complex is extruded from the ruptured follicle into the reproductive tract. In

addition to gonadotrophin action on theca and granulosa cells, the oocyte is also involved in ovulation by inducing cumulus expansion. This occurs to assist the extrusion of the oocyte and suspends it in a structured matrix to facilitate fertilisation. The formation of the matrix and proliferation of cumulus cells at the time of expansion is reliant on GDF-9 and BMP-15 (Pangas and Matzuk 2005; Gueripel *et al.* 2006). Granulosa cell-derived epidermal growth factor (EGF) family members are also required for the resumption of meiosis and cumulus expansion in mice (Park *et al.* 2004).

Ovulation is followed by the luteal phase, characterised by terminal differentiation, or luteinisation, of the remaining follicular cells to form the corpus luteum. This structure consists of steroidogenic cells which produce abundant quantities of progesterone to prepare the uterus for implantation of a fertilised oocyte, and prevent another cycle of follicle recruitment in the case of conception. If fertilisation does not occur, the corpus luteum degenerates to form a fibrous tissue know as the corpus albicans. Thus, timely ovulation of a healthy oocyte is critical to female fertility and the paracrine, autocrine and endocrine signalling that takes place within the follicle throughout its development is fundamental to its success.

Steroidogenesis is essential for maintenance of functional granulosa cells, as well as for the normal endocrine regulation of female physiology. Prior to ovulation the levels of oestrogen and progesterone increase, and during the luteal phase progesterone levels rise even further to support early pregnancy. This process begins with the metabolism of

cholesterol in a highly coordinated enzymatic cascade within the ovary. Androstenedione and testosterone, the predominant androgens found in follicular fluid (McNatty *et al.* 1979), are generated from progesterone in LH-stimulated theca cells. Conversion of androgens to oestrogens, such as estrone and estradiol, takes place in FSH-exposed granulosa cells. Estradiol is the predominant feedback mechanism to the pituitary to reduce the secretion of LH and FSH (Couzinet and Schaison 1993), while progesterone is an essential mediator of ovulation (Robker *et al.* 2000) and the primary secretion of the corpus luteum.

Androgens, including testosterone and its more potent metabolite 5α-dihydrotestosterone (DHT), signal through the androgen receptor (AR). This steroid receptor is a transcription factor located predominantly in the cytoplasm. Upon ligand binding, an AR homodimer forms and rapidly translocates into the nucleus where it binds specific sequences on DNA (androgen response elements, AREs) in order to regulate transcription of genes involved in cell growth, differentiation and survival. In the mammalian ovary, AR is consistently expressed in the granulosa cells, and depending on the species may be expressed in theca cells. It has been suggested that androgens, mediated by AR, play roles in both folliculogenesis and atresia, possibly acting in an autocrine or paracrine manner (Horie *et al.* 1992).

1.3 KITL AND C-KIT EXPRESSION AND SIGNALLING

KITL is encoded by the *Steel* (*Sl*) loci in mice, and is a paracrine growth factor secreted by granulosa cells (Copeland *et al.* 1990; Huang *et al.* 1990; Nocka *et al.* 1990; Zsebo *et al.* 1990). The protein is composed of a short signal peptide and an extracellular domain linked via the transmembrane region to the cytoplasmic domain (Figure 1.3). In most species KITL consists of two membrane-bound isoforms, KITL-1 and KITL-2, depending on alternate splicing of exon 6, and are synthesised as 248 and 220 amino acids respectively (Flanagan *et al.* 1991; Huang *et al.* 1992). While both isoforms are able to be cleaved to soluble forms, KITL-1 is the predominant soluble form due to a proteolytic cleavage site located at amino acid 165 encoded by exon 6 of the KITL gene. Another proteolytic cleavage site, believed to be located on exon 7, allows KITL-2 to be released as a soluble protein but cleavage at this site occurs with much lower efficiency (Figures 1.3 and 1.4) (Huang *et al.* 1992) and therefore, KITL-2 exists primarily as a membrane-bound protein. The proteases and circumstances in which splicing of either isoform occur *in vivo* are unclear. A study examining cleavage of KITL isoforms in COS-1 cells, transfected with the cDNA of either isoform, reported that proteolytic cleavage of both isoforms was induced by the protein kinase C activator, phorbol 12-myristate 13-acetate (PMA), and the calcium ionophore A23187 (Huang *et al.* 1992). The authors suggested that differential regulation of isoform-specific cleavage may occur by activation of distinct proteases by PMA or A23187 that are specific for each isoform, or that one protease may be activated at a higher level than the other resulting in different cleavage rates of each isoform. Another study examining the effect of protease inhibitors on KITL isoform cleavage in transfected

Chinese hamster ovary cell (CHO) lines showed that serine protease inhibitors, diisopropylfluorophosphate (DFP) and 3,4-dichloroisocoumarin (3,4-DCI), inhibited KITL-1 cleavage but had no effect on KITL-2 (Pandiella *et al.* 1992). The cleavage site of KITL-2 contains several lysines, and it was suggested that the lysines act as cleavage sites for trypsin-like enzymes (Pandiella *et al.* 1992). Both studies suggested that while proteolytic cleavage of KITL isoforms involves several proteases with distinct substrate specificities, these proteases are regulated by a common set of activators. Tissue-specific cleavage events and regulation of each KITL isoform remain poorly understood.

When associated with the cell membrane, KITL is predominantly a non-covalently linked dimer with monomers joined by disulphide bonds (Arakawa *et al.* 1991; Kurosawa *et al.* 1996; Lu *et al.* 1996; Tajima *et al.* 1998a), while the majority of circulating KITL in human serum exists in monomeric form (Figure 1.4) (Hsu *et al.* 1997). Dimerisation of both soluble and membrane-bound forms of KITL has been shown to be important for activation of its receptor, c-kit (Arakawa *et al.* 1991; Kurosawa *et al.* 1996; Lu *et al.* 1996; Tajima *et al.* 1998a). The c-kit receptor belongs to the type III family of receptor tyrosine kinases whose members include platelet-derived growth factor (PDGF) receptor-α and β, and colony-stimulating factor (CSF) 1 receptor (Besmer *et al.* 1986; Yarden *et al.* 1987; Qiu *et al.* 1988; Ullrich and Schlessinger 1990; Blume-Jensen and Hunter 2001). Binding of dimeric KITL drives receptor dimerisation followed by intermolecular phosphorylation and activation of either the phosphatidylinositol 3-kinase (PI3K) or MAP kinase (MAPK) signalling cascade (Figure 1.5) (Kurosawa *et al.* 1996; Tajima *et al.* 1998a; Jin *et al.* 2005b; Reddy *et al.* 2005; Liu *et al.* 2007; Yuzawa *et al.* 2007).

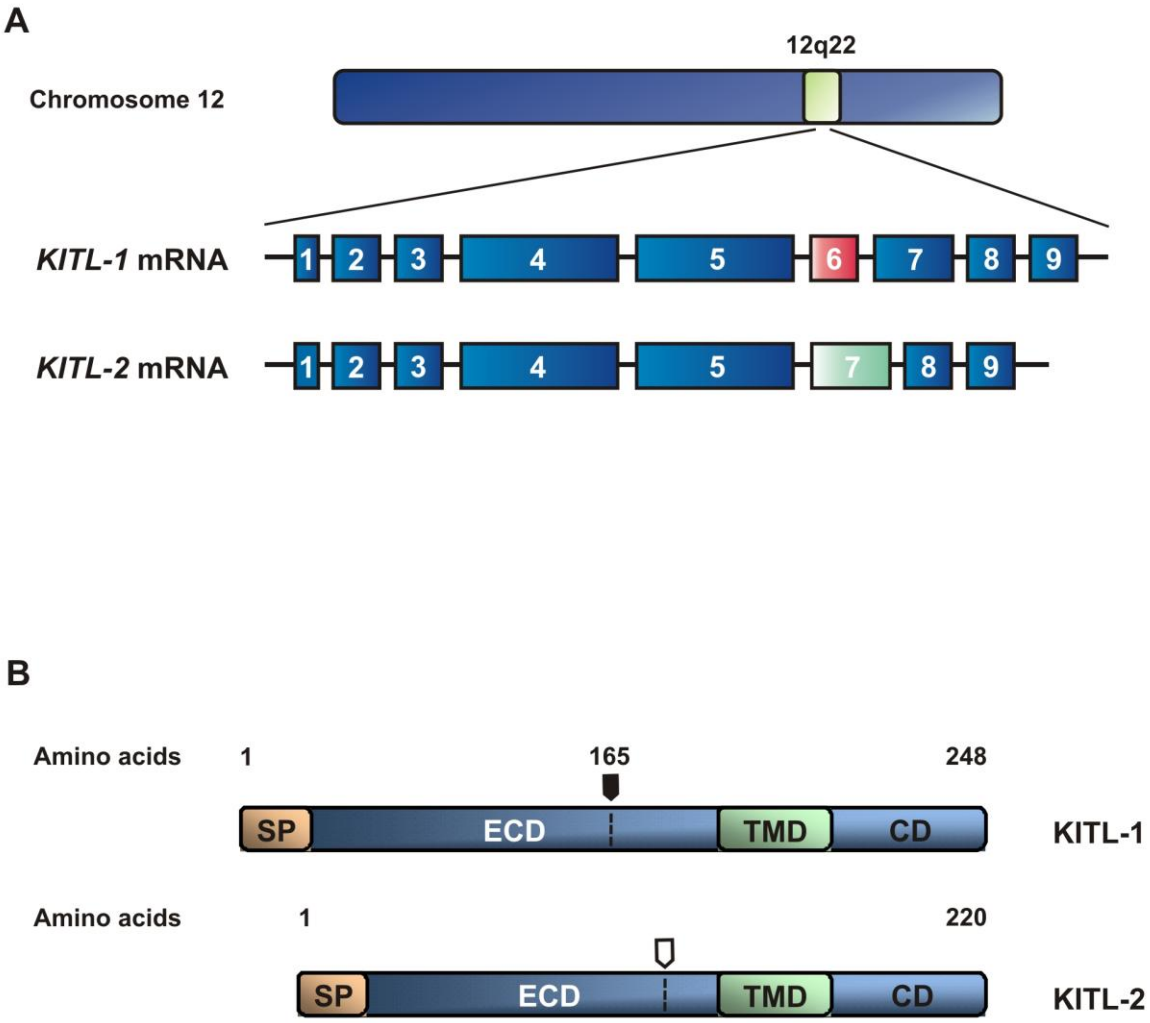

A
12q22
Chromosome 12
KITL-1 mRNA
1
2
3
4
5
6
7
8
9
KITL-2 mRNA
1
2
3
4
5
7
8
9
B
Amino acids
1
165
248
SP
ECD
TMD
CD
KITL-1
Amino acids
1
220
SP
ECD
TMD
CD
KITL-2

Figure 1.3. Schematic depicting the mRNA and protein structures of human KITL isoforms. (A) In humans, the gene encoding *KITL* is located on Chromosome 12, 12q22. Alternate splicing gives rise to *KITL-2*, which lacks exon 6 that encodes the proteolytic cleavage site of *KITL-1* (highlighted in red). KITL-2 contains a different proteolytic cleavage site which is encoded by exon 7 (highlighted in green). (B) KITL-1 is initially synthesised as a membrane-bound protein 248 amino acids in length, and is cleaved at amino acid 165 to form a soluble protein. KITL-2 is a shorter membrane-bound protein at 220 amino acids in length, and contains a different proteolytic cleavage site believed to be located in exon 7 of the KITL gene. Abbreviations: SP, signal peptide; ECD, extracellular domain; TMD, transmembrane domain; CD, cytoplasmic domain.

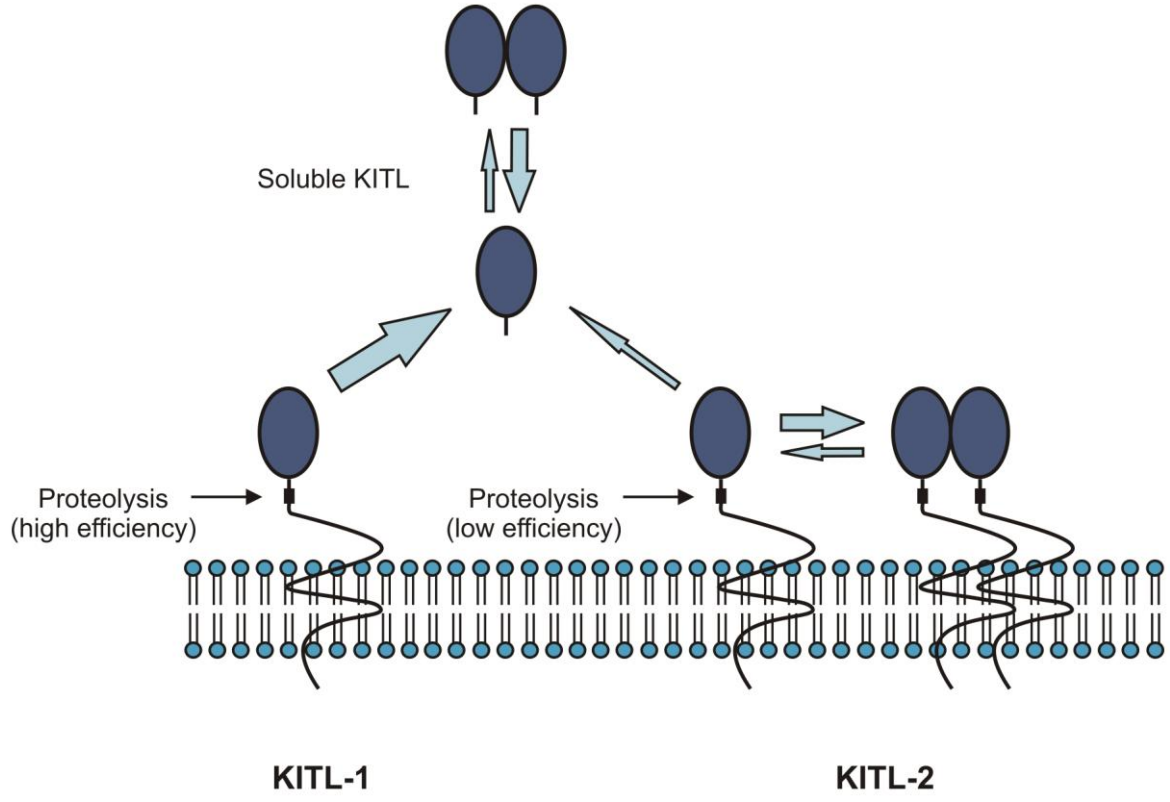


Figure 1.4. Schematic showing the generation of various forms of KITL. Two transmembrane forms, KITL-1 and KITL-2, are generated by alternate mRNA splicing of exon 6. KITL-1 is efficiently cleaved; however, KITL-2 contains a distinct proteolytic cleavage site where cleavage occurs with much lower efficiency, resulting in the isoform being predominantly membrane-bound yet still able to form dimers. Dimerisation of cell-associated KITL is important for homodimerisation and activation of its receptor, c-kit. Soluble KITL is predominantly monomeric.

Modified from original figure (Ashman 1999).

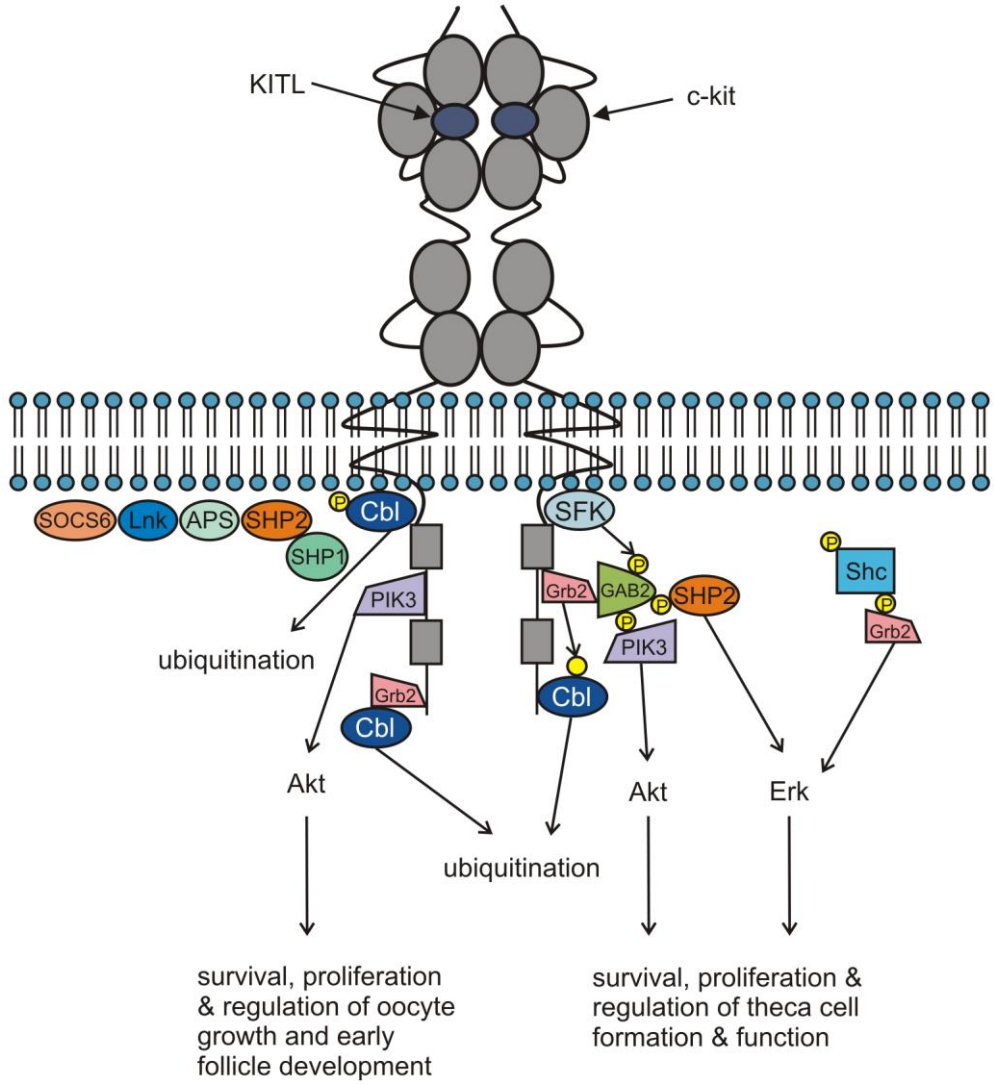


Figure 1.5. Schematic showing the signalling pathways downstream of activated c-kit. Binding of KITL to c-kit stimulates receptor dimerisation and phosphorylation (indicated by a P in a yellow circle) of the receptor interacting proteins, leading to activation of downstream signal transduction pathways including PI3K and MAPK (depicted as the family member ERK). How these signaling pathways generally affect folliculogenesis is also indicated. Modified from original figure (Masson and Ronnstrand 2009).

The c-kit receptor consists of an extracellular ligand-binding domain which is composed of five immunoglobulin-like repeats. The first three repeats act as an independent unit to specifically bind KITL, while the fourth and fifth repeats mediate dimerisation between two c-kit receptors (Yuzawa *et al.* 2007). The cytoplasmic domain contains a protein tyrosine kinase domain and is linked to the extracellular region via a single transmembrane domain (Yuzawa *et al.* 2007).

Different c-kit isoforms in mice and human arise by alternate mRNA splicing. The use of 5' splice donor sites at the exon/intron junction of exon 9 give rise to two c-kit variants which differ by the presence or absence of four amino acids, GNNK (Figure 1.6A) (Reith *et al.* 1991). The role and significance of each isoform in different tissue types remains largely unknown, but a study examining their activation abilities in NIH3T3 fibroblast cells has demonstrated marked differences (Caruana *et al.* 1999). NIH3T3 cells were stably transfected with a construct encoding either the GNNK+ or GNNK− c-kit isoforms. In the presence of recombinant KITL, cells expressing either c-kit isoform were capable of colony formation but only cells expressing the GNNK− isoform lost contact inhibition and demonstrated tumorigenicity in nude mice. While the two c-kit isoforms did not differ in their affinity for recombinant KITL, cells expressing the GNNK− isoform displayed different receptor activation kinetics, including faster and more extensive tyrosine autophosphorylation and faster downregulation via internalisation of the ligand/receptor complex. Downstream signalling, including PI3K activation and c-Akt phosphorylation, were similarly activated by the two isoforms, but activation of the mitogen-activated

protein kinase (MAPK) pathway by GNNK− c-kit was four-fold higher than GNNK+ c-kit. It remains unclear how the presence or absence of just four amino acids in the extracellular domain gives rise to such markedly different activation characteristics, but it has been postulated that this region modulates interaction between c-kit and other membrane-associated proteins. Receptor homodimerisation is unlikely to be affected due to the similar affinity of each isoform for KITL (Caruana *et al.* 1999). The presence and function of GNNK+/− isoforms in the human ovary remains to be elucidated.

Another c-kit isoform is generated by proteolytic cleavage of the membrane-bound c-kit extracellular domain, giving rise to a soluble protein (Figure 1.6B) (Tanikawa *et al.* 1998). This isoform has been detected in human ovarian follicular fluid, human haemopoietic cell lines and human serum. It is unknown which of the other membrane-bound isoforms, GNNK+ or GNNK−, generates soluble c-kit and the functional significance of this isoform is unclear. It was previously assumed that soluble c-kit was a by-product of receptor down-regulation (Brannan *et al.* 1991), but in fact may have a biologically active role by binding to soluble KITL. It has been shown that soluble c-kit bound with higher affinity to soluble KITL in human serum, which may then block interaction of KITL with its cell-surface receptor (Hsu *et al.* 1997). In addition, monomeric KITL was shown to have a much higher affinity for soluble c-kit than the dimeric form of KITL (Hsu *et al.* 1997), indicating that soluble c-kit may function to sequester monomeric KITL in solution, thus preventing KITL dimerisation and interaction with membrane-bound c-kit (Tanikawa *et al.* 1998).

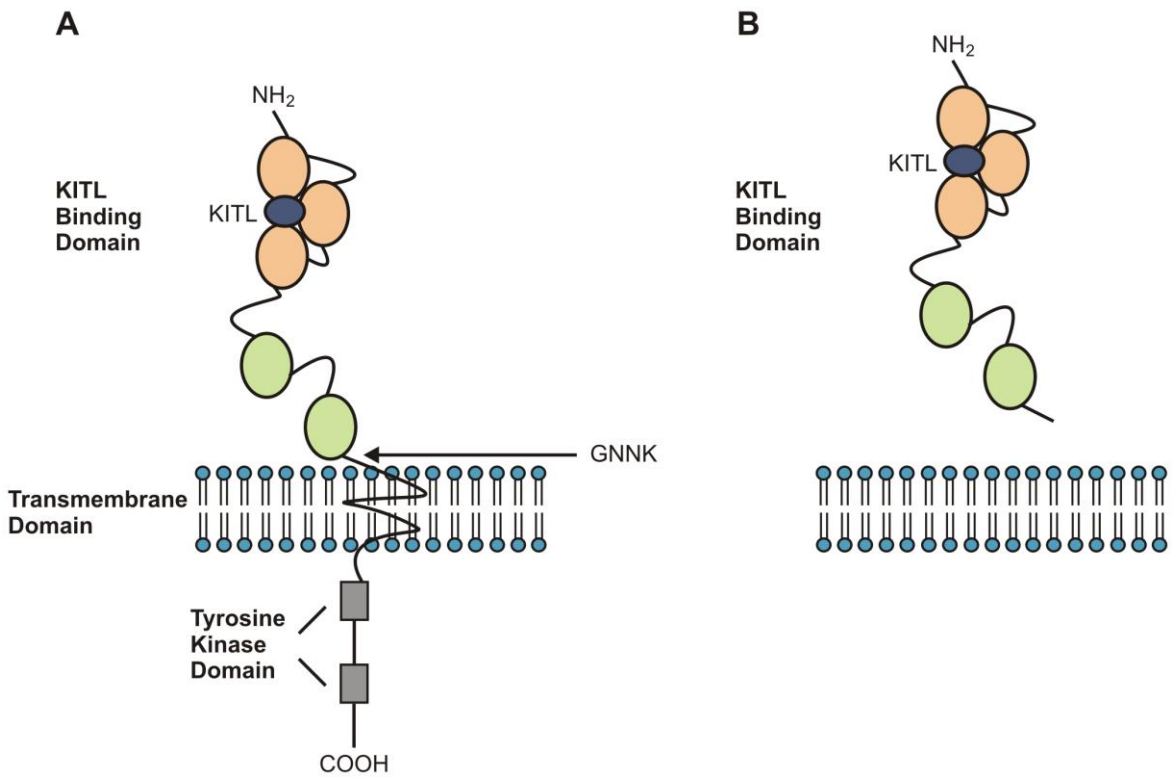


Figure 1.6. Schematic depicting human c-kit protein isoforms. c-kit is composed of an extracellular domain that consists of five immunoglobulin-like loops, the first three of which bind to KITL (orange ovals) while the last two mediate interaction between two c-kit receptors (green ovals). The cytoplasmic region contains a split tyrosine kinase domain characteristic of type III receptor tyrosine kinases. **A** Two isoforms are generated by alternate mRNA splicing and differ by the presence or absence of four amino acids, GNNK, in the extracellular domain. **B** Proteolytic cleavage of the membrane-bound receptor generates soluble c-kit.

Modified from original figure (Ashman 1999).

KITL isoforms feature different activation characteristics, which may be a result of their unique interactions with c-kit. Membrane-bound KITL-1/c-kit complexes were shown to undergo rapid polyubiquitination, internalisation and degradation whereas KITL-2 stimulation caused prolonged receptor activation in a stromal cell line established from fetal mouse liver (Miyazawa *et al.* 1995). Presumably anchorage of KITL-2 in the cell-membrane prevents or impedes internalisation and degradation of the c-kit receptor in a neighbouring cell, resulting in prolonged activation of c-kit. In contrast, c-kit bound to soluble KITL may be more easily degraded. Therefore, KITL-2 signalling may be more efficient and important for stimulation of long-term effects such as cell proliferation and differentiation, while KITL-1 may initiate shorter-term, transient effects. While KITL-2 signalling is limited to cells directly contacting its expressing cells, KITL-1 is a soluble protein and thus is able to exert effects some distance from its expressing cell type. It appears that each isoform may be differentially regulated, as tissue-specific regulation, age and hormone treatment have been shown to alter the ratio of *KITL-1*/*KITL-2* mRNA in mice (Huang *et al.* 1992; Manova *et al.* 1993; Thomas *et al.* 2005). For example, a study comparing *KITL* expression in postnatal mouse gonads up to 12 days old showed equal levels of both isoforms in the ovary, while *KITL-2* was predominantly present in the testes (Manova *et al.* 1993). These studies support the notion of differing actions for each isoform, but the mechanisms and consequences of KITL differential regulation in the ovary remain unclear.

The majority of study into the c-kit/KITL signalling pathway has been performed in animal models, and there is little information available pertaining to the human ovary. Mice in particular have been the main focus of past examination. C-kit protein has been shown to be present in the oocyte at all stages of murine follicular development, with highest expression occurring in primordial, primary and small antral follicles, suggesting a greater role for KITL during early follicle growth (Manova *et al.* 1993; Joyce *et al.* 1999). However, in sheep, oocyte mRNA expression of *c-kit* remains consistent throughout all stages of follicle development, suggesting that KITL signalling is also important or has a similar function during the later stages of folliculogenesis (Tisdall *et al.* 1997). In humans it has been reported that *c-kit* mRNA is present in the oocyte, theca cells and stroma (Tanikawa *et al.* 1998; Ito *et al.* 2001; Abir *et al.* 2004), while protein expression was only evident in the adult oocyte at all follicle stages and only present in granulosa cells of fetal ovaries (Abir *et al.* 2004; Hoyer *et al.* 2005). As the expression of c-kit protein differs between species, the role of KITL in folliculogenesis could vary from species-to-species, and thus further study is needed to establish the expression of KITL and c-kit isoforms in the human ovary.

The essential role of KITL and the particular importance of KITL-2 in reproductive function have been established by *in vivo* mouse models. Deletion of the *KITL* gene, *Sl*, in mice results in embryonic death by anaemia due to the role of *KITL* in hematopoiesis (Copeland *et al.* 1990). A variety of other *KITL* mutations have been generated, including Steel-Panda (Sl^{pan}) and Steel-Contrasted (Sl^{con}) mice (Bedell *et al.* 1995), which are characterised by decreased *KITL* mRNA levels generated by hypomorphic mutations in the

non-coding region of the gene. Female mice were reported to be sterile due to detrimental effects on the initiation and maintenance of folliculogenesis (Bedell *et al.* 1995). The importance of the membrane-bound isoform, KITL-2, was demonstrated by the Steel-Dickie (Sl^{d}) mouse generated by genomic deletions of the transmembrane and cytoplasmic domains (Brannan *et al.* 1991; Flanagan *et al.* 1991). These mutations resulted in production of soluble KITL only, and mice were sterile due to an absence of germ cells. In contrast, exclusive production of KITL-2 (Sl^{KL2}), generated by replacement of exon 6 that is unique to KITL-1 with a PGKneoTRtkpA cassette, resulted in fertile mice shown to be equally viable compared to wildtype (Tajima *et al.* 1998b). Thus, KITL-2 appears to be essential for maintaining normal ovarian function and fertility, and its actions cannot be replaced by soluble KITL-1.

1.3.1 KITL and activation of primordial follicle growth

KITL has been shown to induce primordial follicle activation and thereby initiate folliculogenesis, but this role appears to differ between species. Parrott and Skinner (Parrott and Skinner 1999) first demonstrated that KITL is involved in activation of quiescent primordial follicles in cultured rat ovaries. They reported that recombinant KITL treatment significantly decreased the percentage of quiescent primordial follicles, while primary follicle percentage increased significantly for KITL treated ovaries and this effect became even more dramatic over time. To investigate the role of endogenous KITL on primordial follicle development, rat ovarian organ cultures were treated with ACK-2, a c-kit antibody that strongly inhibits KITL actions by binding to the extracellular domain of c-kit (Parrott and Skinner 1999). Primordial follicle activation occurs spontaneously in

cultured ovarian tissue, and this was completely inhibited by ACK-2. Furthermore, ACK-2 partially inhibited the initiation of primordial follicle development by KITL treatment. The lack of complete inhibition was attributed to a suboptimal concentration of antibody, which may not have been sufficient to completely inhibit the high concentration of exogenous KITL treatment. This role of KITL is supported by another study (Hutt *et al.* 2006), which also showed that KITL promoted primordial follicle activation in cultured mouse ovaries by the addition of recombinant KITL. This effect was abrogated by KITL-neutralising antibody, produced by immunisation of female rats with recombinant mouse KITL.

A study examining the role of c-kit signalling via PI3K in mice demonstrated a specific role for KITL in promoting primary to secondary follicle transition, and showed evidence that KITL is dispensible in primary follicle activation (John *et al.* 2009). Mutant mice were generated containing a knock-in mutation (Kit^{Y719F}) which prevents binding of c-kit to the p85 regulatory subunit of PI3K, thus completely abrogating downstream signalling of c-kit via PI3K. Homozygous Kit^{Y719F} mice were fertile and underwent normal primordial follicle activation, but showed an increased number of morphologically abnormal transitional primary and early secondary follicles. The results of this study indicates that c-kit signalling via PI3K is not essential for primordial follicle activation, but the involvement of other downstream signalling pathways is yet to be fully explored.

The positive effect of KITL on primordial follicle activation has currently not been established in other species, including humans. Hutt et al. (2006) (Hutt *et al.* 2006) reported no effect of KITL on cultured juvenile rabbit ovarian cortical explants which

could be the result of a species difference, or due to the use of mouse recombinant KITL in the culture of rabbit ovaries. This study and one examining cultured adult human ovarian cortical slices found no significant difference between primordial follicle numbers or developmental stages in untreated and recombinant human KITL-treated ovaries (Carlsson *et al.* 2006). In addition, ACK-2 had no effect on the promotion of follicular developmental stages in the human ovary culture (Carlsson *et al.* 2006), while KITL-neutralising antibody did not block spontaneous follicle activation in rabbit ovaries (Hutt *et al.* 2006). These results suggest that KITL may not be essential for primordial follicle activation in rabbit and human ovaries, but further study is required to elucidate the role of KITL in the initiation of human folliculogenesis.

1.3.2 KITL and oocyte growth

Oocyte growth and development is critically dependent on bidirectional communication between granulosa cells and the oocyte, and several lines of evidence implicate the importance of KITL in oocyte growth particularly during early follicle development. Klinger et al. (Klinger and De Felici 2002) demonstrated that initiation of fetal oocyte growth is dependent on KITL, and independent of contact communication with granulosa cells. Fetal mouse oocytes were isolated and cultured in the presence or absence of recombinant mouse KITL, which resulted in a doubling of oocyte diameter compared to control (Klinger and De Felici 2002). This effect was completely attenuated by the addition of ACK-2, indicating that increased oocyte growth was a result of KITL/c-kit signalling. After 4 days, no additional increase in size was observed, and oocytes were then seeded on granulosa cell monolayers obtained from adult mouse ovaries and treated with

or without KITL. A significant increase in size was observed for oocytes cultured in the presence of exogenous KITL, showing that contact communication with granulosa cells is needed for further oocyte growth. KITL production by mouse granulosa cells has been demonstrated by another study to dramatically decrease in culture (Thomas *et al.* 2008). Therefore, the absence of continued oocyte growth when cultured on granulosa cells, without the addition of exogenous KITL, suggests that a certain level of KITL expression is required to support oocyte growth. This is supported by the phenotype of Sl^{pan} and Sl^{con} mice, which produce decreased levels of *KITL* mRNA and contain follicles arrested at the primary stage (Bedell *et al.* 1995).

The role of KITL in preantral oocyte growth is supported by other studies, and again it appears that the actions of KITL may be species-specific. Recombinant KITL treatment has been shown to increase oocyte diameter in isolated sheep primordial follicles (Muruvi *et al.* 2005) and cultured ovarian cortical slices from mice and rabbits (Hutt *et al.* 2006). Addition of KITL-neutralising antibody mitigated the effects of recombinant KITL in the mouse and rabbit tissue, but neutralising antibody was not used in the sheep study. Interestingly, while a similar increase in size was seen for early primary, primary and growing primary follicles in rabbit tissue, little effect was observed for these follicle types in mouse tissue compared to control (Hutt *et al.* 2006). There was no effect of recombinant KITL on oocyte diameter of preantral follicles for either species. Collectively, these findings indicate that KITL plays an important role in initiating the oocyte into the growth phase, but KITL-mediated stimulation of growth beyond the primordial stage may only occur in some species.

The relative biopotency of soluble KITL-1 and membrane-bound KITL-2 in oocyte growth was examined by co-culturing mouse oocytes with fibroblasts stably expressing either isoform (Thomas *et al.* 2008). KITL-2 was found to be the principal isoform involved in oocyte growth, as growth occurred only in oocytes cultured in the presence of KITL-2. Oocyte growth was inhibited by suppressing KITL-2 action via two c-kit inhibitors, Gleevec and Ack-2, which have different means of receptor inhibition. Interestingly, addition of exogenous KITL-1 suppressed the growth of oocytes cultured in the presence of KITL-2-producing fibroblasts. Clearly, KITL-2 plays an important role in the growth of mouse oocytes *in vitro*, but these actions may be species-specific. In contrast to mouse expression, measurement of KITL isoforms in goat ovaries showed that KITL-1 mRNA was expressed only in preantral follicles, whereas antral follicles expressed both isoforms (Silva *et al.* 2006). It appears that relative expression of the two KITL isoforms, and hence their differential functions, differ between species and the expression of each isoform in the human ovary and their role in human oocyte growth remains to be elucidated.

1.3.3 KITL and theca layer formation and growth

A critical aspect of folliculogenesis is the formation of the theca layer, which is responsible for the secretion of steroid hormones, particularly androgens. During early follicle development, ovarian stromal cells are recruited and organised into distinct theca cell layers around primary follicles. This has been proposed to involve initial proliferation of stromal cells and subsequent initiation of functional differentiation to theca cells, with recruitment driven by a granulosa-cell derived factor (Roberts and Skinner 1990). There is

evidence indicating that KITL is an important granulosa cell-secreted factor mediating theca layer formation (Parrott and Skinner 2000). Bovine stromal stem cell proliferation was found to be significantly stimulated by recombinant KITL in a dose-dependent manner, and theca layer thickness in relation to total follicle diameter was significantly increased by KITL treatment. These results suggest that KITL may play a role in establishing theca cell layers *in vivo*, either by recruiting theca cells from undifferentiated stromal cells, or by promoting proliferation of theca cells that are already recruited. KITL/c-kit blocking antibodies were not used in this study, which would have provided further support for the role of KITL in theca cell function. In addition to these results, Parrott and Skinner (Parrott and Skinner 1997) also demonstrated that KITL directly regulates bovine theca cell proliferation in a dose-dependent manner. In humans, c-kit protein in theca cells has not been reported thus far. Even if KITL does not act directly on stromal cells or theca cells, KITL may play an important indirect role in theca cell formation by stimulating production of basic fibroblast growth factor (bFGF) in oocytes, as shown in the rat ovary (Nilsson and Skinner 2004). Oocyte-derived bFGF could then have a direct action on stromal cells surrounding early developing follicles (Nilsson and Skinner 2004), but the expression and role of bFGF in the human ovary also remains to be elucidated.

1.3.4 KITL and steroidogenesis

In addition to promoting theca cell recruitment and growth, KITL plays a role in theca cell differentiation. Soluble c-kit in human follicular fluid was found to strongly correlate with testosterone and androstenedione concentrations (Tanikawa *et al.* 1998). This suggests that

the KITL/c-kit system may be involved in human androgen biosynthesis, and this role is supported by several animal studies. By examining mRNA expression in theca cells from immature rats, Huang et al. (Huang *et al.* 2001) showed that KITL, in combination with IGF-1, upregulated expression of genes enabling theca cells to undertake androgen biosynthesis. These genes include *StAR*, a mitochondrial protein that regulates cholesterol transport within the mitochondria for subsequent steroid hormone biosynthesis, and *CYP11A* and *3β-HSD*, a cholesterol side-chain cleavage enzyme and dehydrogenase which catalyse steroid hormone production. Several other studies have also reported a role for KITL in steroidogenesis. Parrott and Skinner (1997) (Parrott and Skinner 1997) showed that KITL stimulated androstenedione production in cultured bovine theca cells which suggests that differentiation of theca cells is directly regulated by KITL, and that steroid production can be stimulated by KITL in the absence of gonadotropins. Support for this role of KITL is provided in a study by Reynaud et al. (2000) (Reynaud *et al.* 2000), who examined testosterone output from cultured mouse follicles. While testosterone production was not affected by KITL in serum-free conditions, KITL significantly increased testosterone output in the presence of 5% fetal calf serum. This result may be explained by the requirement for IGF-1 as reported by Huang et al. (Huang *et al.* 2001). Based on these studies, it can be concluded that KITL plays an important role not just in formation of theca cell layers and theca cell growth, but also in theca cell differentiation and steroidogenic capacity.

1.3.5 KITL and inhibition of apoptosis

Oocyte and somatic cell survival is required for follicle development and ovulation to occur, and previous studies have reported strong anti-apoptotic effects of KITL in both animals and humans. Jin et al. (Jin *et al.* 2005a) demonstrated this effect using cultured whole rat ovaries, where a significant reduction in the percentage of apoptotic oocytes was observed in ovaries treated with recombinant KITL compared to control. Addition of ACK-2 in KITL-treated cultures abolished this effect, resulting in a dramatic increase in the percentage of apoptotic oocytes. Therefore, these results suggest that KITL is able to elicit an anti-apoptotic effect in the oocytes of primordial follicles via the c-kit receptor. Inhibition of apoptosis was also observed in another study examining later stages of follicle development. Reynaud et al. (Reynaud *et al.* 2000) used rabbit polyclonal c-kit antibody SC1494 to block KITL/c-kit interactions in cultured preantral mouse follicles. A dramatic effect was observed for SC1494-treated follicles, in which death of the oocyte occurred, with subsequent degeneration and disconnection from surrounding granulosa cells. This anti-apoptotic effect of KITL on ovarian follicles has also been demonstrated *in vivo*, when the c-kit blocking antibody, ACK-2, was injected into female mice (Yoshida *et al.* 1997). A dramatic reduction in the number of preantral follicles was observed due to induction of follicular atresia, in addition to preovulatory follicle atresia and ovarian failure. These results contrast with an *in vitro* study of cultured mouse follicles where blockade of KITL/c-kit interactions, using an antibody known as SC1494, had no effect on follicular death (Reynaud *et al.* 2000). Reynaud et al. (Reynaud *et al.* 2000) demonstrated a dramatic effect of KITL on the survival of cultured oocytes as discussed previously, but administration of the SC1494 blocking antibody did not affect follicle survival. The

authors suggested that the discrepancy between their *in vitro* results and those of Yoshida et al. (Yoshida *et al.* 1997) may be due to indirect actions of KITL *in vivo* via c-kit located on the hypothalamus and pituitary (Kovacs *et al.* 1996; Zhang and Fedoroff 1997), through which KITL may mediate gonadotrophin-secretion. Importantly, survival of human follicles has been shown to be mediated by KITL during early follicle development (Carlsson *et al.* 2006). Cultures of ovarian cortical slices treated with ACK-2 showed significantly increased proportions of atretic follicles compared to control and furthermore, addition of ACK-2 into the culture media of isolated primordial follicles resulted in several cases of atresia. These results indicate that the survival of human preantral follicles is likely to be controlled by KITL/c-kit interactions.

1.3.6 KITL/c-kit and polycystic ovary syndrome

An underlying hypothesis within this thesis is that disruption of the KITL/c-kit system could explain the defects in folliculogenesis that are characteristic of polycystic ovary syndrome (PCOS). Previously, I demonstrated abnormally increased KITL protein levels in polycystic ovaries (PCO) compared to non-PCO (Figure 1.7) (Tuck *et al.* 2007; Tuck *et al.* 2010a). Granulosa cells and the oocyte of preantral follicles, and granulosa, theca cells and the oocyte of antral follicles all contained increased KITL immunostaining intensity. PCO is a characteristic of PCOS, one of the most prevalent endocrine disorders affecting women of reproductive age (Norman *et al.* 2007).

Approximately 1 in 15 women worldwide are afflicted by this disorder, which is a major cause of female infertility due to lack of ovulation. PCOS is a complex disorder affecting

several body systems, with abnormalities in reproductive, metabolic and cardiovascular functions. Consequently, PCOS is associated with several health complications such as infertility, menstrual irregularity, hirsutism, acne, obesity and insulin resistance. In addition, a higher risk of developing type II diabetes and cardiovascular disease has been established in women with PCOS (Legro *et al.* 1999; Rizzo *et al.* 2008). The aetiology of PCOS remains unknown, but the emerging picture implicates both environmental influences and genetic factors (Norman *et al.* 2007).

Due to the range of clinical features and the complexity of PCOS, diagnosis is based on the assessment of three main characteristics. According to current consensus criteria (Rotterdam 2003), these are hyperandrogenism, polycystic ovaries (PCO) and oligoanovulation. For PCOS to be diagnosed, two of these three criteria must be met. Hyperandrogenism is the most consistent characteristic of PCOS, with approximately 60-80% of women with the disorder presenting with elevated circulating testosterone levels (Chang *et al.* 2005). Hyperthecosis, or thickened thecal cell layers, is also observed in PCOS (Gilling-Smith *et al.* 1994). Isolated theca cells from polycystic ovaries produce excess androgen under basal and LH-stimulated conditions (Gilling-Smith *et al.* 1994). Polycystic ovaries are detected by transvaginal ultrasound and characterised by significantly increased numbers of growing follicles including primary, secondary and antral (Webber *et al.* 2003; Maciel *et al.* 2004). Primary follicles in particular are found in greater numbers (Maciel *et al.* 2004), and one study reported a proportional decrease in the percentage of primordial follicles compared to normal ovaries (Webber *et al.* 2003). It has

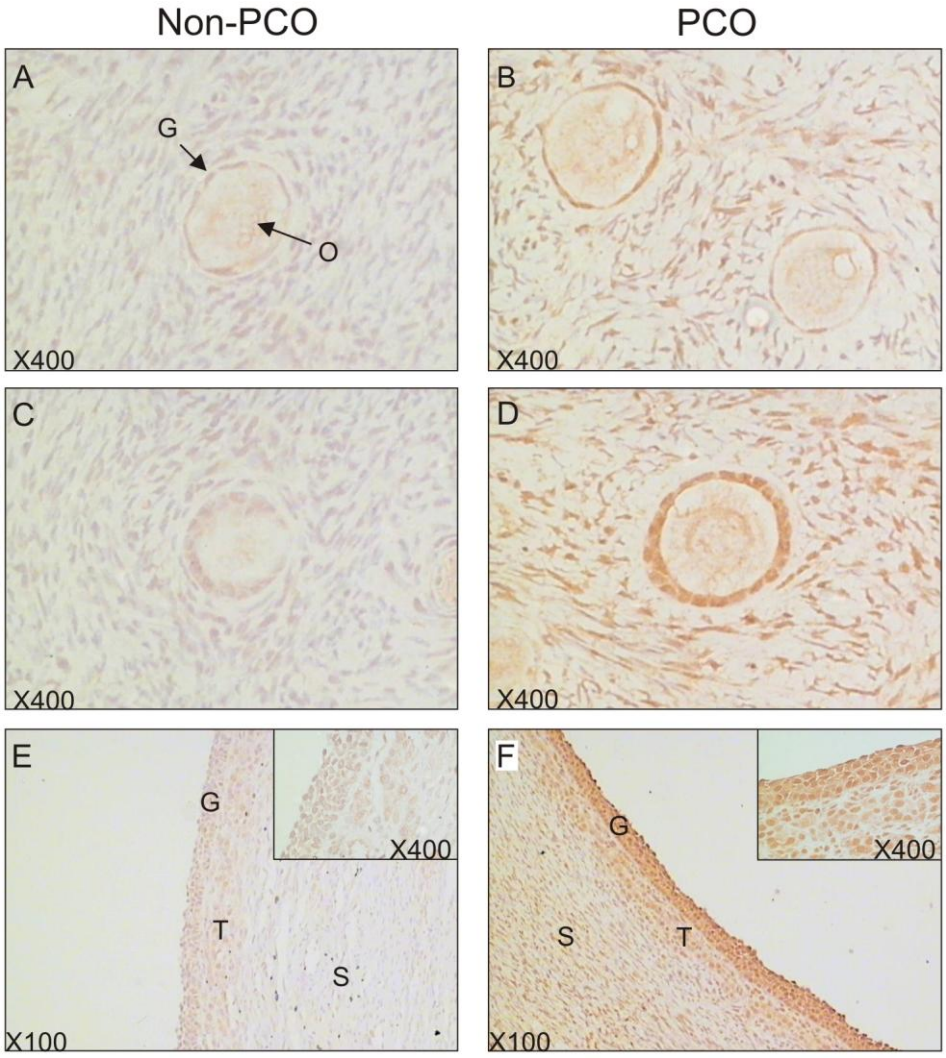


Figure 1.7. Immunohistochemical staining of KITL in human non-PCO and PCO tissues. (**A,B**) primordial follicles; (**C,D**) primary follicles; (**E,F**) antral follicles. G, granulosa cells; O, oocyte; T, theca; S, stroma.

(Tuck *et al.* 2007; Tuck *et al.* 2010a; Tuck *et al.* 2010b)

been reported that the rate of atresia throughout *in vitro* culture of human follicles was significantly decreased in PCOS tissue (Webber *et al.* 2007). This suggests that the large follicle pool evident in PCOS is due to reduced rates of atresia, but this has not been definitively proven. Oocyte growth has also been reported to be abnormal in anovulatory PCOS ovaries, which contain enlarged oocytes that initiate growth before primordial follicles have reached the primary stage (Stubbs *et al.* 2007). Abnormal androgen signalling has been implicated as a possible mechanism underlying polycystic ovaries, with several studies reporting a positive correlation between serum testosterone and follicle number in women with PCOS (Pesce *et al.* 1993; Jonard *et al.* 2003; Welt *et al.* 2006). Several lines of evidence also implicate abnormal androgen and insulin signalling as the cause of chronic anovulation, an important feature of PCOS that results in decreased fertility, and oligomenorrhea or amenorrhea (Norman *et al.* 2007).

Vendola et al. (1998) examined the effects of androgen on follicle growth and survival in primate ovaries (Vendola *et al.* 1998). They reported that early stages of follicular growth were stimulated by short-term androgen treatment consisting of low and high doses (20 µg/kg to 4 mg/kg), seemingly independent of gonadotropins or cycle stage. Testosterone treatment significantly increased total follicle number, with a 2.5-fold increase after 3 days of treatment, and a 4.5-fold increase after 10 days compared to control. In addition, granulosa cell proliferation was significantly increased in testosterone-treated animals for preantral to large antral follicles, while thecal layer thickness and thecal cell proliferation were also increased. Survival of small antral follicles was also promoted, with a significant

reduction in granulosa cell apoptosis for testosterone-treated animals compared to controls. Although testosterone can be aromatised to estrogen, which signals through the estrogen receptor, the androgenic effects observed in the above study were also observed in animals treated with the non-aromatisable testosterone metabolite 5α-dihydrotestosterone (DHT), thus implicating AR signalling as the primary mechanism. The authors suggested that these findings support the argument that polycystic ovaries are the result of androgen stimulation of early follicle development. This explanation is further supported by a human study, in which long-term testosterone treatment in female-to-male transsexuals results in ovaries with a PCO-like morphology (Bosinski *et al.* 1997). While it appears that there is good evidence for the involvement of abnormal androgen signalling in PCOS, the molecular mechanism involved as well as which genes are regulated by AR in the ovary are unknown.

A recent study describing the ovarian phenotype of AR null (ARKO) female mice identified *KITL* as a candidate AR-regulated gene (Shiina *et al.* 2006). Female ARKO mice have apparently normal folliculogenesis, but develop premature ovarian failure (POF) at eight weeks of age. It was reported that several genes involved in folliculogenesis were downregulated at 8 weeks of age, one of which was *KITL*. Upon examination of 3-week-old ARKO ovaries, which appeared to have normal follicle development, *KITL* was the only gene that remained significantly downregulated. It was suggested that downregulation of *KITL* may result in impaired follicle development at a later age, as seen in the eight-week-old mice. In order to determine possible AR regulation of the *KITL* gene, a luciferase reporter assay with mouse and human *KITL* promoters was used. It was observed that

transactivation of these promoters was significantly induced by DHT treatment in KGN cells, a human ovarian granulosa tumour cell line (Nishi *et al.* 2001). Therefore, *KITL* is an excellent candidate for potential AR-regulation in ovarian granulosa cells. Since androgens are secreted in excess in PCOS, it is likely that AR signalling is abnormal stimulated, but this remains to be proven. Perturbation of AR signalling in PCOS could result in dysregulated *KITL* gene expression resulting in the increased protein levels I previously observed in PCO, giving rise to the abnormalities present in PCOS.

1.4 SUMMARY

In summary, folliculogenesis within the mammalian ovary is a highly complex and coordinated process critical to female fertility. Many aspects of this fundamental process remain to be fully understood particularly in the human ovary. Thus, much of our current understanding of the molecular and cellular biology of human folliculogenesis is inferred on the findings of animal studies. Several of these animal studies have identified the KITL/c-kit system to be a vital regulator of follicular development and fertility, playing several distinct roles throughout folliculogenesis. These actions include the activation of primordial follicles, promotion of oocyte growth and survival, mediating theca layer formation and androgen production, and inhibition of atresia. Species differences in the expression and function of KITL and c-kit illustrate the importance of examining this system in the human ovary to further our understanding of the molecular mechanisms underlying human fertility and infertility disorders. One such disorder, PCOS, remains poorly understood and is a major cause of female infertility. Evidence from my previous study suggests that KITL is a candidate factor contributing to PCOS-associated ovarian phenotypes, but regulation of KITL gene expression in the human ovary remains to be explored. Understanding human ovarian disease and disorders, such as PCOS or other causes of female infertility, relies on the elucidation of the fundamental molecular and cellular signalling systems throughout follicle development, such as KITL and c-kit.

1.5 OBJECTIVES OF THIS THESIS

The overall objective of this thesis was to contribute further knowledge to the understanding of basic human ovarian physiological function by examining the expression and localisation of KITL and c-kit in the human ovary, and identifying factors that might regulate KITL gene expression in human granulosa cells.

This was achieved by addressing the following specific aims:

1. To investigate KITL and c-kit isoform expression in human ovarian tissues and to examine expression and localisation of c-kit throughout follicle development.

2. To investigate the regulation of KITL gene expression in human granulosa cells by endocrine and intraovarian factors.

CHAPTER 2

Materials and Methods

Chapter 2: Materials and Methods

2.1 MATERIALS

The suppliers of all material used throughout this thesis are indicated below.

Reagent	***Reagent Supplier***
2-Mercaptoethanol, 98+%	*Sigma (St Louis, MO, USA)*
5α-dihydrotestosterone (DHT)	*Sigma (St Louis, MO, USA)*
Agarose, analytical grade	*Promega Corporation (Madison, WI, USA)*
Albumin from Bovine Serum	*Sigma (St Louis, MO, USA)*
Amersham Hyperfilm ™ ECL	*GE Healthcare Ltd (Buckhamshire, UK)*
Anti-alpha Tubulin Rabbit Polyclonal Antibody	*Sapphire Bioscience (Waterloo, NSW, Australia)*
Anti-mouse IgG/HRP-conjugated	*DakoCytomation (Glostrup, Denmark)*
Anti-rabbit IgG/HRP-conjugated	*DakoCytomation (Glostrup, Denmark)*
AR N-20 Rabbit Polyclonal Antibody	*Santa Cruz Biotechnology (Santa Cruz, CA, USA)*
Big Dye ™ Buffer	*Applied Biosystems (Boston, MA, USA)*
Big Dye ™ Terminator	*Applied Biosystems (Boston, MA, USA)*
Bovine Serum Albumin (BSA)	*Sigma (St Louis, MO, USA)*
Bradford Protein Dye Reagent	*Biorad (Hercules, CA, USA)*
Bromophenol Blue	*Sigma (St Louis, MO, USA)*
Cells (KGN)	*Kyushu and Kurume Universities, Japan*
Charcoal	*Asia Pacific Specialty Chemicals (Seven Hills, NSW, Australia)*
Citric Acid Monohydrate	*Sigma (St Louis, MO, USA)*
Criterion ™ XT MOPS Running	*Biorad (Hercules, CA, USA)*

Buffer (20x)	
Criterion ™ XT Precast Gel 4-12% Bis-Tris	*Biorad (Hercules, CA, USA)*
dATP, dCTP, dGTP, dTTP	*Promega Corporation (Madison, WI, USA)*
Deoxynucleotides Triphosphates (dNTPs) (10 mM) each	*Promega Corporation (Madison, WI, USA)*
Developer and Rapid Fixer	*AGFA (Mortsel, Belgium)*
Dextran	*Amersham Biosciences (Buckinghamshire, England)*
DMSO	*BDH Laboratory Supplies (Vic, Australia)*
Dulbecco's Modified Eagle's Medium Nutrient Mixture F-12 Ham	*Sigma (St Louis, MO, USA)*
Dulbecco's Phosphate Buffered Saline (DPBS) (1x)	*Invitrogen (San Diego, CA, USA)*
Ethanol (molecular biology grade)	1) For qPCR: *Ambion Inc. (Austin, TX, USA)* 2) For other applications: *Ajax Finechem (Seven Hills, NSW, Australia)*
Ethidium Bromide	*Pharmacia Biotech (Boston, MA, USA)*
Experion RNA HighSens Reagents and Chips	*Biorad (Hercules, CA, USA)*
Experion RNA StdSens Reagents and Chips	*Biorad (Hercules, CA, USA)*
Foetal Bovine Serum (FBS)	*Sigma (St Louis, MO, USA)*
Glycerol	*Sigma (St Louis, MO, USA)*
Glycogen	*Ambion Inc. (Austin, TX, USA)*
Goat Serum	*Sigma (St Louis, MO, USA)*
HCl	*Ajax Finechem (Seven Hills, NSW, Australia)*
HSP 90 Mouse Monocloncal Antibody	*Santa Cruz Biotechnology (Santa Cruz, CA, USA)*
Hybond ™ –C Extra Nitrocellulose	*Amersham Biosciences (Buckinghamshire,*

Transfer Membrane	*England)*
Hydrochloric Acid	*Chem Supply (Gillman, SA, Australia)*
Hydrogen Peroxide 30%	*Chem Supply (Gillman, SA, Australia)*
iScript cDNA synthesis kit	*Biorad (Hercules, CA, USA)*
iQ SYBR Green Supermix	*Biorad (Hercules, CA, USA)*
Isopropanol (molecular biology grade)	*Ajax Finechem (Seven Hills, NSW, Australia)*
Kanamycin	*Sigma (St Louis, MO, USA)*
KCL	*Ajax Chemicals (Auburn, NSW, Australia)*
KOH	*Ajax Chemicals (Auburn, NSW, Australia)*
Luria Broth Components	*Becton Dickinson (Sparks, MD, USA)*
Methanol	*Ajax Finechem (Seven Hills, NSW, Australia)*
$MgCL_2$	*Ajax Chemicals (Auburn, NSW, Australia)*
50 mM $MgSO_4$ for PCR	*Invitrogen (San Diego, CA, USA)*
NaCl	*Sigma (St Louis, MO, USA)*
NaOH	*Riedel-de Haen (Seelze, Germany)*
Nuclease-free water	*Ambion Inc. (Austin, TX, USA)*
Nuclon Lab-Tek II Chamber Slide	*Lab-Tek (Naperville, IL, USA)*
Opti-MEM Reduced Serum Medium	*Invitrogen (San Diego, CA, USA)*
Paraformaldehyde	*Merck (Darmstadt, Germany)*
Passive Lysis 5x buffer	*Promega Corporation (Madison, WI, USA)*
Penicillin-Streptomycin Solution	*CSL Biosciences (VIC, Australia)*
Pentraxin 3 Mouse Monoclonal Antibody	*Enzo Life Sciences (Farmingdale, NY, USA)*
Pfu DNA Polymerase, including 10x buffer	*Promega Corporation (Madison, WI, USA)*
Phosphate Buffered Saline (PBS) (1x)	*Sigma (St Louis, MO, USA)*
Polyclonal Goat Anti-Rabbit IgG Biotinylated	*DakoCytomation (Glostrup, Denmark)*

Polyclonal Rabbit Anti-Human CD117, c-kit Antibody	*DakoCytomation (Glostrup, Denmark)*
Ponceau S	*Sigma (St Louis, MO, USA)*
Precision Plus Protein ^{TM} Standards Dual Colour	*Biorad (Hercules, CA, USA)*
Protease Inhibitor Cocktail Tablet	*Roche Applied Science (Penzberg, Germany)*
QIAquick Gel Extraction Kit	*QIAGEN (Hilden, Germany)*
QIAquick PCR Purification Kit	*QIAGEN (Hilden, Germany)*
QIAprep Spin Miniprep Kit	*QIAGEN (Hilden, Germany)*
Recombinant Human Bone Morphogenetic Protein 15 (rhBMP-15) (5 µg)	*R&D Systems (Minneapolis, MN, USA)*
Recombinant Human Follicle Stimulating Hormone (rhFSH)	*Puregon, N.V. Organon (Oss, Netherlands)*
Recombinant Mouse Growth Differentiation Factor 9 (rmGDF-9) (10 µg)	*R&D Systems (Minneapolis, MN, USA)*
Restriction Digest Enzymes, including 10x Buffer and 100x BSA	*New England Biolabs (Beverly, MA, USA)*
RNase-Free DNase Set	*QIAGEN (Hilden, Germany)*
RNeasy® Micro Kit	*QIAGEN (Hilden, Germany)*
RT-PCR Grade Water	*Ambion Inc. (Austin, TX, USA)*
Shrimp Alkaline Phosphatase	*Roche Applied Science (Penzberg, Germany)*
Skim Milk Powder	*Diploma (Melbourne, VIC, Australia)*
Spongostan Dental Sponges 1cm^3	*Johnson & Johnson Medical Ltd (Skipton, UK)*
Stem Cell Factor Antibody	*Millipore (Billerica, MA, USA)*
Streptavidin/HRP	*DakoCytomation (Glostrup, Denmark)*
SuperSignal® West Dura Extended Duration Substrate	*Thermo Scientific (Rockford, IL, USA)*
SuRE/Cut Restriction Buffer	*Roche Applied Science (Penzberg, Germany)*

T4DNA Ligase including 10x buffer	*Roche Applied Science (Penzberg, Germany)*
Taq DNA Polymerase	*New England Biolabs (Beverly, MA, USA)*
Taqman® Gene Expression Assays	*Applied Biosystems (Boston, MA, USA)*
Taqman® Gene Expression Mastermix	*Applied Biosystems (Boston, MA, USA)*
TE buffer ph 7.0	*Ambion Inc. (Austin, TX, USA)*
Tris	*Sigma (St Louis, MO, USA)*
Triton-X 100	*Sigma (St Louis, MO, USA)*
Trypsin-EDTA 0.25%	*Invitrogen (San Diego, CA, USA)*
Tween®20	*Sigma (St Louis, MO, USA)*
Whatman Filter	*Whatman International Ltd (Maidstone, England)*
Water	Sterilised reverse osmosis (RO) water
Xylene	*Chem Supply (Gillman, SA, Australia)*

2.2 BUFFERS AND SOLUTIONS

The composition of buffers and solutions used throughout this thesis are described below. Unless indicated otherwise, solutions were made using sterile RO water and stored at room temperature, and pH levels were adjusted using concentrated NaOH and HCl. Solutions needing sterilisation were autoclaved at 103 kPa, 121°C for 20 min on a Tomy High Pressure Steam Steriliser ES-315 (Tomy-Tech USA Inc., CA, USA).

Citrate Buffer

1.05 g Citrate Acid Monohydrate

Made up to 500 mL with water

pH 6.5

Dextran Coated Charcoal (DCC)

5 g Charcoal

0.5 g Dextran

20 mL Glycerol

Made up to 1 L with TE buffer

Mixed overnight with rotation

Ethidium Bromide 10 mg/mL

0.2 g Ethidium Bromide

20 mL Water

Store in the dark

Loading Dye (6x) for agarose gels

50 mL Glycerol

40 mL 0.5 M EDTA (pH 8.0)

0.25 g Bromophenol blue

0.25 g Xylene cyanol

Made up to 100 mL with water

Stored at 4°C

Phosphate-Buffered Saline (PBS) 10x for immunohistochemistry

4.25 g NaCl

71.5 g K2PO4

12.5 g KH_2PO_4

Made up to 5 L with water

pH 7.3

Ponceau Stain 10x

2 g Ponceau S

30 g Trichloracetic Acid

30 g Sulfosalycylic Acid

Made up to 100 mL with water

RIPA Lysis Buffer

0.1211 g 10 mM Tris

0.8766 g 150 mM NaCL

0.0292 g 1 mM EDTA

1 mL 1% Triton X-100

Made up to 100 mL with water

pH 7.4

1 tablet Complete Mini Protease Inhibitor/10 mL RIPA Lysis Buffer

Stored at -20℃

Running Buffer 10x

75 g Tris

360 g Glycine

25 g SDS

Made up to 2.5 L with water

TAE Buffer 50x

242 g Tris

57.1 mL Glacial acetic acid

100 mL 0.5 M EDTA Ph 8.0

Made up to 1 L with water

TBS 10x

151.5 g Tris

219 g NaCl

Made up to 2.5 L with water

pH to 7.4

TBST

5 mL Tween®20

2.5 mL TBS

TE

1.211 g Tris

0.588 g EDTA

900 mL water

Made up to 1 L with water

pH to 8.0

Sterilised

Transfer Buffer 10x

77.5 g Tris

360 g Glycine

Made up to 2.5 L with water

Stored at 4°C

Transfer Buffer 1x

800 mL Methanol

400 mL 10x Transfer buffer

Made up to 2.8 L with water

Stored at 4°C

Tris 1 M, pH 8.8

60.57 g Tris

500 mL water

pH to 8.8

Tris 1 M, pH 6.8

60.57 g Tris

500 mL water

pH to 6.8

2.3 METHODS

2.3.1 Experimental Models

The human granulosa tumour cell line, KGN, was derived from a 63-year-old female human (*Homo sapiens*) patient with an invasive ovarian granulosa cell carcinoma (Nishi *et al.* 2001). KGN cells represent granulosa cells from the preantral stage, and is the only human granulosa cell line to maintain functional FSH signalling.

Human cumulus cells and mural granulosa cells represent specialised granulosa cells from preovulatory follicles. Cumulus cells have previously been demonstrated by work in this laboratory to express high levels of androgen receptor (AR) protein, and thus were used in studies examining androgen signalling.

Human ovarian tissues, consisting of premenopausal paraffin-embedded archival tissue and snap-frozen tissue, contained follicles at all stages of development intact in their surrounding stroma and represented women with or without polycystic ovaries (PCO). Further details are described below.

2.3.2 Human ovarian tissue collection

2.3.2.1 Archival tissue

Formalin-fixed, paraffin-embedded ovarian tissues were obtained from hospital archives owned and operated by the Institute of Medical and Veterinary Science (IMVS; Adelaide, South Australia) under ethics approvals from the Royal Adelaide Hospital, the Central Northern Adelaide Health Service, and the University of Adelaide. Electronic pathology

records were extensively searched for the following terms, alone or together: “hysterectomy”; “oophorectomy”; “ovary”; “PCOS”; “polycystic”; “hyperthecosis”; “wedge resection”; or “Stein-Leventhal”. Records corresponding to premenopausal women who had surgery for benign gynaecological conditions were collated and the associated tissue blocks were retrieved where possible. Tissue morphology was independently assessed by two pathologists employed by the IMVS (Prof Tom Dodd and Dr Shalini Jindal) using 3 μm sections of tissue blocks stained with haematoxylin and eosin. PCO morphology was determined by the presence of sub-capsular fibrosis, multiple healthy antral follicles in the ovarian cortex and evidence of thecal hypertrophy. Eight cases of PCO were identified and in 2 of these, the pathology reports state a previous diagnosis of polycystic ovary syndrome (PCOS). In the other 6 cases of PCO, an association with PCOS could not be determined. Six ovarian tissues from women of a similar age were selected and designated non-PCO as they lacked any histological feature of PCO and appeared morphologically and histologically normal with the exception of the presence of benign inclusion cysts in two cases. Examples of PCO and non-PCO tissues are depicted in Figure 2.1.

A follicle was classified as primordial if it consisted of an oocyte surrounded by a single layer of flattened granulosa cells, and classified as primary if the oocyte was surrounded by a single layer of cuboidal granulosa cells. A secondary follicle had two or more layers of granulosa cells without an antrum, and an antral follicle had a large cavity, several layers of granulosa cells around the antrum and oocyte and a defined basal lamina and thecal layer. Large atretic follicles were classified by having an oocyte with an irregular

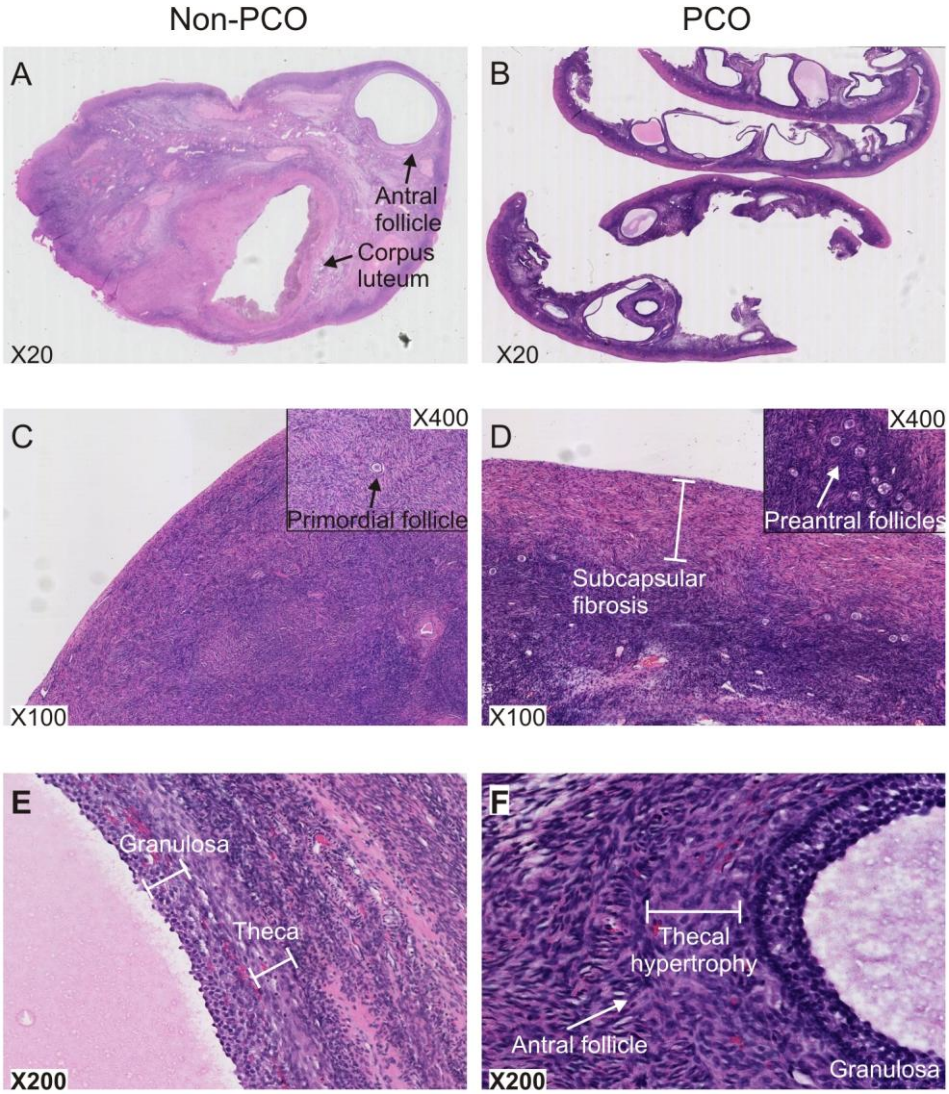

Non-PCO
PCO
A
Antral follicle
Corpus luteum
X20
B
X20
C
X400
Primordial follicle
X100
D
X400
Preantral follicles
Subcapsular fibrosis
X100
E
Granulosa
Theca
X200
F
Thecal hypertrophy
Antral follicle
Granulosa
X200

Figure 2.1. Haematoxylin and eosin stained sections of non-PCO (**A-E**) and PCO (**B-F**). An antral follicle and corpus luteum are visible in a whole section of non-PCO (**A**), and several antral follicles are present in a whole section of PCO (**B**). One primordial follicle is present in a representation of a non-PCO cortex region (**C**; inset), while subcapsular fibrosis is present in the PCO cortex representation (**D**) as well as several clustered preantral follicles (**D** inset). A normal theca layer is present in a non-PCO antral follicle (**E**) in contrast to thecal hypertrophy visible in a PCO antral follicle (**F**). Original magnifications: X20 (**A,B**); X100 (**C,D**); X200 (**E,F**). Bars denote regions of tissue as indicated.

membrane or a thinning layer of granulosa cells with pyknotic nuclei. Stages of atresia were assessed by the level of degradation of the granulosa cell layer and the appearance and definition of the basal lamina and theca layer. Corpus albicans were classified as distinct, large solid white areas resembling scar tissue and containing discrete areas of stromal cells. Germinal inclusion cysts were classified as fluid-filled cavities of various sizes situated in the cortex, surrounded by one or more layers of cuboidal or rectangular epithelial cells.

2.3.2.2 Fresh tissue

Fresh pieces of human ovarian cortex were obtained from consenting premenopausal and postmenopausal women having oophorectomy as part of gynaecological surgery at the Royal Adelaide Hospital, South Australia, Australia. The study was approved by the ethics committees of the Royal Adelaide Hospital and the University of Adelaide. Tissue was immediately snap frozen upon collection and stored in liquid nitrogen.

2.3.2.3 Human granulosa cells

Following informed consent, granulosa cell (GC) subtypes from pre-ovulatory follicles were collected from women who were receiving assisted reproduction at infertility clinics including Repromed (Dulwich, South Australia) and Flinders Reproductive Medicine (Bedford Park, South Australia). Patients were diagnosed by Prof Robert Norman or Dr Marcin Stankiewicz as having PCOS based on the international Rotterdam diagnostic criteria (2004a; 2004b) or as not having any feature of PCOS (Controls). The study was approved by the ethics committees of the Women's and Children's Hospital, the University

of Adelaide, and the Flinders Medical Centre. All women underwent a standard hyperstimulation protocol with gonadotropin-releasing hormone (GnRH) down-regulation and subsequent gonadotrophin stimulation prior to oocyte retrieval. Two GC subtypes were collected as two separate pools for each patient as previously described (Robker *et al.* 2009); these were luteinised mural granulosa cells (MGC) that originate from the follicle wall and cumulus cells (CC) that originate from the cumulus-oocyte complex.

2.3.3 Analysis of follicular fluid hormones

Follicle fluid was removed from follicular aspirates that were centrifuged at 1500 *g* for 10 minutes, and stored at -80°C. Total testosterone levels were measured by isotope dilution-liquid chromatography-tandem mass spectrometry (SA Pathology, Flinders Medical Centre, South Australia), which has a sensitivity of 0.5 nmol/liter, with an intraassay coefficient of 4.8% and interassay coefficient of less than 10%. Free androgen index (FAI) was calculated as testosterone/sex hormone binding globulin (SHBG) X 100, where SHBG levels were measured by Radio Immunoassay (RIA) kit (Orion Diagnostics, Espoo, Finland). The assay was performed according to the manufacturer's protocol, and had an intraassay coefficient of variation of 4.0% and interassay coefficient of 5.5%.

2.3.4 Cell Culture

The human granulosa tumour cell line, KGN, was maintained in a 1:1 mixture of Dulbecco's Modified Eagle' Medium (DMEM) and Ham's F-12 Nutrient Mixture supplemented with 1% penicillin-streptomycin and 10% heat-inactivated foetal bovine serum (HI-FBS).

Human cumulus cells, obtained from female human patients undergoing assisted reproductive technology (see section 2.3.2.3), were washed three times upon collection with Opti-MEM medium supplemented with 5% dextran-coated charcoal-stripped foetal bovine serum (DCC-FBS) and maintained in the same medium.

All cells were maintained at 37°C with 5% CO_2. All procedures involving cell lines and primary cells were performed under aseptic conditions in a laminar flow cabinet. Receptor expression of ligands used throughout this thesis are listed in Table 1.

2.3.4.1 Passaging cells

KGN cells were maintained in T125 sterile tissue culture flasks and were passaged at regular intervals, when cells were approximately 80% confluent. Culture medium was aspirated from the flasks and cells were washed with 10 mL pre-warmed 1x phosphate buffered saline (PBS). PBS was aspirated and 4 mL 0.25% trypsin-EDTA (1x) was added and cells incubated at 37°C for approximately 5 minutes until the cells began to detach. DMEM/Ham's F-12 medium (10 mL) was added and the cell suspension centrifuged for 5 minutes at 1500 rpm. The supernatant was discarded and the cell pellet resuspended in 10 mL medium containing serum and counted using a haemocytometer. Cells were either passaged into a new flask with fresh medium or seeded into the appropriate culture dish at the specific density indicated for each experiment.

2.3.4.2 Freezing cells

Cells were detached using trypsin, as outlined above, and the cells resuspended at $2x10^6$ cells/mL in medium containing serum. Freezing mix was prepared on ice containing 40%

DMSO, 40% serum and 20% growth medium. Cells were placed on ice and 500 µL freezing mix was added dropwise per 500 µL cell suspension. The cells were mixed gently and 1 mL aliquots were prepared in cryovials. Cryovials were placed into a controlled rate freezing unit containing isopropanol at -80°C overnight and cells were either stored at -80°C or in liquid nitrogen.

2.3.4.3 Thawing cells

Stored vials containing KGN cells were removed from frozen storage and cells were rapidly thawed in a 37°C water bath. The thawed cells were added to 9 mL pre-warmed media in a 15 mL tube. Cells were centrifuged for 5 minutes at 1500 rpm. The supernatant was discarded and the cell pellet resuspended in fresh medium and seeded into a T125 culture flask.

2.3.4.4 Charcoal Stripping of Foetal Bovine Serum

Dextran-coated charcoal (DCC), 50 mL per 50 mL tube, was centrifuged for 30 min at 4000 rpm and the supernatant was discarded. FBS (50 mL) was added to each 50 mL tube containing the charcoal pellet and incubated for 2 hours at room temperature with rotation. Tubes were centrifuged for 30 minutes at 4000 rpm and the supernatant poured into fresh tubes containing fresh charcoal pellets and incubated for 2 hours at room temperature with rotation. The tubes were centrifuged for 30 minutes at 4000 rpm and the resulting charcoal stripped serum was filter sterilised and stored at -20°C.

Table 1. Expression of receptors in KGN cells and human cumulus cells.

Receptor	KGN	CC
c-kit	Y	−
AR	Y	Y
FSHR	Y	Y
FGFRII	−	−
BMPR2	Y	Y
ALK5	Y	Y
ALK6	−	Y

Y indicates receptor is expressed, − indicates receptor is not expressed.

2.3.5 Preparation of Steroid Stocks

5α-dihydrotestosterone (DHT) was prepared in 100% ethanol to a concentration of 10^{-2}M and stored in glass vials at -20°C. Concentrated stock was diluted in culture medium as required.

2.3.6 Preparation of rhGDF-9 and rmBMP-15

Recombinant human BMP-15 and recombinant mouse GDF-9 were obtained as lyophilised proteins. Reconstitution was performed at 100 µg/ml in sterile 4 mM HCl containing 0.1% BSA. Samples were aliquoted to avoid repeated freeze-thaw cycles and stored at -20°C. Concentrated stock was diluted in culture medium as required.

2.3.7 RNA extraction and generation of cDNA

Total RNA was extracted from KGN cells using an RNeasy Mini Kit, and extracted from cumulus cells using an RNeasy Micro Kit according the manufacturer's protocol. Samples were stored at -80°C in RLT buffer and thawed for 5 minutes in a 37°C dry heat block and briefly vortexed prior to the addition of 70% ethanol. RNA was eluted in 14 µl (RNeasy Micro Kit) or 30 µl (RNeasy Mini Kit) TE buffer (pH 7.0). RNA was quantitated using the ND-1000 Nanodrop spectrophotometer (NanoDrop Technologies Inc, Wilmington, DE, USA) (KGN cells), or using an Experion Automated Electrophoresis System (cumulus cells) to assess the quality and integrity of the samples. Samples were quantitated on the Experion Automated Electrophoresis System according to the manufacturer's protocol for Eukaryote Total RNA Assay, and samples that did not achieve an RNA Quality Indicator (RQI) value of at least 7 out 10 were discarded.

cDNA was generated from total RNA from cumulus cells (100 ng) and KGN cells (500 ng) respectively using an iScript cDNA Synthesis Kit (Biorad, Hercules, CA, USA) containing oligo (dT) and random primers according to the manufacturer's protocol. Briefly, the appropriate volume of RNA for each sample was added to a mastermix of 1x iScript Reaction Mix, iScript reverse transcriptase and nuclease-free water. After 5 minutes at room temperature, samples were incubated for 30 minutes at 42°C and the reaction was terminated at 85°C for 5 minutes. Samples were stored at 4°C and diluted for quantitative real-time PCR (qRT-PCR) reactions using nuclease-free water.

2.3.8 Quantitative Real-Time PCR

Target cDNA amplification using Taqman Gene Expression Assays (Applied Biosystems, Boston, MA, USA) was performed on a CFX384 Touch Real-Time PCR Detection System (Biorad) in a total reaction volume of 10 µl using the following PCR conditions: 1 cycle at 50°C for 2 min, 1 cycle at 95°C for 10 min, 40 cycles at 95°C for 15 sec and 60°C for 1 min. Target cDNA amplification using QuantiTect Gene Expression Assays (Qiagen) and primers purchased from Geneworks (Adelaide, SA, Australia) were performed on an iQ5 iCycler (Biorad) using the following PCR conditions: 1 cycle at 95°C for 3 min, 40 cycles at 95°C for 15 sec, 60°C for 15 sec, 72°C for 30 sec, 95°C for 1 min, 55°C for 1 min. Melt curve analysis occurred at 60°C for 10 sec.

qRT-PCR data was analysed using the $\Delta\Delta C_T$ method (Livak and Schmittgen 2001) for quantitation relative to a calibrator, which was the mean value of 3 Control experimental replicates in each study. Data analysed using this method was expressed as the fold change relative to the calibrator.

2.3.9 Western Blot

2.3.9.1 Generation of human tissue lysates

A piece of frozen tissue, approximately $1cm^3$, was ground to a fine powder using a mortar and pestle. Liquid nitrogen ensured that samples remained frozen at all times. Each sample was placed in 200 µL of RIPA buffer and homogenised by passing the sample through a 25 gauge needle at least five times, followed by centrifugation at 10,000 rpm for 10 min at 4°C. Total protein concentration was measured by a Bradford protein assay at 595 nM and protein samples were prepared by the addition of 6x loading dye, denaturation at 100°C and centrifugation for 1 minute.

2.3.9.2 Generation of cell lysates

Cells harvested for Western immunoblotting were lysed using RIPA buffer including protease inhibitor cocktail tablets, followed by centrifugation at 10,000 rpm for 10 min at 4°C. Concentration of protein was determined using a Bradford protein assay at 595 nM.

2.3.9.3 Visualisation of target proteins

Protein samples were prepared by adding 6x load dye, denaturing at 100°C for 5 minutes then centrifuging for 1 minute. Proteins (20 µg) were then electrophoresed on Criterion XT precast gels in 1x XT MOPS running buffer at 150 volts for a total running time of 60-90 minutes. The Precision Plus Protein Standards Dual Colour molecular weight marker was run alongside protein samples. Proteins were transferred to a Hybond-C nitrocellulose membrane under wet transfer conditions, using a Criterion Blotter according the manufacturer's instructions. Briefly, the transfer was set up in cold 1x transfer buffer with

the gel and membrane stacked between Whatman filter paper, and transferred in cold 1x transfer buffer with at 400 mA for 60-90 minutes. Protein transfer was checked by staining the membrane for 1 minute in Ponceau stain, followed by brief rinsing in TBST. Membranes were blocked in TBST containing 3% skim milk powder for 1 hour, followed by incubation with a primary antibody (anti-SCF, 1:1000; anti-c-kit 1:1000; anti-PTX3; 1:1000; anti-HSP 90 1:1000; anti-alpha Tubulin 1:1000) for 1 hour to overnight. The appropriate HRP-conjugated secondary antibody (1:2000) was added for 30 minutes. Each primary and secondary antibody was diluted in TBST containing 1% skim milk powder. Membranes were washed 3 times for 10 minutes in TBST following incubation with each antibody. All steps were performed with gently rotation at room temperature, except for overnight incubations which were performed at 4°C. Bound antibody was visualised using SuperSignal West Dura Extended Duration Substrate, according to the manufacturer's instructions, and exposed to Amersham Hyperfilm ECL for the appropriate time. The film was developed in a dark room using standard developing and fixative reagents. Densitometry analysis of immunoblot was performed by measuring band intensity using the AlphaImager 2200 gel documentation system (Alpha Innotech, San Leandro, CA, USA).

2.3.10 Immunohistochemistry

Standard immunohistochemistry was performed on tissue sections (3 μm) as previously described (Ricciardelli *et al.* 2005), except antigen retrieval was performed using a Decloaking Chamber (Biocare Medical, Concord, CA, USA) rather than a microwave following the manufacturer's protocol in 10 mM citrate buffer (pH 6.5). Quenching of

endogenous peroxidise activity (6% H_2O_2 in methanol for 5 minutes at room temperature) was performed followed by blocking of non-specific antigens with 5% goat serum for 1 hour at room temperature. Tissues were stained simultaneously with a 1:300 dilution of c-kit (CD117) antibody overnight at 4°C. Human gastrointestinal stromal tumour (GIST) was used as a positive control tissue, and omission of the primary antibody was used as a negative control in GIST and ovarian tissue. Visualisation of c-kit immunoreactivity was peformed using a standard immunoperoxidase reaction. Biotinylated anti-rabbit antibody (1:400), streptavidin-HRP complex (1:500) and diaminobenzidine tetrahydrochloride was used to generate an insoluble brown deposit. Stained sections were scanned using the NanoZoomer image system (Hamamatsu, Japan) set at a magnification of 40X.

2.3.11 Immunocytochemistry

Cells cultured in 8-well Lab Tek II Chamber Slides were fixed in 4% paraformaldehyde for 10 minutes at room temperature then washed twice for 5 minutes in PBS. Chambers were subsequently removed and slides immersed in pre-chilled methanol for 3 minutes at -20ºC, followed by 1 minute in pre-chilled acetone at -20°C. Immunohistochemistry was performed as described above with the exception of deparaffinising the slides and subjecting the cells to antigen retrieval and blocking.

CHAPTER 3

Characterisation of c-kit and Kit Ligand Expression and Localisation in the Adult Human Ovary

Chapter 3: Characterisation of c-kit and Kit Ligand Expression and Localisation in the Adult Human Ovary

3.1 INTRODUCTION

A major aim of this thesis was to characterise the expression and localisation of c-kit in the human ovary, as well as the expression of KITL isoforms throughout folliculogenesis. *KITL* and its receptor *c-kit* have been predominantly studied in the mouse, and many aspects of their expression and function have also been elucidated in other animal models including rat, rabbit, chicken, goat and cow (Driancourt *et al.* 2000). While these studies have been valuable in giving an indication of the function and importance of the KITL/c-kit system in ovarian function, species differences have been identified and there is very little known about their roles and expression in the human ovary. Human studies to date have largely been limited to fetal ovaries due to the difficulty in obtaining adult ovarian tissue samples, resulting in a paucity of knowledge about KITL and c-kit during a woman's reproductive lifespan.

Previous work from this laboratory characterised KITL protein expression and localisation throughout adult human folliculogenesis and showed abnormally high levels in PCO compared to non-PCO (Tuck *et al.* 2007; Tuck *et al.* 2010a; Tuck *et al.* 2010b), as shown in Chapter 1 (section 1.3.6). Cytoplasmic immunostaining was present in granulosa cells, oocytes and surrounding stroma of preantral follicles, and in the granulosa, theca layer and oocytes of antral follicles. Higher levels of protein were observed in granulosa cells of

antral follicles compared to preantral follicles. Staining was markedly more intense in all cell types, except the theca layer, in PCO compared to non-PCO, suggesting dysregulation of KITL in this disorder may contribute to the phenotypic characteristics of PCO. These could include abnormal oocyte growth, increased follicle and stromal density, thickened thecal layers and excess thecal cell androgen biosynthesis as suggested by the range of actions ascribed to KITL in these cell types, which have been demonstrated in various animal models (Parrott and Skinner 1997; Yoshida *et al.* 1997; Parrott and Skinner 1998b; Parrott and Skinner 1999; Parrott and Skinner 2000; Reynaud *et al.* 2000; Hutt *et al.* 2006; Thomas and Vanderhyden 2006; Thomas *et al.* 2008). KITL expression was also present in human granulosa and theca cells of atretic follicles, and interestingly, appeared quite intense in the epithelial cells of germinal inclusion cysts, suggesting involvement in ovarian cystic pathology. These staining patterns differ to several animal models in which KITL localisation is more discreet, present only in granulosa cells and some areas of stroma. These differences in localisation may suggest different roles for KITL in the human ovary, and thus identification of c-kit-expressing cells is required to begin understanding KITL signalling in the human ovary.

KITL isoforms have been shown to have different functions and importance throughout follicle development. *KITL-2* null mice were completely infertile due to an absence of germ cells, while *KITL-1* null mice were fertile and equally as viable as wildtype mice indicating that *KITL-2* is essential for normal folliculogenesis and fertility (Brannan *et al.* 1992; Tajima *et al.* 1998b). Indeed, a study examining isolated mouse oocytes showed that KITL-2 is the principal isoform required for oocyte survival and growth in vitro (Thomas

et al. 2008). Isolated oocytes were co-cultured on transfected fibroblasts expressing either KITL-1 or KITL-2, and oocyte growth occurred only in the presence of KITL-2-expressing fibroblasts. Spontaneous germinal vesicle breakdown (GVBD) was inhibited by the presence of KITL-2 but not KITL-1, and the addition of meiosis inhibitors did not prevent spontaneous GVBD in the presence of KITL-1 suggesting that GVBD was a result of oocyte degeneration rather than meiotic progression (Thomas *et al.* 2008). Studies examining mRNA expression of each isoform have shown almost exclusive expression of *KITL-1* in the granulosa cells of bovine antral follicles whereby levels increased with follicle growth (Parrott and Skinner 1997). *KITL-1* and *KITL-2* levels are similar in small antral follicles in mice, but at the cessation of oocyte growth and the beginning of antral follicle growth the levels of *KITL-1* increases (Parrott and Skinner 1997; Thomas *et al.* 2005). Protein isoform expression has not been examined in animal ovarian follicles including human, and thus the ovarian protein expression and localisation of KITL-1 and KITL-2 in the human ovary remains to be investigated.

Two studies examining c-kit protein localisation in adult human ovaries show conflicting results. Abir *et al.* reported c-kit staining to be present exclusively in oocytes of some preantral follicles, but did not examine follicles at the antral stage due to samples consisting only of biopsies (Abir *et al.* 2004). Another study by Carlsson *et al.* showed staining to be present in some oocytes and granulosa cells of preantral follicles, and an absence of staining in all cell types of antral follicles (Carlsson *et al.* 2006). This study is quite different to observations in a number of animal studies examining mice and sheep, which express high levels of c-kit protein in oocytes throughout folliculogenesis. A

summary of c-kit expression in non-human and human ovaries is shown in Table 1. While some species differences are known to exist, such as constant levels of c-kit expression in oocytes of aging sheep versus declining levels in oocytes of aging mice (Manova *et al.* 1993; Tisdall *et al.* 1997), it is unlikely that human antral follicles are totally devoid of c-kit protein expression. This concept is supported by a study examining human follicular fluid, which measured levels of soluble c-kit and showed a significant correlation with follicle fluid volume and follicle fluid hormone concentrations including estradiol, testosterone and androstenedione (Tanikawa *et al.* 1998). Since soluble c-kit is produced by hydrolysis of the ectodomain of the membrane-bound receptor (Yee *et al.* 1993; Brizzi *et al.* 1994), its presence in follicular fluid may suggest that c-kit protein is expressed by preovulatory antral follicles, or may possibly accumulate from circulating soluble c-kit (Kanbe *et al.* 2001). Furthermore, this study also reported c-kit mRNA expression to be present in isolated oocytes and granulosa cells from human preovulatory follicles. Our study showing that KITL protein is expressed by multiple cell types within antral follicles suggests that c-kit would also be present in order for KITL signalling to be transmitted in target follicular cells. Clearly, c-kit protein expression in human adult ovaries requires further investigation to determine localisation throughout follicle development.

Little is known about the expression of *c-kit* isoforms in the mammalian ovary and their specific functions remain unknown. *GNNK+* and *GNNK−* have been shown to coexist in a variety of non-reproductive tissues and to date, only one study has examined their expression in the ovary. Isolated granulosa and theca cells from bovine antral follicles were shown to express both mRNA isoforms (Koch *et al.* 2009). A study performed using

a fibroblast cell line, NIH3T3, transfected with each *c-kit* isoform demonstrated that GNNK− features faster receptor phosphorylation and downregulation following activation by ligand compared to GNNK+, as well as greater activation of the MAPK pathway (Caruana *et al.* 1999). The presence of each isoform in the human ovary remains unknown.

To address current gaps in knowledge about the KITL/c-kit signalling pathway in human ovaries, the specific aims for this chapter were to characterise the expression and localisation of c-kit protein in the human ovary throughout follicle development, and to examine the expression of *c-kit* mRNA isoforms in human ovarian cortex. It also sought to characterise KITL protein isoform expression in the ovary at different stages of follicle development, and to compare *KITL* mRNA levels in cumulus cells from women with or without PCOS to support previous work showing different KITL protein levels in PCO compared to non-PCO.

Table 1. Summary of data reported on expression of c-kit mRNA and protein in the ovary of non-human models and c-kit protein expression in the human ovary.

		Primordial		**Primary**		**Secondary**		**Antral**		
		GC	OOC	GC	OOC	GC	OOC	TH	GC	OOC
Non-Human	mRNA	−	+	−	+	−	+	+	+	+
Non-Human	Protein	−	+	−	+	−	+	+	−	+
Human	Protein	+	+	+	+	+	+	−	−	−

GC: granulosa cells; OOC: oocyte; TH; theca cells. + indicates the presence of mRNA or protein; − indicates the absence of mRNA or protein.

(Horie *et al.* 1993; Manova *et al.* 1993; Motro and Bernstein 1993; Laitinen *et al.* 1995; Clark *et al.* 1996; Ismail *et al.* 1996; Ismail *et al.* 1997; Parrott and Skinner 1997; Parrott and Skinner 1998a; Parrott and Skinner 1998b; Tanikawa *et al.* 1998; Joyce *et al.* 1999; Parrott and Skinner 1999; Driancourt *et al.* 2000; Joyce *et al.* 2000; Parrott and Skinner 2000; Reynaud *et al.* 2000; Kang *et al.* 2003; Abir *et al.* 2004; Brankin *et al.* 2004; Nilsson and Skinner 2004; Thomas *et al.* 2005; Carlsson *et al.* 2006; Gilchrist *et al.* 2006; Hutt *et al.* 2006; Thomas and Vanderhyden 2006; Thomas *et al.* 2008)

3.2 MATERIALS AND METHODS

3.2.1 Human tissue collection

3.2.1.1 Archival tissue

Formalin-fixed, paraffin-embedded ovarian tissues originating from premenopausal women having surgery for benign gynaecological conditions were obtained from the Institute of Medical and Veterinary Sciences (IMVS) tissue archives, with ethics approval from The University of Adelaide, and morphology examined as described in Chapter 2 (section 2.3.2.1). Briefly, PCO morphology was determined by the presence of sub-capsular fibrosis, multiple healthy antral follicles in the ovarian cortex and evidence of thecal hypertrophy. Eight cases of PCO were identified and in 2 of these, the pathology reports state a previous diagnosis of PCOS. In the other 6 cases of PCO, an association with PCOS could not be determined. Six ovarian tissues from women of a similar age were selected and designated non-PCO as they lacked any histological feature of PCO and appeared morphologically and histologically normal with the exception of the presence of benign inclusion cysts in two cases. Examples of PCO and non-PCO tissues are depicted in Chapter 2 (section 2.3.2.1, figure 2.1). Follicle classifications are described in Chapter 2 (2.3.2.1). The ages of patients are listed in Table 2.

Formalin-fixed, paraffin-embedded human gastrointestinal stromal tumours (GISTs) were obtained from the IMVS tissue archives for use as c-kit positive controls in immunohistochemistry.

3.2.1.2 Fresh tissue

Fresh pieces of premenopausal human ovarian cortex were obtained as described in Chapter 2 (section 2.3.2.2) for use in Western blot and qPCR analysis.

3.2.1.3 Human granulosa cells

Granulosa cell subtypes from pre-ovulatory follicles were collected from women who were receiving assisted reproduction at infertility clinics in Adelaide as described in Chapter 2 (section 2.3.2.3). Two GC subtypes were collected as two separate pools for each patient as previously described (Robker *et al.* 2009); these were luteinized mural granulosa cells (MGC) that originate from the follicle wall and cumulus cells (CC) that originate from the cumulus-oocyte complex.

3.2.2 Analysis of KITL and c-kit mRNA in Primary Granulosa Cells

RNA was extracted from mural granulosa cells and cumulus cells using Tri Reagent (Sigma, St Louis, MO, USA) according to the manufacturer's protocol, with the exception of using an overnight precipitation step at -20°C. Deoxyribonuclease treatment was performed to eliminate any genomic DNA contamination using Ambion DNA-free. Total RNA extraction from cumulus cells from women with or without PCOS was performed using an RNeasy Micro Kit (Qiagen, Hilden, Germany) as described in Chapter 2 (section 2.3.7) with on-column DNase treatment and stored at -80°C. Quantitation of RNA is described in General Methods Section_, and cDNA was generated from 100 ng of RNA as described in General Methods using an iScript cDNA Synthesis Kit (Biorad, Hercules, CA, USA) containing oligo (dT) and random primers.

Quantitative real-time PCR reactions for *KITL* and its housekeeping gene *RPL19* were performed using 2x iQ SYBR Green Supermix as outlined by the manufacturer (Biorad). Primers for each gene were commercially obtained (Qiagen) and diluted as per the manufacturer's instructions using Nuclease-Free Water. Target cDNA was amplified as described in Chapter 2 (section 2.3.8).

PCR to detect c-kit isoforms was performed using a primer pair designed to show both isoforms as previously described (Caruana *et al.* 1999). Target cDNA was amplified as described in section Chapter 2 (section 2.3.8), and PCR products were visualised on a 4% agarose gel to detect products 93 and 81 bp in size.

3.2.3 Western Blot

Western blots to specifically detect c-kit and KITL in human ovarian tissue and follicle cell-types were performed as described in Section 2.3.9. The same anti-c-kit polyclonal antibody (DakoCytomation) used for immunohistochemistry was utilised for Western blot to show specificity of the antibody, as a specific peptide for competition studies was unavailable. Several polyclonal KITL antibodies were tested and optimised using human ovarian tissue, human breast and KGN cell lysates and from these, a rabbit polyclonal antibody was chosen based on its use in a previous study examining KITL expression in human glioma cells (Sun *et al.* 2006). Quantitative comparison of KITL proteins in cumulus versus mural granulosa cell lysates could not be reliably performed by Western blot due to the large amount of albumin present in the cumulus cell lysate *(data not shown)*, which formed the bulk of the protein loaded onto the gel.

3.2.4 Immunohistochemistry

Immunohistochemistry to specifically detect c-kit in paraffin-embedded human ovarian tissue was performed as described in Chapter 2 (section 2.3.10). A range of c-kit antibodies were tested and optimised using human ovarian tissue and GIST as a positive control. From these, CD117 rabbit polyclonal antibody (DakoCytomation, Glostrup, Denmark) was chosen as it was the antibody that provided a pattern of staining in GIST most similar to that used for diagnosis as shown in previous studies (Wong and Melegh 2009; Novelli *et al.* 2010), and had low background staining in ovarian test sections. One section per patient was stained for c-kit, and the number of each follicle type observed in non-PCO and PCO groups are detailed in Table 2.

Table 2. Tissue characteristics and numbers of each follicle type observed in non-PCO and PCO ovarian sections used for c-kit immunostaining.

Ovarian Morphology	Age (yr)	Primordial	Primary	Secondary	Antral	Corpus Albicans
Non-PCO	30-41	30	23	0	5	4
PCO	29-45	372	174	4	14	2

3.3 RESULTS

3.3.1 KITL mRNA levels in human preovulatory granulosa cells

Levels of total *KITL* mRNA were significantly higher in cumulus cells compared to mural granulosa cells ($P<0.05$) (Figure 3.1A). *KITL* mRNA levels were compared in cumulus cells obtained from women with or without PCOS, and no difference was observed between groups (Figure 3.2B).

3.3.2 KITL protein isoforms in the human ovary throughout folliculogenesis

To examine KITL protein isoforms throughout follicle development, Western blot was performed on several ovarian cell types representing different stages of follicle development as well as ovarian cortex which contained several preantral follicles. The human granulosa tumour cell line, KGN, represents preantral follicles, and mural granulosa cells (MGC) and cumulus cells (CC) represent preovulatory follicles. KGN cell lysates showed two bands 36 kD and 33 kD in size, which correspond to KITL-1 and KITL-2 respectively. Lysates derived from primary tissue showed an additional, unknown 29 kD band (Figure 3.2A). Levels of KITL-2 appeared higher in KGN cells and cortex, while levels of KITL-1 appeared greater in MGC and CC lysates. To determine the specificity of each band, the KITL primary antibody was incubated overnight with its specific peptide produced from the sequence provided by the antibody's manufacturer (Cell Signalling Technology, Inc., Danvers, MA, USA; Auspep, West Melbourne, VIC, Australia). The unknown 29 kD band was abolished along with the two KITL isoform bands following peptide competition (Figure 3.2B), suggesting that it is also a specific

KITL band, albeit larger than the secreted form of KITL which is known to be approximately 18 kD in size. These results suggest that KITL-2 is the predominant isoform expressed during early stages of follicle development, while KITL-1 is predominantly expressed in large antral or preovulatory follicles.

3.3.3 c-kit protein and mRNA isoforms in the human ovary

To examine whether *GNNK c-kit* mRNA isoforms are present in the human ovary, PCR was performed on snap frozen ovarian cortex from women between 40-50 years of age using a specific primer pair designed to show each isoform (Caruana *et al.* 1999). cDNA from the human leukaemic cell line, K562, was used as a positive control (Xie *et al.* 2010). Figure 3.3A shows the presence of two bands in cortex from three different patients as well as K562 cells, which correspond to *GNNK*+ (93 bp) and *GNNK*− (81 bp) isoforms. *GNNK*− levels appear to be higher than GNNK+ in all samples. Collectively, these results indicate that cells within human ovarian cortical tissue expresses both mRNA isoforms of *c-kit*.

To determine whether human ovarian cortex and the granulosa tumour cell line, KGN, express c-kit protein, ovarian tissue and KGN cell lysates were examined by Western blot. A human leukaemic cell lysate, CCRF-HSB-2, and a human breast tissue lysate were used as positive controls. Ovary and breast tissues contained a single 200 kD band, while KGN cells did not show a band of any size (Figure 3.3B). CCRF-HSB-2 showed a 200 kD and 150 kD band as expected, with each band proposed by previous studies to be glycosylated and unglycosylated c-kit respectively (Reith *et al.* 1991; Koch *et al.* 2009).

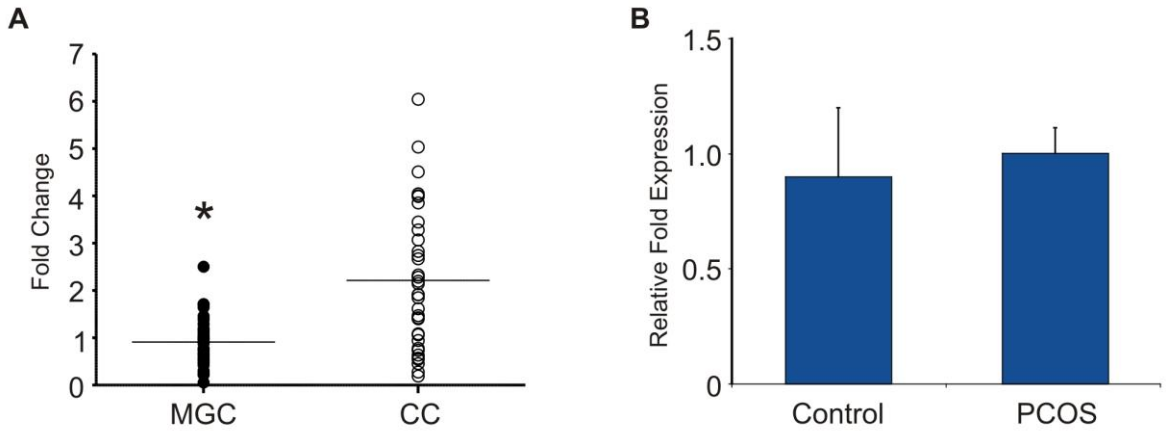


Figure 3.1: Comparison of *KITL* steady-state mRNA levels in mural granulosa and cumulus cells from non-PCOS patients, and in cumulus cells from patients with or without PCOS. (A) *KITL* mRNA levels are significantly lower in MGC compared to CC from individual patients (* P<0.05) (n = 33). (B) *KITL* mRNA levels are not different in cumulus cells obtained from women with or without PCOS. Error bars represent the standard deviation of the mean for: Control (n=11); PCOS (n=6). qPCR data was normalised to reference gene *RPL19*.

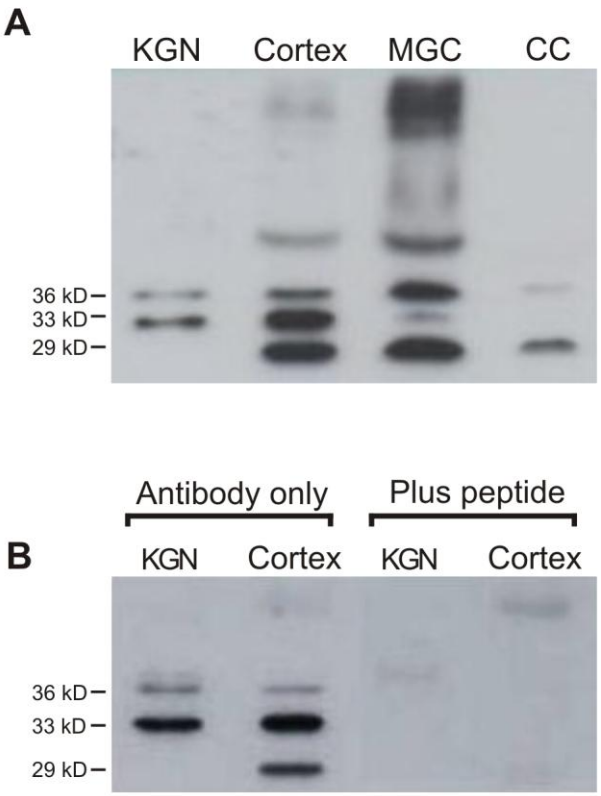


Figure 3.2: Western blot of KITL protein isoforms in human ovarian tissues that represent different stages of follicle development and comparative levels of *KITL* mRNA in granulosa cells. (A) KGN cells represent preantral stages; mural granulosa and cells (MGC) and cumulus cells (CC) represent the pre-ovulatory stage. Ovarian cortex contained some preantral follicles. Specific bands for KITL-1 (36kD) and KITL-2 (33kD) are present in all samples. An unknown 29kD band appears in the primary tissues but is absent in KGN cells. (B) Overnight incubation of the primary KITL antibody with its specific peptide shows specificity for 2 bands in KGN cells (36kD and 33kD) and 3 bands in human cortex (36kD, 33kD and 29kD).

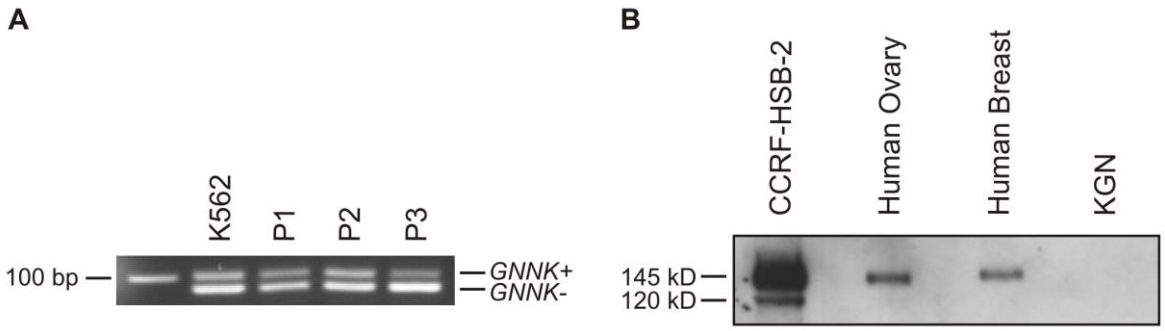


Figure 3.3: The presence of *c-kit* mRNA isoforms and protein in human cortex. (A) c-kit isoforms, *GNNK+* and *GNNK−*, were both present in ovarian cortex from three premenopausal women. Human leukaemia cell line K562 was used as a positive control and contains both isoforms. (B) Western blot depicting a single 145 kD band in human ovary and breast. c-kit is absent in KGN cells. CCRF-HSB-2 was used as a positive control and contains a smaller 150kD c-kit band.

These results suggest that the antibody is specific to c-kit, and protein visualised by immunohistochemistry may be primarily glycosylated.

3.3.4 c-kit immunostaining in gastrointestinal stromal tumours

Human GISTs were used for antibody optimisation of immunohistochemistry and as a positive control for the c-kit polyclonal antibody, based on studies assessing different c-kit antibodies for use in diagnostic immunohistochemistry techniques for GIST (Wong and Melegh 2009; Novelli *et al.* 2010). In this chapter, diffuse cytoplasmic immunostaining was present in all 3 GISTs in addition to sporadic, moderate membrane staining on some cells, as shown by representative images in Figure 3.4 (A, B) using the rabbit CD117 antibody (Dako). Staining patterns matched those presented in previous studies for use in GIST diagnosis. Negative control consisted of omission of the primary antibody, which did not show any immunostaining (Figure 3.4C).

Human ovaries were then stained with c-kit to identify KITL target cells throughout human follicle development, and also to determine whether levels of c-kit are different in PCO compared to non-PCO as has been previously shown for KITL (Tuck *et al.* 2007; Tuck *et al.* 2010a; Tuck *et al.* 2010b). c-kit immunostaining was present on the oolemma of 78% non-PCO primordial follicles and 69% primary follicles (Figure 3.5) (Table 3), and also present within the oocyte of the majority of follicles with or without staining of the oolemma. Some follicles also showed diffuse cytoplasmic staining in all granulosa cells surrounding the oocyte, including 31% of primordial follicles and 33% of primary follicles (Table 3). Figure 3.5 shows representative images depicting the variation of staining within

the cohort, with some follicles within individual ovaries showing less intense, diffuse staining (left-hand panels A and C), and others showing much more intense immunostaining (right-hand panels B and D). Some diffuse cytoplasmic staining was present in stromal cells surrounding the follicles. No secondary follicles were found within the non-PCO cohort.

c-kit immunostaining intensity did not appear to be visually different in preantral follicles of PCO compared to non-PCO (Figure 3.6). However, a higher percentage of preantral follicles had staining present on the oolemma than non-PCO preantral follicles, including 94% of primordial follicles and 96% of primary follicles (Table 3). Oolemma staining was present in 100% of secondary follicles. All preantral follicles were observed to have staining within the oocyte. A lower percentage of preantral follicles were observed to have diffuse cytoplasmic staining in granulosa cells compared to non-PCO, and included 11% of primordial follicles, 8% of primary follicles and 75% of secondary follicles (Table 3). Figure 3 depicts the variation in staining intensity within individual ovaries of the PCO cohort, from less intense (left-hand panels A, C, E) to much stronger immunostaining (right-hand panels B, D, F). Some diffuse cytoplasmic staining was present in stromal cells surrounding the follicles. Collectively, these results suggest that KITL signalling may occur via c-kit in human preantral oocytes and granulosa cells and surrounding stroma.

Non-PCO antral follicles showed diffuse, weak cytoplasmic staining within granulosa cells and the thecal layer (Figure 3.7A, B). No membrane staining was evident in either cell

type. The cells in the basal lamina region were negative in all antral follicles present. There were no oocytes present in any of the antral follicles for examination. Corpus albicans or adjacent stroma did not show any c-kit immunostaining (Figure 3.7C, D).

c-kit immunostaining did not appear visually different in PCO antral follicles compared to non-PCO (Figure 3.8). Diffuse, cytoplasmic staining was present in granulosa cells and the theca layer, and oocytes and their oolemma contained positive staining (Figure 3.8A-D). Figure 3.8 shows images of a small (A, B) and large (C, D) antral follicle within an individual PCO, showing variations in staining intensity between different follicles. Corpus albicans or adjacent stroma did not show any c-kit immunostaining (Figure 3.8E-F). These results suggest that c-kit protein levels are normal in PCO antral follicles, and that granulosa cell, theca cells and the oocyte are targets for KITL signalling.

Immunostaining was also examined in atretic follicles from the PCO cohort, as the ovaries contained a larger number of atretic follicles at various stages of atresia than the non-PCO cohort. A follicle in the early stages of atresia with an intact granulosa layer, basal lamina and theca layer showed diffuse cytoplasmic c-kit staining in the granulosa and theca cells (Figure 3.9A). Cells in the basal lamina appeared negative. In a follicle with a degrading granulosa layer, c-kit staining remained present in the cytoplasm of apoptotic granulosa cells and theca cells (Figure 3.9B). An absence of staining in the basal lamina was observed. c-kit immunostaining was present in theca cells and the few remaining granulosa cells of a follicle which lacked distinction between the areas of remaining granulosa cells,

basal lamina and theca layer (Figure 3.9C). A follicle in the final stages of atresia, lacking a granulosa layer, basal lamina and defined theca layer, appeared mostly negative with some faint cytoplasmic staining in remaining theca cells (Figure 3.9D). Collectively, these results suggest that the KITL/c-kit system may still have some role in granulosa and theca cells of atretic follicles, or remains persistent, until each cell type has undergone apoptosis and disappeared.

3.3.5 c-kit immunostaining in other ovarian structures

The presence of c-kit protein was examined in other structures present in the ovary, including areas of stroma, germinal inclusion cysts and ovarian epithelium. Regions of stroma within the cortex, some within the vicinity of follicles, showed distinct cytoplasmic staining that was absent in the medulla (Figure 3.10A, B). The epithelial layer of germinal inclusion cysts contained cytoplasmic staining in all cells (Figure 3.10C, D), and intact ovarian epithelial cells showed either very faint or absent cytoplasmic staining (Figure 3.10E). These results suggest that KITL/c-kit signalling may be important in dense stromal regions adjacent to developing follicles. KITL signalling may play a role in the development or function of germinal inclusion cysts.

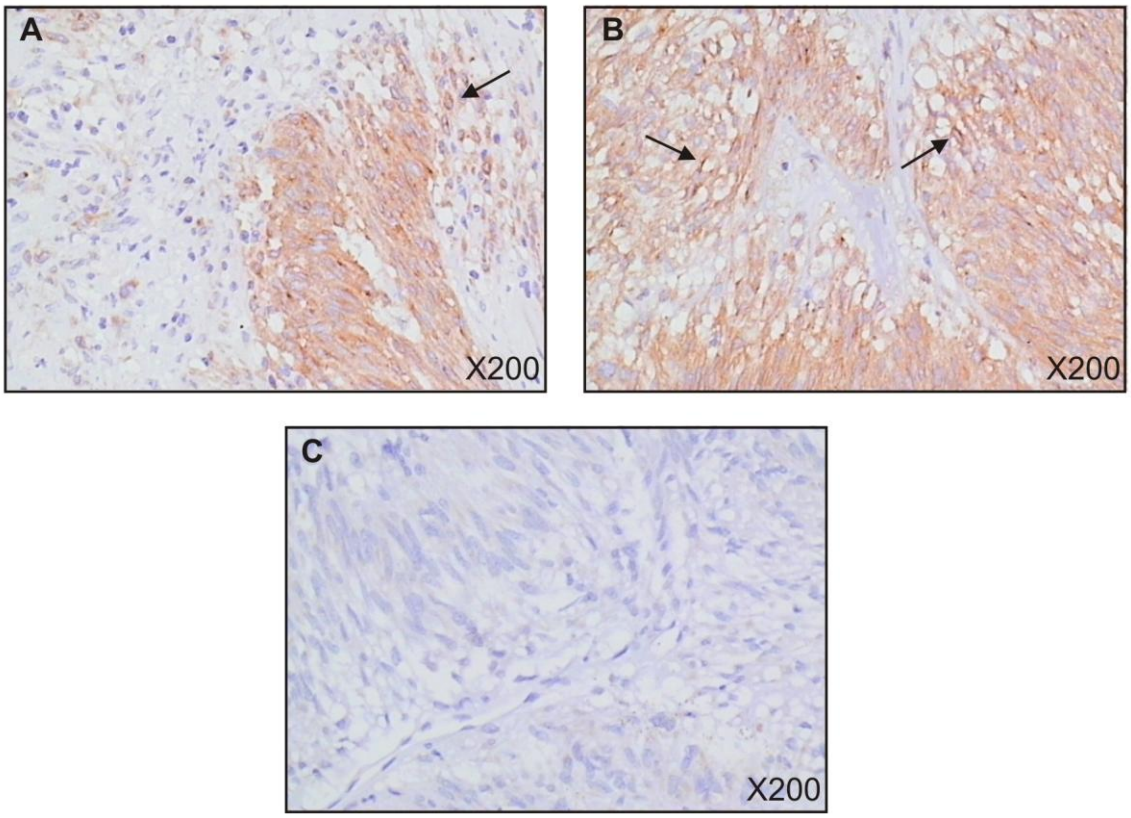


Figure 3.4: c-kit immunostaining in human gastrointestinal stromal tumour (GIST) used as a positive control. (A, B) Cytoplasmic and membrane staining present in distinct areas. (C) Negative consisted of omission of the primary antibody. Arrows indicate cells with membrane staining.

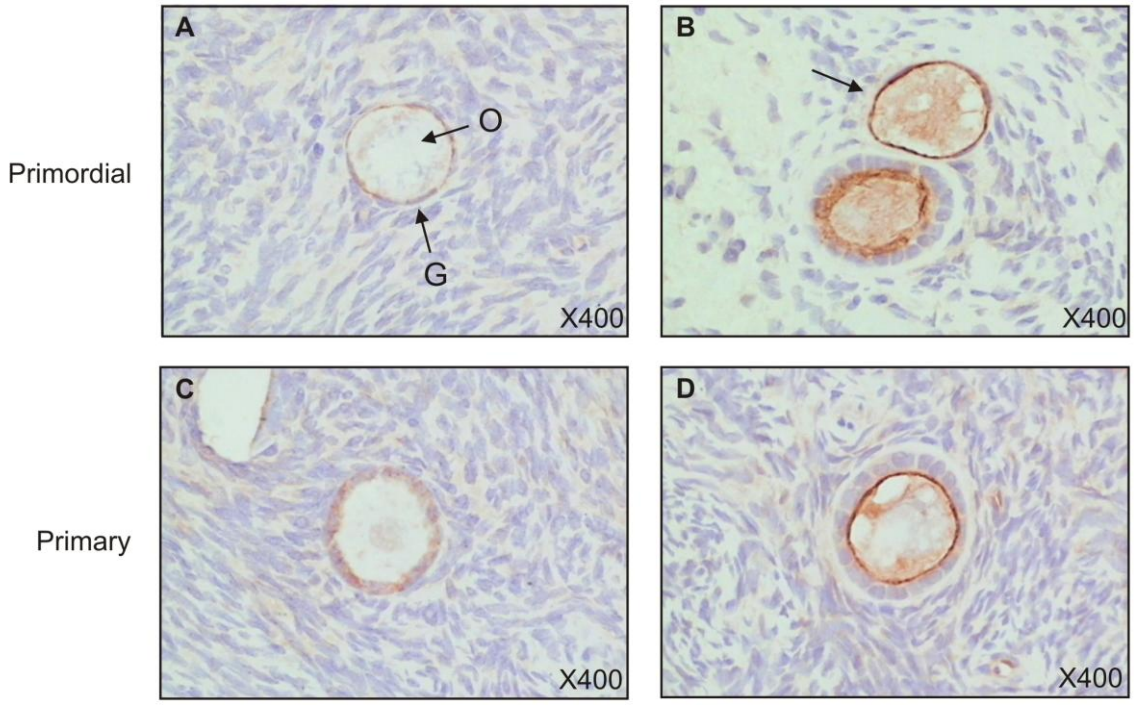


Figure 3.5: c-kit immunostaining is present in granulosa cells and on the oolemma of non-PCO preantral follicles. Representative images show the range of staining intensities for each follicle type including primordial (A, B) and primary (C, D). O: oocyte; G: granulosa cells.

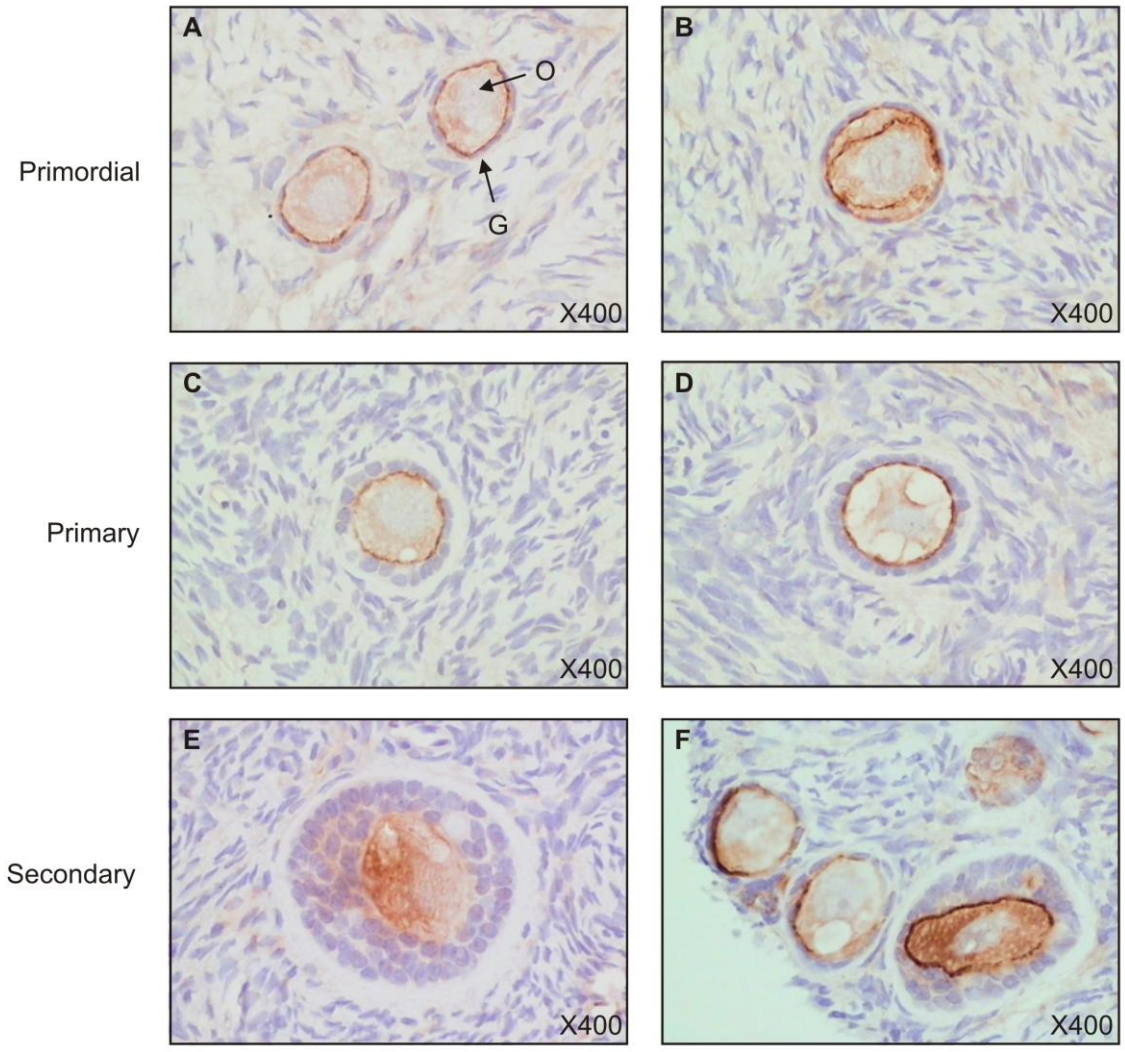


Figure 3.6: c-kit immunostaining is present in granulosa cells and on the oolemma of PCO preantral follicles. Representative images show the range of staining intensities for each follicle type including primordial (A, B), primary (C, D) and secondary (E, F). O: oocyte; G: granulosa cells.

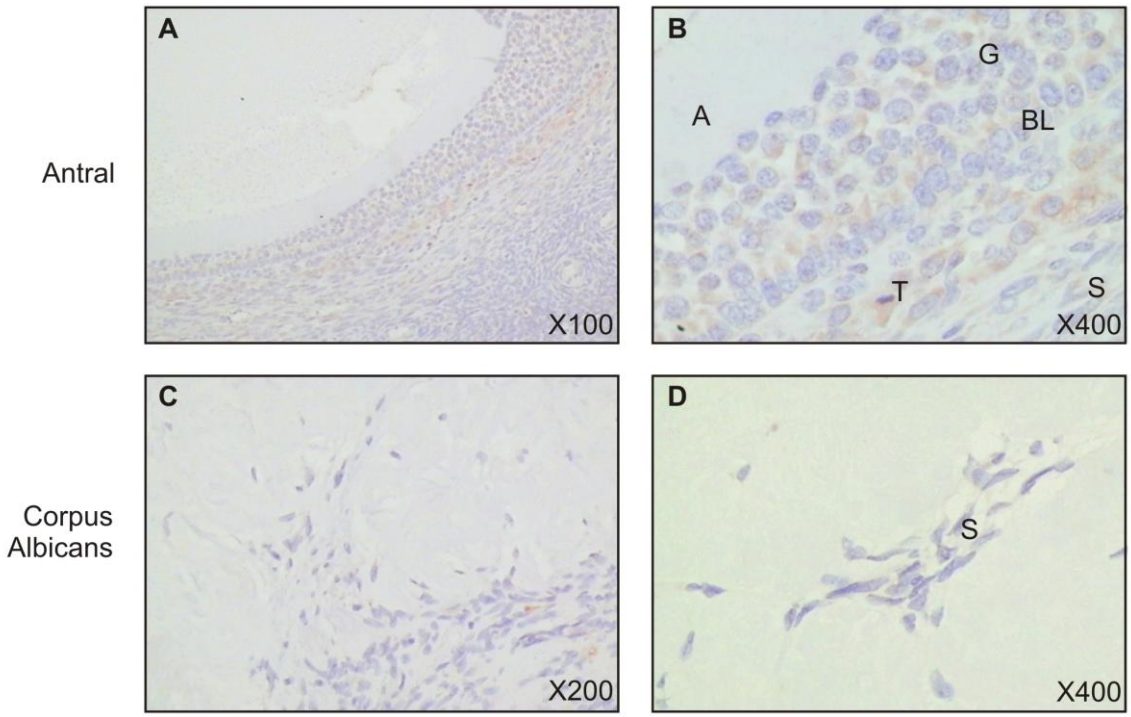


Figure 3.7: c-kit immunostaining is present in granulosa cells and the theca layer of antral follicles of non-PCO, and is absent in corpus albicans. Representative images at different magnifications of an antral follicle (A, B) and a corpus albicans (C, D). A: antrum; G: granulosa cells; BL: basal lamina; T: theca layer; S: stroma.

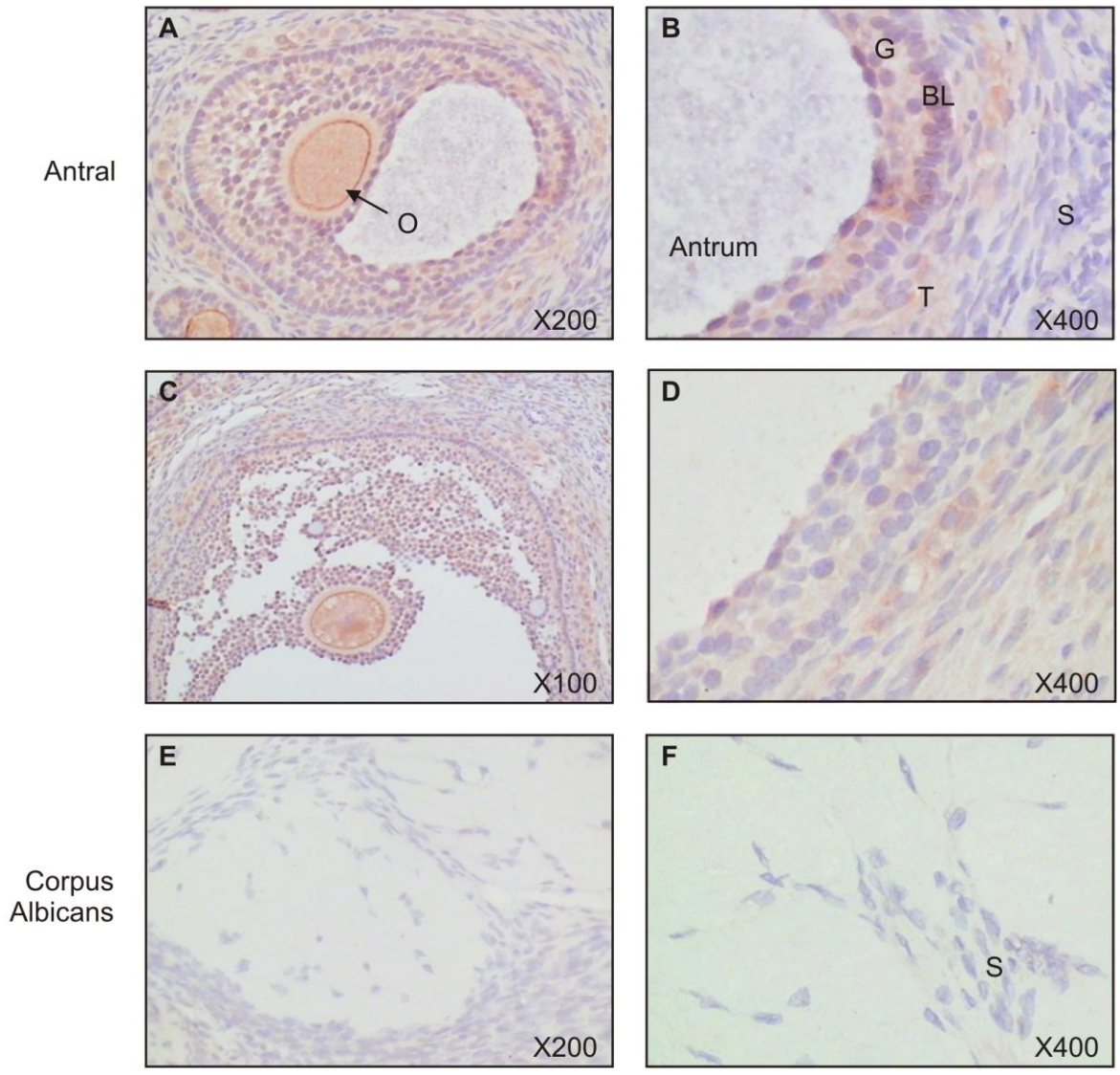


Figure 3.8: PCO antral follicles express c-kit in the granulosa and theca layer. Representative images of a small antral follicle containing an oocyte (A, B) and a large antral follicle also containing an oocyte (C, D). Staining not present in corpus albicans, as shown in representative images at different magnifications (E, F). A: antrum; G: granulosa cells; BL: basal lamina; T: theca layer; S: stroma.

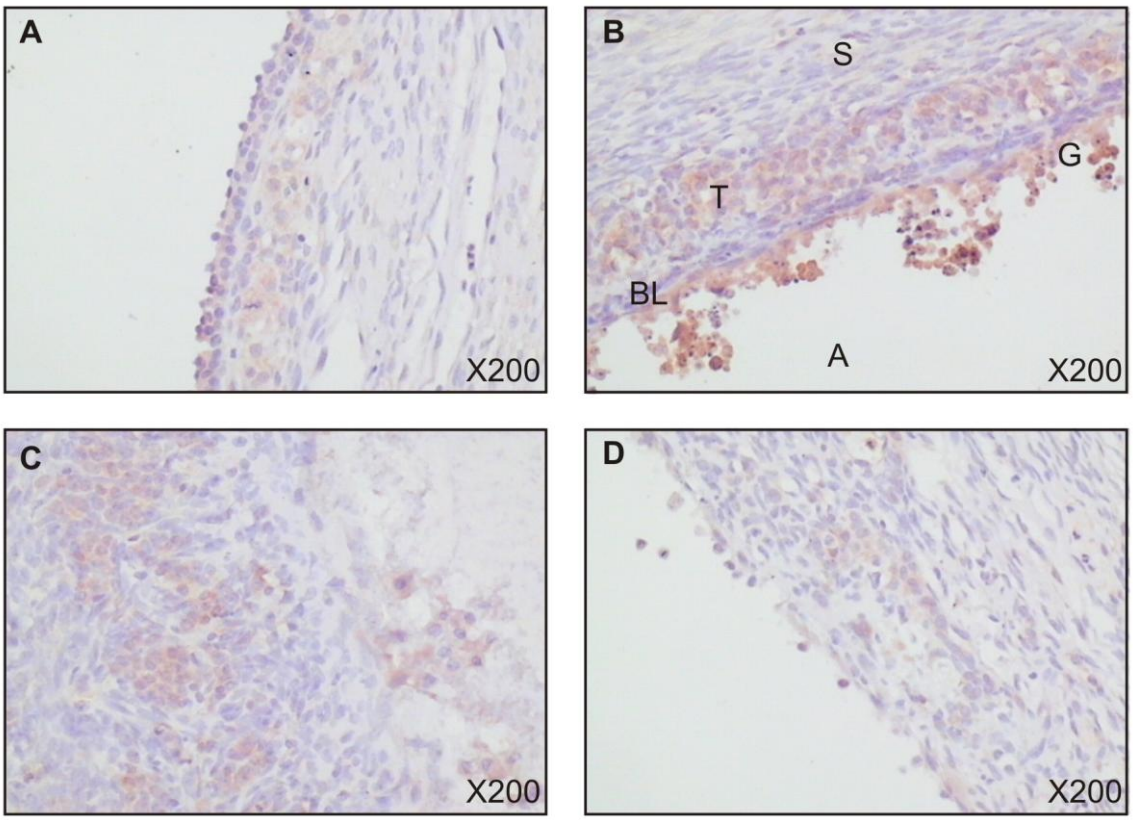


Figure 3.9: c-kit immunostaining is present in granulosa and theca cells of follicles in various stages of atresia. (A) Intact granulosa layer, theca layer and basal lamina. (B) Disassociating granulosa cell layer undergoing apoptosis. (C) Atretic follicle with little remaining of the granulosa layer and no distinction between the basal lamina and theca layer. (D) Granulosa layer, basal lamina and theca layer have almost completely degraded. A: antrum; G: granulosa cells; BL: basal lamina; T: theca layer; S: stroma.

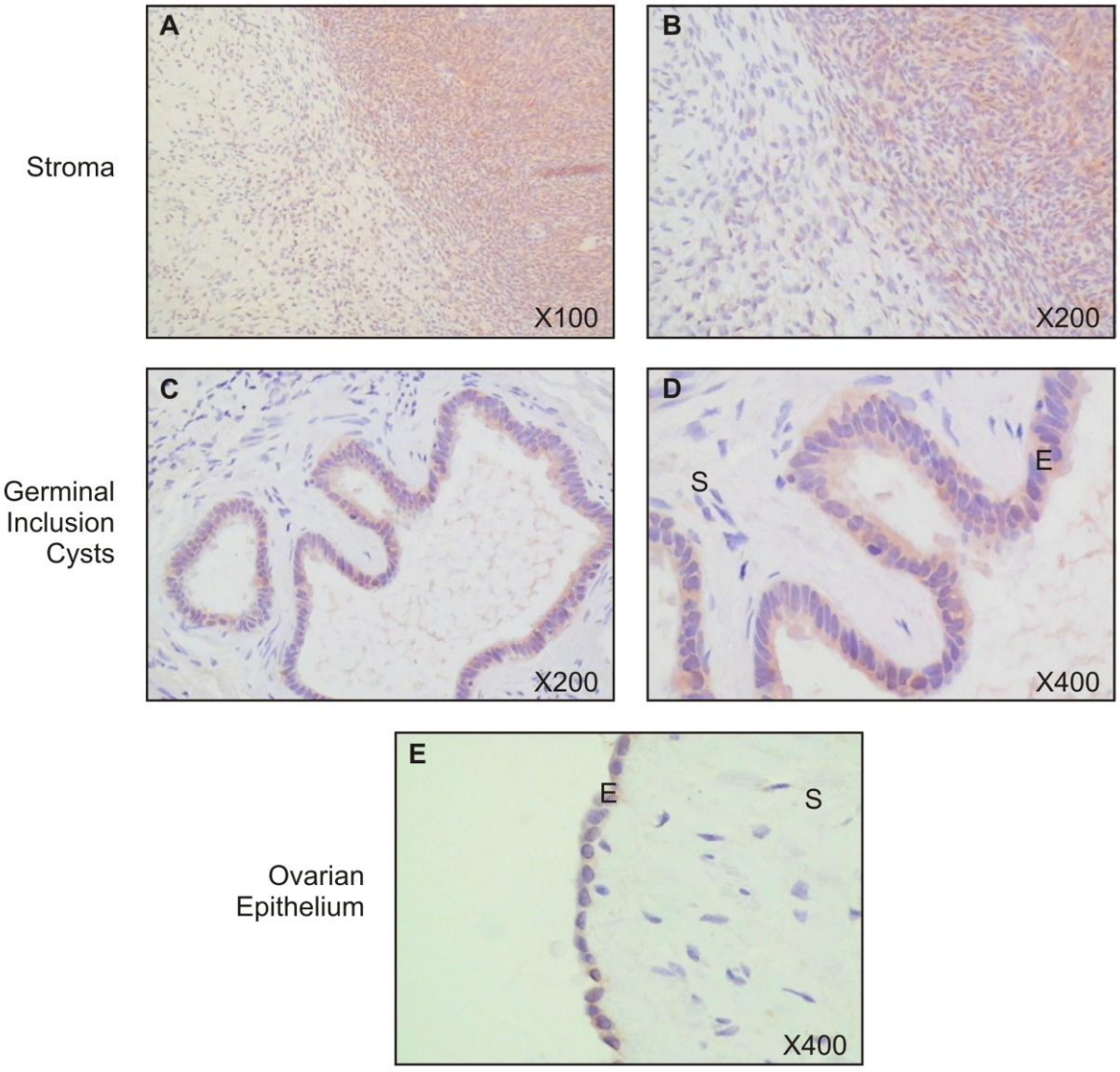


Figure 3.10: c-kit immunostaining is present in the stroma of the cortex region, germinal inclusion cysts and ovarian epithelium. Distinct areas of staining were present in stroma, shown at different magnifications (A, B). Epithelial cells of germinal inclusion cysts showed diffuse, cytoplasmic staining and is depicted at different magnifications (C, D). Ovarian epithelium contained very faint cytoplasmic staining (E). E: epithelium; S: stroma.

Table 3. Total percentage of preantral follicles with c-kit staining in the oolemma or granulosa cells (GC).

		Primordial	Primary	Secondary
Non-PCO	Oolemma	78%	69%	N/A
	GC	31%	33%	N/A
PCO	Oolemma	94%	96%	94%
	GC	11%	8%	75%

3.4 DISCUSSION

The results presented in this chapter suggest that KITL-2 is the predominant isoform expressed by granulosa cells during early follicle development, while KITL-1 is primarily expressed by follicles in later stages of development. In addition, this chapter has shown that KITL human follicular target cells may include granulosa, theca and the oocyte, and that both *c-kit* mRNA isoforms are present in human ovarian tissues. Furthermore, levels of c-kit protein visually appeared no different in PCO compared to non-PCO.

This study was able to obtain fresh, adult human ovarian tissue, and thus was able to be the first to examine *c-kit* mRNA isoform expression in the human ovary. Both *GNNK+* and *GNNK−* mRNA isoforms were found to be present, but it remains unknown which cell types express each isoform since PCR was performed on RNA extracted from pieces of whole tissue. Due to the small differences between each isoform, consisting of the presence or absence of four amino acids, no antibody is currently available that can specifically detect each protein isoform for immunohistochemistry or show individual bands on a Western blot. PCR results suggest that the *GNNK−* isoform is expressed more abundantly than *GNNK+*. In NIH3T3 fibroblast cells, *GNNK−* causes faster receptor phosphorylation and internalisation, and more extensive activation of the MAPK pathway than *GNNK+* (Caruana *et al.* 1999). The significance of the two isoforms in the ovary is unknown, and further study examining *c-kit* isoforms in individual cell types is required, for example by isolating cell types via laser capture microdissection of snap frozen ovarian tissue.

C-kit protein was identified via western blot in human ovarian tissue and its size suggests post-translation modification of the protein. The estimated molecular weight for c-kit on Ensembl Human (http://asia.ensembl.org/Homo_sapiens/Transcript/ProteinSummary?db=core;g=ENSG00000157404;r=4:55524085-55606881;t=ENST00000288135) is 109 kD, indicating that c-kit protein is present in the human ovary has been modified. The exact nature and purpose of this remains unknown, including whether this modification has a unique function in the ovary.

This thesis characterised c-kit protein expression and localisation throughout adult human follicle development and revealed that unlike in the mouse, c-kit protein is expressed by granulosa cells at all stages of follicle development, suggesting an autocrine function for KITL in human granulosa cells. c-kit protein has also been reported to be weakly present in some granulosa cells of goat follicles (Silva *et al.* 2006), but the staining observed in this thesis was moderate and uniformly present throughout all granulosa cells of antral follicles and some preantral follicles. Studies examining human fetal ovaries have shown the presence of *c-kit* mRNA in pregranulosa cells of unformed follicles and granulosa cells of primordial follicles, in addition to some weak immunostaining of these cell types (Abir *et al.* 2004; Hoyer *et al.* 2005; Carlsson *et al.* 2006). Staining observed in this thesis suggests that c-kit expression may be increased or more ubiquitously expressed in granulosa cells of adult ovaries for the purposes of unknown autocrine mechanisms. The established roles of KITL in promoting oocyte growth and thecal growth, differentiation and survival in animal models (Parrott and Skinner 1997; Parrott and Skinner 1998b;

Parrott and Skinner 2000; Hutt *et al.* 2006; Thomas *et al.* 2008) may suggest similar functions are present in human granulosa cells. Interestingly, not all preantral follicles were found to express c-kit in their granulosa cells. Reasons for this observation are unknown, and one could speculate that KITL autocrine actions promote better follicle and oocyte development, conferring a selective advantage over follicles not expressing c-kit at early stages. Further study is required to elucidate the roles of KITL and c-kit in human granulosa cells and their impact on follicle development and viability.

c-kit protein was found to be present in the theca layer of all antral follicles, suggesting the actions of KITL on the theca layer remain conserved in the human ovary. In contrast, a study examining c-kit immunostaining in human adult ovaries did not show any c-kit protein in the theca layer (Carlsson *et al.* 2006). This is most likely due to the primary antibody used, ACK-2, which has been utilised primarily to inhibit c-kit in *in vivo* mechanistic studies rather than for immunohistochemistry. The antibody used in this thesis was carefully selected from a range of antibodies that were extensively tested, and has also been established in studies to be the most optimal and accurate antibody for use in diagnosis of GIST (Wong and Melegh 2009; Novelli *et al.* 2010). The sensitivity of the antibody, in addition to applying it to tissues overnight at 4°C instead of 30 minutes at room temperature, enabled thecal c-kit protein to be detected in this chapter. No blocking peptide was available, but the presence of a single band on Western blot indicates the antibody is specific. An essential role for KITL in thecal layer formation and function in bovine models (Parrott and Skinner 1997; Parrott and Skinner 1998b; Parrott and Skinner 2000) indicates a similar involvement is likely to be present in the human ovary.

Evidence suggests that KITL is a key factor involved in recruitment of undifferentiated stromal cells for formation of the theca layer. Several mutations in mice at the Steel locus, including Sl^{pan}, Sl^{con} and Sl^{t}, causes decreased mRNA expression and results in arrested follicular development at the time when surrounding stromal cells are being recruited to differentiate into thecal cells (Kuroda *et al.* 1988; Huang *et al.* 1993; Bedell *et al.* 1995). There is evidence that thecal cell function is disrupted by the Sl^{t} mutation (Kuroda *et al.* 1988), and a bovine study demonstrated that KITL can directly promote theca growth and differentiation (Parrott and Skinner 2000). A striking c-kit cytoplasmic staining pattern was observed in this thesis in dense areas of stroma usually adjacent to growing follicles and was clearly absent in sparser areas without follicles present. Cytoplasmic staining may be evidence of internalised c-kit, which occurs as part of receptor downregulation and degradation after activation by ligand (Miyazawa *et al.* 1995). This staining pattern may suggest that KITL also acts as a stromal recruitment factor in the human ovary promoting theca differentiation and growth. Abnormally elevated KITL levels in PCO could then result in thickened thecal layers caused by increased recruitment and stimulation of growth. c-kit expression in stroma may also suggest communication of granulosa cells with stroma via KITL, perhaps as part of the bidirectional paracrine signalling from surrounding stromal cells that supports healthy follicle development (Driancourt *et al.* 2000).

This is the first study to identify c-kit expression in atretic follicles and germinal inclusion cysts in either animals or humans. c-kit is expressed by granulosa and theca cells

throughout atresia until cell layers have undergone total degradation, similar to KITL as previously shown, and it is unclear what function the KITL/c-kit system would have in dying follicles. c-kit expression in the epithelial cells of germinal inclusion cysts suggests involvement of KITL in some aspect of their growth and development. Previously, strong KITL immunostaining was shown in these cysts indicating the presence of an autocrine mechanism (Tuck *et al.* 2007). Germinal inclusion cysts are believed to be formed by invaginations of the ovarian epithelial layer (Radisavljevic 1977), which we have shown to express KITL and some c-kit protein (Tuck *et al.* 2007; Tuck *et al.* 2010a; Tuck *et al.* 2010b). Whether KITL is involved in the formation and development of these cysts is unknown.

Previously, it was found that KITL protein levels were abnormally high in PCO compared to non-PCO as shown in Chapter 1 (1.3.6). This thesis did not observe any visual difference in the intensity of c-kit immunostaining; however, the percentage of preantral follicles with positive staining observed on the oolemma or in granulosa cells was found to be different. More PCO preantral follicles, including primordial and primary, had c-kit staining present on the oolemma than non-PCO preantral follicles. Studies show that KITL is an important mediator in oocyte growth in the mouse (Thomas and Vanderhyden 2006; Thomas *et al.* 2008), and the observation in this chapter suggests that more oocytes in PCO preantral follicles are targets for KITL signalling than non-PCO oocytes. Oocytes of preantral follicles in PCOS feature abnormally increased growth, and the results of this chapter may suggest that KITL is involved in the promotion of abnormal oocyte growth in PCO. Expression of c-kit by oocytes, granulosa cells, the theca layer and stroma support

the possibility that increased KITL levels may feasibly contribute to phenotypes such as enlarged oocytes, increased growing follicle numbers, hyperthecosis and excess androgen production. Interestingly, this chapter observed a much lower percentage of PCO preantral follicles with c-kit staining present within granulosa cells compared to non-PCO. Reasons for this are unclear, and it suggests that granulosa cells of the majority of preantral follicles in PCO are not targets for KITL signalling, and contain less internalised c-kit receptor than non-PCO. These observations, together with the previous finding of increased KITL protein levels (Tuck *et al.* 2007; Tuck *et al.* 2010a), collectively suggest that the KITL/c-kit system is potentially involved in the abnormalities of the PCOS ovary.

Interestingly, a comparison of *KITL* mRNA levels in cumulus cells from women with or without PCOS showed no difference between the two groups. This is in stark contrast to the more intense KITL immunostaining observed in all granulosa cells of PCO compared to non-PCO (Tuck *et al.* 2007; Tuck *et al.* 2010a; Tuck *et al.* 2010b). As cumulus cells were obtained from hyperstimulated ovaries, this may explain why no difference was observed. Hyperstimulation is known to affect abnormal levels of hormones and factors present in the PCOS ovary (Norman *et al.* 2007), and thus basal levels of KITL measured in cumulus cells obtained from PCOS patients in fertility clinics may not represent levels present *in vivo* prior to hyperstimulation. A comparison of *KITL* mRNA levels in mural granulosa cells and cumulus cells showed that cumulus cells contained higher levels and greater inter-patient variation. The significance of this is unclear, and it may suggest a greater role for KITL in communication with the oocyte in large antral follicles than functions involving the antrum or theca layer.

KITL is known to exist as two isoforms in most species except the chicken, which has six identified isoforms (Wang *et al.* 2007). This study showed the presence of an additional 29 kD KITL band in Western blot of primary tissue lysates, which peptide competition indicated to be specific. Soluble KITL, which is a cleavage product of membrane-bound KITL, is approximately 18 kD in size (Driancourt *et al.* 2000) indicating that the additional 29 kD band is too large to be a soluble product. Its presence exclusively in primary tissue, and not the KGN cell line, could suggest it is a cleavage product as other cell types may secret a factor that gives rise to an additional soluble protein. However, due to its size, it could possibly be a third KITL isoform, previously undiscovered in human ovaries. Interestingly, Ensembl Human (http://asia.ensembl.org/Homo_sapiens/Transcript/ProteinSummary?db=core;g=ENSG00000049130;r=12:88890359-88974238;t=ENST00000378535) includes a transcript of a third *KITL* splice variant which encodes a protein of similar size to one identified in this thesis, suggesting that this protein may be a third isoform. Future study would attempt to identify the band by liquid chromatography-electrospray ionisation ion-trap (LC-eSI-IT) mass spectrometry (MS), and may require immunoprecipitation of tissue lysates enabling purified KITL protein to be cut from a Coomassie-stained gel and identified without contamination of the many other proteins present that are of an approximate size to KITL. Currently, no firm conclusion can be made as to the identity of the unknown 29 kD band.

Cells representative of preantral follicles, KGN cells, showed higher levels of KITL-2, which suggests a greater role for KITL during early follicle development. KITL-2 is the essential isoform required for fertility and normal folliculogenesis (Brannan *et al.* 1992; Tajima *et al.* 1998b), and is known to be required for mouse oocyte growth and survival in vitro (Thomas *et al.* 2008). Its anchorage in the cell membrane is believed to be responsible for its prolonged receptor activation compared to KITL-1, suggesting KITL-2 signalling may be more efficient and promotes long-term effects (Miyazawa *et al.* 1995). Binding of soluble KITL-1 to c-kit results in faster receptor internalisation and degradation, suggesting initiation of shorter-term, transient effects (Miyazawa *et al.* 1995). In this thesis, granulosa cells from preovulatory follicles showed higher levels of KITL-1. There is little evidence that KITL plays an important or essential role in large or preovulatory follicles, except one study which showed evidence that KITL may be involved in nuclear maturation of preovulatory oocytes by promoting first polar body extrusion (Ye *et al.* 2009). Other established roles for KITL occur during earlier follicle development (Driancourt *et al.* 2000), and the results shown in this thesis support the notion that KITL may be more important during early human folliculogenesis.

In summary, this chapter has shown evidence that the KITL/c-kit system is involved in human folliculogenesis by demonstrating the presence of the receptor throughout follicle development as well as KITL protein isoforms. Higher levels of KITL-2 in cells representative of early follicle development suggests that KITL has a greater role during early stages compared to antral or preovulatory follicles, which expressed higher levels of KITL-1. Demonstration of an additional KITL protein in primary tissue may suggest the

presence of a third isoform in the human ovary, or production of a cleaved protein which is absent in KGN cells. c-kit protein localisation in granulosa cells suggests there is unique, unknown autocrine function for KITL in the human ovary. Finally, c-kit expression patterns in the oolemma and granulosa cells of PCO preantral follicles were found to be different to non-PCO antral follicles, further supporting a potential role for KITL and c-kit in PCOS ovarian phenotypes such as abnormal oocyte growth. Chapters 4 and 5 explore regulation of *KITL* gene expression in human granulosa cells.

CHAPTER 4

Investigation of Direct Androgen Receptor-Mediated Regulation of Kit Ligand Gene Expression in Human Granulosa Cells

Chapter 4: Investigation of Direct Androgen Receptor-Mediated Regulation of Kit Ligand Gene Expression in Human Granulosa Cells

4.1 INTRODUCTION

One of the major objectives of this thesis was to investigate regulation of *KITL* gene expression in human granulosa cells by ovarian-secreted factors. This chapter focuses on androgens which are of interest due to the identification of *KITL* as a candidate androgen receptor (AR) regulated gene (Shiina *et al.* 2006), and their high circulating levels in women with PCOS (Norman *et al.* 2007).

KITL has been well-characterised in mice and shown to stimulate follicle activation, growth, and survival (Driancourt *et al.* 2000; Thomas and Vanderhyden 2006). Abnormally increased KITL levels in PCO could explain several of the associated phenotypic abnormalities of this disorder. PCO is a diagnostic feature of women with polycystic ovary syndrome (PCOS) and other less common disorders of female androgen excess. Compared to normal ovaries, PCO are enlarged and have an increased follicle density, particularly at preantral stages of development (Norman *et al.* 2007). Several mechanisms have been postulated to explain the increase in developing follicle numbers in PCO, including increased primordial follicle activation, increased numbers of growing versus non-growing primary follicles and increased capacity for follicles to survive (Franks *et al.* 2008). The number of activated primordial follicles and the growth and survival of

the resulting preantral follicles within mammalian ovaries is mainly coordinated by interplay between inhibitory and stimulatory intra-ovarian regulatory molecules, and KITL is one such stimulatory factor that is involved in all these processes.

Transcriptional regulation of *KITL* in human granulosa cells has so far been shown to be influenced by follicle stimulating hormone (FSH), human chorionic gonadotropin (hCG) and activin A (Laitinen *et al.* 1995; Coutts *et al.* 2008). AR has also been identified as a candidate transcriptional regulator of *KITL*, where a study showed that *AR* null mouse ovaries and WT ovaries of mice treated with an anti-androgen had reduced expression of *KITL* mRNA (Shiina *et al.* 2006). Furthermore, the mouse and human *KITL* promoter was shown to be stimulated by androgen treatment via *in vitro* luciferase assays (Shiina *et al.* 2006). This was of interest as we have previously shown abnormally increased KITL protein levels in human polycystic ovaries (PCO) (section 1.3.6). The strong clinical association between female serum androgen levels and the incidence and severity of PCO suggests a primary role for androgens in disrupting the normal processes that regulate folliculogenesis. The mechanism through which androgens may disrupt follicle development is unknown, and there are currently no characterised AR regulated genes in the human ovary. Therefore, this chapter investigated whether *KITL* is an AR-regulated gene in human granulosa cells, including human cumulus cells and the human granulosa tumour cell line KGN.

4.2 MATERIALS AND METHODS

4.2.1 Cell Culture

KGN cells were maintained in DMEM/Ham's F12 medium (Sigma, St Louis, MO, USA) supplemented with 10% fetal bovine serum (FBS) in an atmosphere of 37°C with 5% CO_2, as described in Chapter 2 (section 2.3.4). For all treatments, cells were seeded in DMEM/Ham's F12 medium containing 10% DCC-FBS overnight. For experiments examining protein levels, medium was changed to fresh medium prior to treatment. Human cumulus cells were chosen as several studies have demonstrated higher expression of AR mRNA and protein in human (Koks *et al.* 2010; Grondahl *et al.* 2012) and rat (Szoltys *et al.* 2010) cumulus cells compared to mural granulosa cells in individual follicles. Cumulus cells were obtained from women undergoing assisted reproductive technology at Flinders Medical Centre following informed consent, and maintained in Opti-MEM medium (Gibco, Carlsbad, CA, USA) supplemented with 2% DCC-FBS in an atmosphere of 37°C with 5% CO_2 as described in Chapter 2 (section 2.3.4). Total time in culture, including treatments, did not exceed 30 hours to limit their time in separation from the oocyte and to avoid possible luteinisation.

ZR-75-1 human breast cancer cell line was maintained in RPMI-1640 medium (Sigma) supplemented with 10% FBS in an atmosphere of 37°C with 5% CO_2. Cells were seeded in RPMI-1640 phenol-red-free medium supplemented with 10% DCC-FBS.

4.2.2 DHT treatments

DHT concentrated stock was prepared as described in Chapter 2 (section 2.3.5). Concentrated stock was diluted in culture medium as required for treatments, and absolute ethanol was diluted in the same manner for vehicle control.

4.2.3 RNA extraction and generation of cDNA

RNA was extracted from KGN cells using an RNeasy Mini Kit (Qiagen, Hilden, Germany), and extracted from cumulus cells using an RNeasy Micro Kit (Qiagen) as described in Chapter 2 (section 2.3.7), and stored at -80°C. Quantitation of RNA is described in Chapter 2 (section 2.3.7), and cDNA was generated from 100 and 500 ng of RNA from cumulus cells and KGN cells respectively as described in General Methods using an iScript cDNA Synthesis Kit (Biorad, Hercules, CA, USA) containing oligo (dT) and random primers.

4.2.4 Quantitative Real-Time PCR

Quantitative real-time PCR (qRT-PCR) reactions for total *KITL* levels, *Ar* levels and reference gene *RPL19* were performed using 2x iQ SYBR Green Supermix as outlined by the manufacturer (Biorad). Primers for *KITL* and *RPL19* were commercially obtained (Qiagen) and diluted as per the manufacturer's instructions using Nuclease-Free Water. Primers for *AR* were taken from (Bieche *et al.* 2001) and were purchased from Geneworks (Adelaide, SA, Australia). Sequences of *AR* primers are as follows: CCTGGCTTCCGCAACTTACAC (forward); GGACTTGTGCATGCGGTACTC (reverse). Target cDNA was amplified on an iQ5 iCycler (Biorad) using the following PCR conditions: 1 cycle at 95°C for 3 min, 40 cycles at 95°C for 15 sec, 60°C for 15 sec,

72°C for 30 sec, 95°C for 1 min, 55°C for 1 min. Melt curve analysis occurred at 60°C for 10 sec.

Quantitative real-time PCR reactions for *KITL-1*, *KITL-2*, *PTX3* and reference gene *RPL19* were performed using Taqman Gene Expression Assays (Applied Biosystems, Boston, MA, USA) as described in Chapter 2 (section 2.3.8). The sequence for the custom-made *KITL-2* assay is as follows: forward primer, TGAGAAAGGGAAGGCCAAAA; reverse primer, AGAAAACAATGCTGGCAATGC; probe, ACTCCAGCCTACACTGG.

4.2.5 Western Blot

Western blotting to specifically detect KITL isoforms, AR or tubulin was performed on KGN cell lysates and ZR-75-1 cell lystates as described in Chapter 2 (section 2.3.9). The specific AR N-20 rabbit polyclonal antibody (Santra Cruz Biotechnology, Santa Cruz, CA, USA) was diluted to a concentration of 1:1000 in TBST containing 1% skim milk powder and incubated with the membrane at 4°C overnight.

4.2.6 Immunocytochemistry

Immunocytochemistry to detect AR protein in cells cultured in 8-well Lab Tek II Chamber Slides was performed as described in Chapter 2 (section 2.3.11). AR N-20 rabbit polyclonal antibody was diluted to a concentration of 1:1000 in phosphate-buffered saline (PBS) and applied to paraformaldehyde-fixed cells overnight at 4°C.

4.2.7 Analysis of follicular fluid hormones

Follicle fluid was removed from follicular aspirates that were centrifuged at 1500 *g* for 10 minutes, and stored at -80°C. Total testosterone levels were measured by isotope dilution-liquid chromatography-tandem mass spectrometry (SA Pathology, Flinders Medical Centre, South Australia), which has a sensitivity of 0.5 nmol/liter, with an intraassay coefficient of 4.8% and interassay coefficient of less than 10%. Free androgen index (FAI) was calculated as testosterone/sex hormone binding globulin (SHBG) X 100, where SHBG levels were measured by Radio Immunoassay (RIA) kit (Orion Diagnostics, Espoo, Finland). The assay was performed according to the manufacturer's protocol, and had an intraassay coefficient of variation of 4.0% and interassay coefficient of 5.5%. Laboratory work was performed by Lisa Akison and Brenton Bennett in the Research Centre for Reproductive Health (Adelaide) (Robker *et al.* 2009).

4.2.8 Correlation of total KITL mRNA in MGC and CC to follicular fluid measures

Firstly, qPCR data was analysed as described in Chapter 2 (section 2.3.8). The calibrator was an arbitrary sample that was run on every plate, and all data was expressed as fold change relative to that calibrator. Correlation between follicular fluid measures and mRNA data was then performed using GraphPad Prism 5 for Windows (GraphPad Software, Inc.).

4.2.9 Analysis of soluble KITL levels in conditioned media

Conditioned media was centrifuged at 1500 *g* for 10 minutes, and stored at -80°C. Soluble KITL levels were measured using Enzyme Linked Immunosorbent Assay (ELISA) (R&D Systems, Minneapolis, MN, USA), as outlined by the manufacturer. Briefly, standards and samples were added to the plate containing a monoclonal KITL antibody and any unbound

substances were washed away. An enzyme-linked polyclonal antibody specific for KITL was added, followed by a wash and addition of substrate solution. Colour development, which occurs in proportion to the amount of KITL bound to the monoclonal antibody, was performed and after stopping the reaction colour intensity was measured at 540nm on an EL808 Ultra Microplate Reader (Bio-Tek Instruments, Winooski, VT, USA).

4.2.10 Statistical analysis

GraphPad Prism 5 for Windows (GraphPad Software, Inc.) statistical software was used to analyse all data. Statistical differences were determined by one-way ANOVA with Tukey's post-hoc test, and a p-value of <0.05 was considered statistically significant.

4.3 RESULTS

4.3.1 AR expression and localisation in response to DHT treatment in human cumulus cells

To begin to determine the role of androgens in regulating *KITL* expression in the human ovary, *AR* mRNA levels in human cumulus cells were examined. The response of *AR* to DHT treatment was measured by qPCR and as expected, treatment with either 1 or 10 nM DHT had no effect on mRNA levels compared to control (Figure 4.1A). The localisation of AR protein within cumulus cells and its responsiveness to DHT was measured by immunocytochemistry. Cells were either cultured overnight in steroid-depleted conditions prior to treatment, or cultured for 4 days to allow any remaining nuclear ligand-bound AR to return to the cytoplasm. Surprisingly, cells displayed constitutive nuclear localisation for AR after overnight and 4 day culture, no apparent change was observed after treatment with 10 nM DHT for 24 hours (Figure 4.1).

4.3.2 Effect of DHT treatment on *KITL* mRNA in human cumulus cells

To determine whether AR directly affects *KITL* gene expression, cumulus cells were treated with 1 or 10 nM DHT. Treatment for 6 hours had no effect on *KITL-1* and *KITL-2* mRNA expression (Fig. 4.2A). A comparison between CC obtained from women with and without PCO showed no difference in total *KITL* levels either before or after 1nM DHT treatment for 6 hours (Fig. 4.2B). These results suggest that AR does not directly regulate *KITL* gene expression in cumulus cells from preovulatory follicles, and *KITL* mRNA levels

in cells obtained from women with PCO does not respond differently to DHT treatment compared to non-PCO.

4.3.3 Comparison of human granulosa cell *KITL* mRNA levels and androgenic follicular fluid measures

This chapter then investigated whether there was a correlation between *KITL* mRNA levels in preovulatory granulosa cells and androgenic profiles in follicular fluid from the follicles from which they were derived. No significant correlations were found between total mRNA levels of *KITL* and follicular fluid levels of total testosterone or the free androgen index (FAI) (Fig. 4.3).

4.3.4 AR protein levels and localisation in response to DHT treatment in KGN cells

The role of androgens in regulating *KITL* gene expression in the ovary was then investigated using another model of human granulosa cells, the KGN cell line. AR protein levels were firstly examined by Western blot, and cells treated with 10 nM had increased stabilisation of AR protein levels compared to control (Fig. 4.4A). AR localisation within KGN cells and its responsiveness to DHT was then examined by immunocytochemistry. KGN cells displayed diffuse cytoplasmic and nuclear immunoreactivity for AR under both overnight and 4 day steroid-depleted conditions, and treatment with 10 nM DHT for 24 hours resulted in increased in nuclear staining intensity in both instances (Fig. 4.4). This is consistent with stabilisation and increased nuclear localisation of AR in the presence of ligand.

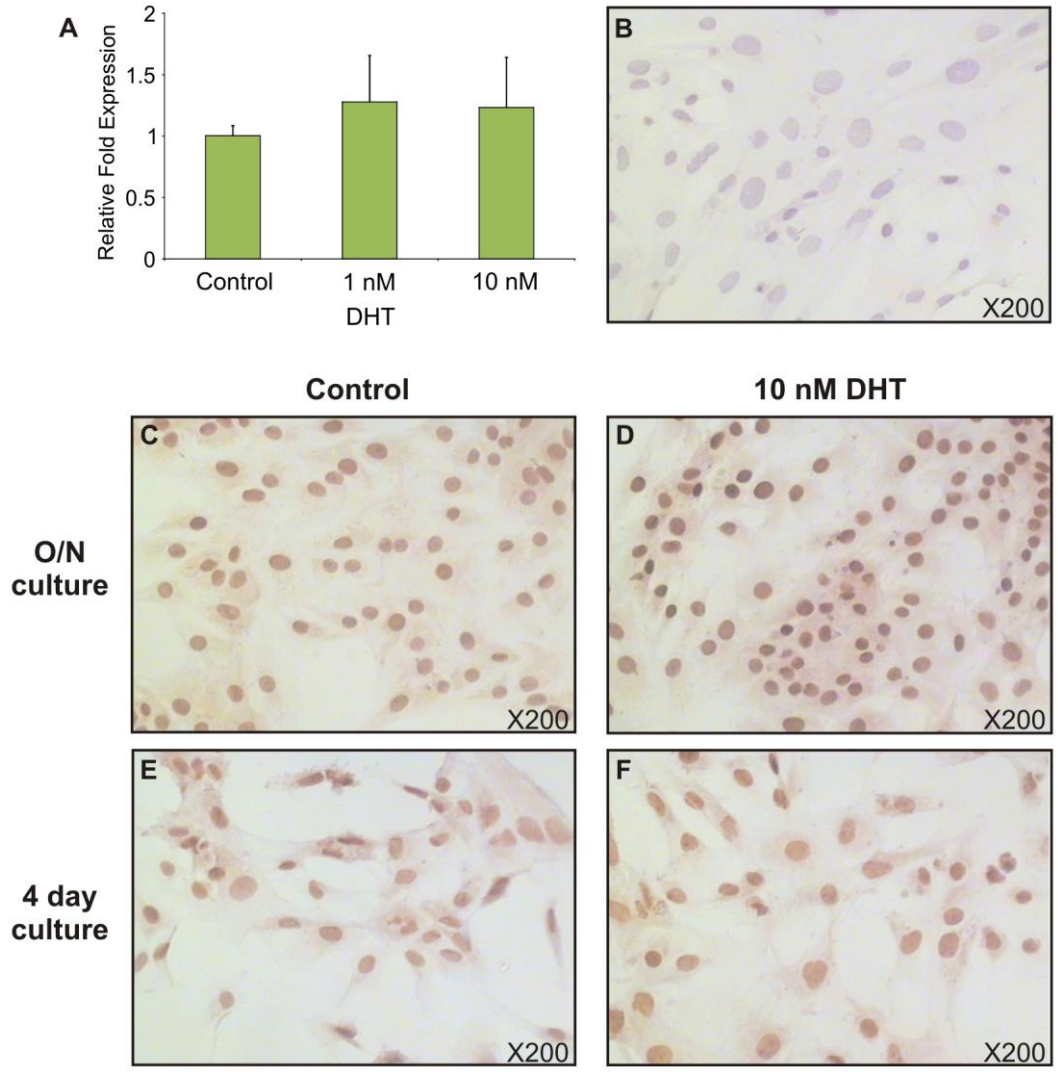


Figure 4.1: AR expression and localisation in human cumulus cells. (A) *AR* steady-state mRNA levels in cells were treated with DHT for 6 hours. (B) Cells cultured and treated with 10 nM DHT used as a negative control for immunocytochemistry, which consisted of omission of the primary antibody. Cells were cultured overnight (C, D) or for 4 days (E, F) in steroid-depleted conditions prior to treatment with 10 nM DHT for 24 hours. Images shown are representative of 3 independent experiments.

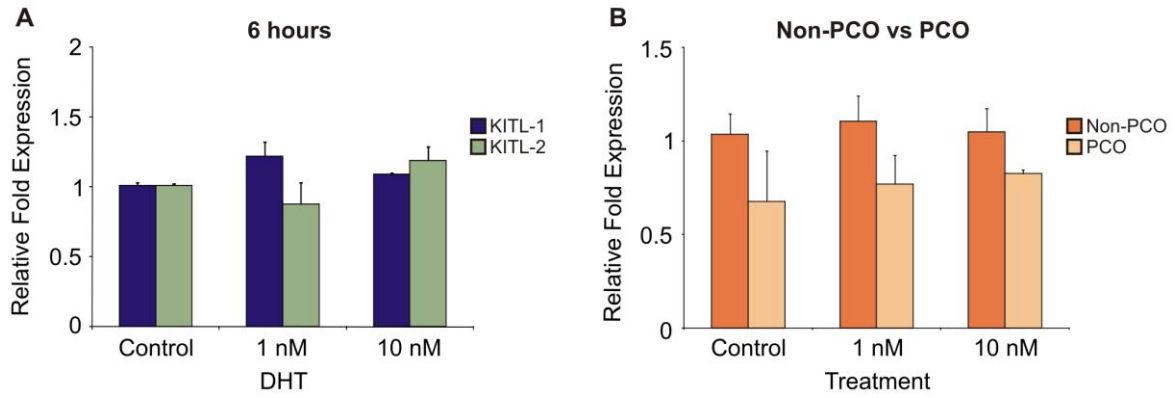


Figure 4.2: *KITL* steady-state mRNA levels do not change in human cumulus cells exposed to DHT. Cells were treated with DHT for 6 hours (A). Levels of *KITL* were compared in CC from women with or without PCO (B). qPCR data was normalised to reference gene *RPL19*. Error bars represent the standard deviation of the mean for 3 replicate experiments in (A). Error bars depicted in (B) represent the standard deviation of the mean for: non-PCO, n=10; PCO, n=5.

4.3.5 Effect of DHT treatment on KITL mRNA and protein levels in KGN cells

To determine if nuclear translocated AR increases *KITL* expression, KGN cells were treated with 1 or 10nM DHT. Treatment for 6 or 24 hours did not alter mRNA levels of *KITL-1* or *KITL-2* (Fig. 4.5). KITL protein levels were also unaffected by exposure of KGN cells to DHT for 24 or 48 hours, although as shown in Figure 4, androgen treatment increased steady-state levels of AR protein (Fig. 4.6A). Treatment with AR antagonists, bicalutamide and hydroxyflutamide, alone or in combination with DHT had no effect on KITL-1 or KITL-2 protein levels after 24 or 48 hours (Fig. 4.6A). As expected, bicalutamide and hydroxyflutamide had no effect on levels of AR protein (Fig. 4.6A). Levels of KITL protein isoforms and total KITL were quantified by densitometry which confirmed there was no change with treatment (Fig. 4.6 B, C).

A 24 hour dose-response was then performed with 1, 10, 100 or 1000 nM DHT alone or in combination with 1 or 10 µM bicalutamide for 24 hours and KITL protein levels were once again found to be unchanged by treatment, (Fig. 4.7A) and which was confirmed by densitometry (Fig. 4.7B). *KITL-2* gene expression in breast cancer cell line, ZR-75-1, has been demonstrated in this laboratory to be regulated by AR (Ochnik 2011) and was used in this thesis as a positive control for DHT treatment. Figure 4.8C shows increased KITL-2 expression after 10 nM treatment at 48 hours compared to control and 1 nM DHT treatment, which did not appear different to control. This data indicates that DHT treatment was effective in regulating KITL expression in a cell line previously used to examine AR

regulation, and supports the results in this chapter showing no effect of DHT treatment on KITL mRNA or protein levels in KGN or cumulus cells.

Soluble KITL protein levels in conditioned media were also found to be unaffected by 1 nM DHT treatment alone or in combination with 1 μM bicalutamide for 48 hours, but treatment with 10nM DHT caused a significant decrease in KITL levels ($P<0.05$) (Fig. 4.8A). No difference was found in KITL levels at 72 hours after treatment with 1 or 10 nM DHT alone or in combination with 1 μM bicalutamide (Fig. 4.8B).

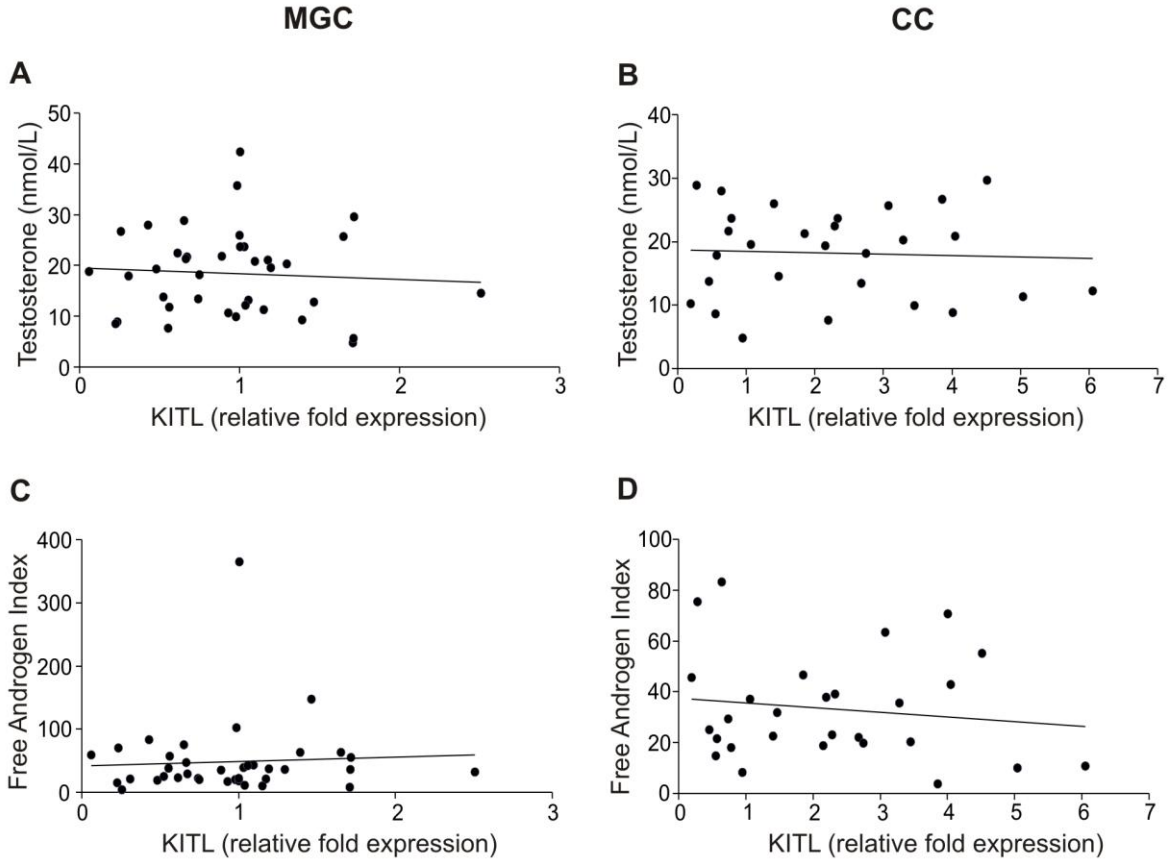


Figure 4.3: Comparison of *KITL* mRNA expression in mural granulosa cells (MGC) and cumulus cells (CC) from preovulatory human follicles to androgen content of the follicular fluid from which they were derived. Total testosterone was measured in follicular fluid by isotope dilution-liquid chromatography-tandem mass spectrometry and correlated to total *KITL* mRNA levels in MGC (A) and CC (B) from matching follicles (r = 0.0046 for MGC; r = 0.0022 for CC). Free androgen index (FAI) was calculated as testosterone/sex hormone binding globulin (SHBG) (measured by Radio Immunoassay or RIA) X 100, and correlated to total *KITL* mRNA levels (r = 0.0195 for MGC; r = 0.0037 for CC).

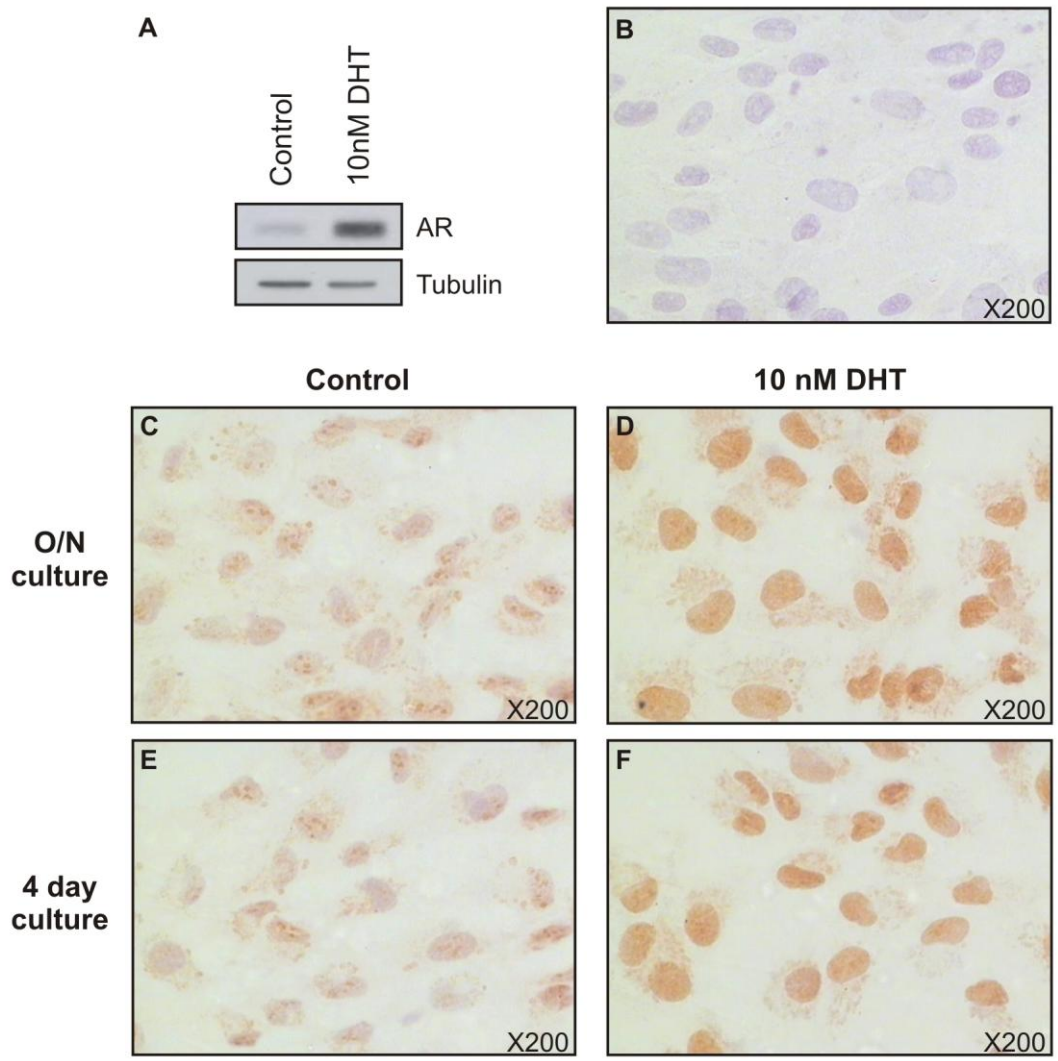


Figure 4.4: AR levels and localisation in KGN cells. (A) Steady-state AR protein levels in cells treated with or without 10 nM DHT. (B) Cells cultured and treated with 10 nM DHT used as a negative control for immunocytochemistry, which consisted of omission of the primary antibody. Cells were cultured overnight (C, D) or for 4 days (E, F) in steroid-depleted conditions prior to treatment with 10 nM DHT for 24 hours. Images shown are representative of 3 independent experiments.

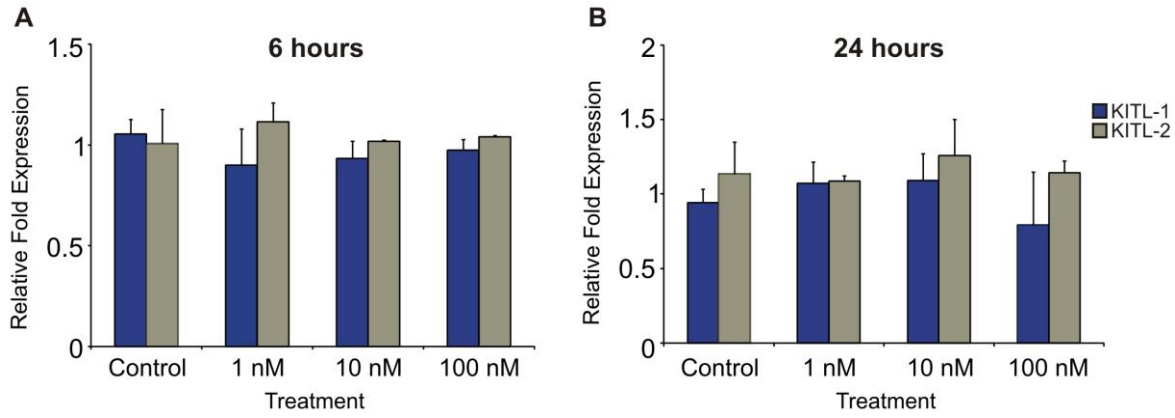


Figure 4.5: *KITL* steady-state mRNA levels do not change in KGN cells exposed to DHT. Treatments were performed for 6 (A) or 24 (B) hours. qPCR data was normalised to reference gene *RPL19*. Error bars represent the standard deviation of the mean for 3 replicate experiments.

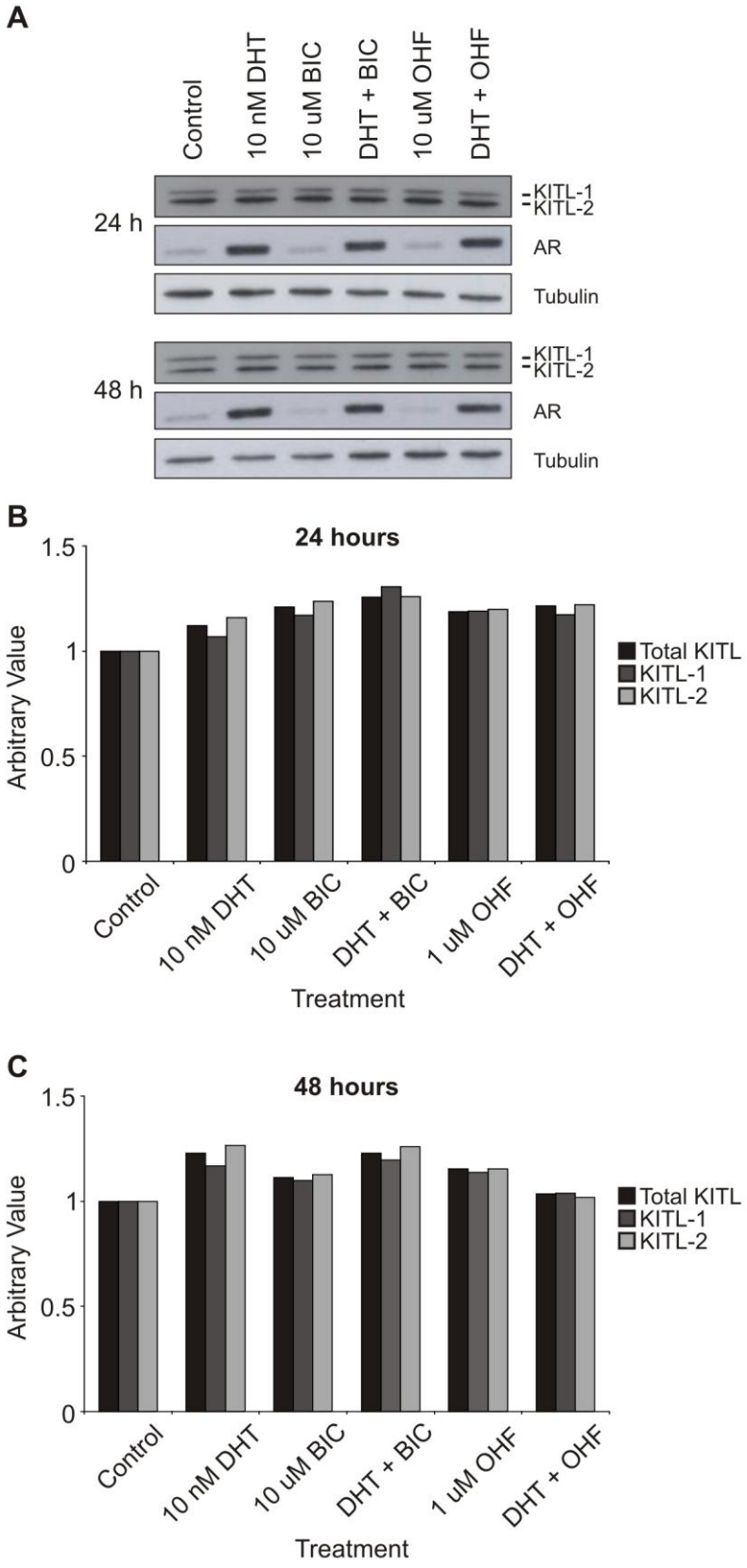

A
Control
10 nM DHT
10 uM BIC
DHT + BIC
10 uM OHF
DHT + OHF
24 h
KITL-1
KITL-2
AR
Tubulin
48 h
KITL-1
KITL-2
AR
Tubulin
B
24 hours
Arbitrary Value
1.5
1
0.5
0
Total KITL
KITL-1
KITL-2
Control
10 nM DHT
10 uM BIC
DHT + BIC
1 uM OHF
DHT + OHF
Treatment
C
48 hours
Arbitrary Value
1.5
1
0.5
0
Total KITL
KITL-1
KITL-2
Control
10 nM DHT
10 uM BIC
DHT + BIC
1 uM OHF
DHT + OHF
Treatment

Figure 4.6: Expression of KITL and AR protein levels in KGN cells exposed to DHT, bicalutamide (BIC) or hydroxyflutamide (OHF). (A) Western blots depicting KITL-1, KITL-2 and AR at 24 or 48 hours. Tubulin was used as a loading control. (B, C) Densitometry performed on KITL isoforms represented in (A), normalised for tubulin.

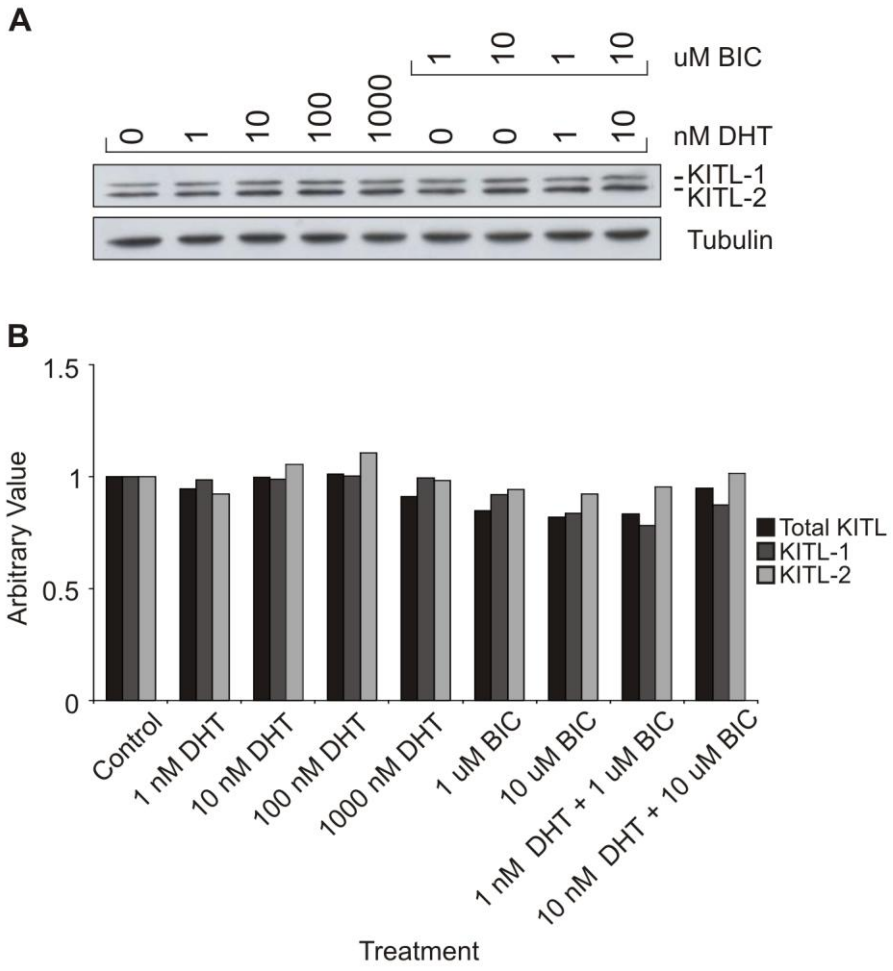

A
uM BIC
1
10
1
10
nM DHT
0
1
10
100
1000
0
0
1
10
KITL-1
KITL-2
Tubulin
B
Arbitrary Value
1.5
1
0.5
0
Total KITL
KITL-1
KITL-2
Control
1 nM DHT
10 nM DHT
100 nM DHT
1000 nM DHT
1 uM BIC
10 uM BIC
1 nM DHT + 1 uM BIC
10 nM DHT + 10 uM BIC
Treatment
C
0
1
10
nM DHT
KITL-2
Tubulin

Figure 4.7: Expression of KITL protein levels in KGN cells exposed to various concentrations of DHT and bicalutamide (BIC). (A) Western blots depicting KITL-1 and KITL-2 at 24 hours. Tubulin was used as a loading control. (B) Densitometry performed on KITL isoforms represented in (A), normalised for tubulin. (C) ZR-75-1 breast cancer cell line was used as a positive control for DHT treatment and has increased KITL-2 levels after 48 hours of DHT treatment. Tubulin was used as a loading control.

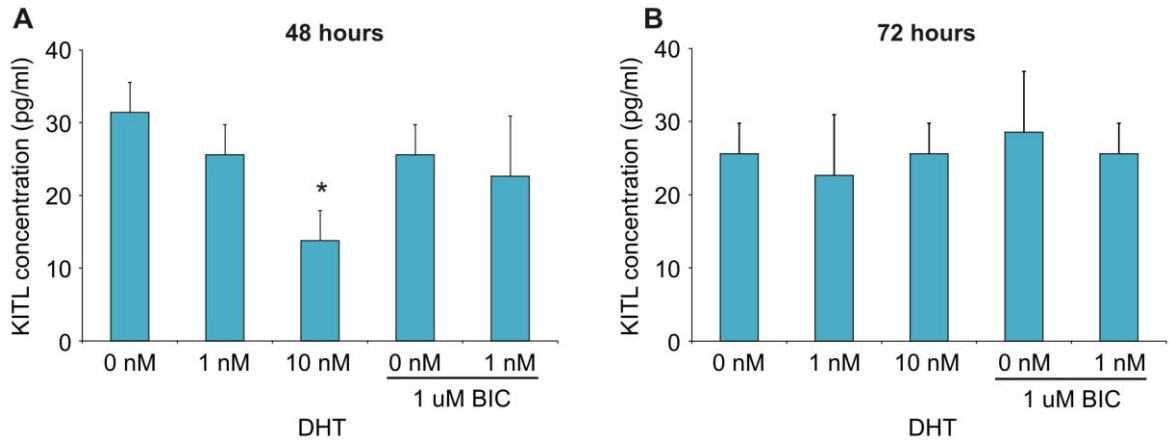


Figure 4.8: Expression of soluble KITL protein levels in conditioned media from KGN cells. DHT and bicalutamide (BIC) treatments were performed for 48 (A) and 72 (B) hours. * denotes group is significantly different to 0 nM DHT ($P<0.05$, One-Way ANOVA). Error bars represent the standard deviation of the mean for 3 replicate experiments.

4.4 DISCUSSION

KITL has previously been identified as a candidate AR-regulated gene in the ovary, as Shiina et al. (Shiina *et al.* 2006) showed that treatment with DHT could activate a reporter gene construct containing a human or mouse *KITL* promoter, inserted into the pGL3-basic vector, in transfected KGN cells. However, we have found no evidence for an effect of DHT on endogenous steady state *KITL* mRNA or protein levels in KGN cells or in cultured primary human CC, suggesting that *KITL* is not directly regulated by androgen in the human ovary. This finding was further supported by the observation that levels of *KITL* mRNA in pre-ovulatory CC or MGC did not correlate with androgenic follicular fluid measurements. The exception was the observation that soluble KITL levels were significantly lower after treatment with 10 nM DHT at 48 hours, which was the only change observed in this chapter after treatment with androgen after extensive investigation. This may suggest that this result may not be indicative of a true effect whereby DHT regulates soluble KITL levels, and would require further investigation in future study.

The results presented in this chapter may be a more accurate representation of transcription occurring in granulosa cells *in vivo*. Shiina *et al.* demonstrated an effect using a transfected reported gene construct (Shiina *et al.* 2006), which is a non-chromatinised DNA template and thus is highly artificial and may not recapitulate transcription in the native chromatin context. *In vivo*, transcription always occurs in the context of chromatin which is structurally complex and affects the initiation and completion of transcription (Lorch *et al.* 1992; Li *et al.* 2007; Oduro *et al.* 2008). Chromatin structure is tightly regulated by several

mechanisms such as histone modification and chromatin remodeling, where promoter sites can be exposed or sequestered by nucleosomes. Transcription of a transfected promoter construct, such as that used by Shiina *et al.*, occurs completely differently from transcription in the native chromatin context which is maintained in the model used in this study.

The working models used in this chapter have limitations which may not enable *KITL* regulation by androgens to be observed. Both models are maintained in culture without the presence of an oocyte, which communicates with granulosa cells in the ovary via paracrine signalling for the mutual mediation of growth and maturation. Granulosa cells cultured in isolation will be limited in their function without the influence of the oocyte. Futhermore, KGN cells are a granulosa tumour cell line, and thus will have some significant differences to granulosa cells *in vivo*. Certain signalling pathways and expression of genes may be modified or non-functional in these cells, which may affect how *KITL* gene expression is regulated by certain factors. Additionally, KGN cells are representative of undifferentiated granulosa cells at early stages of follicle development, limiting observations to a narrow period of follicle development. Cumulus cells on the other hand are representative of terminally differentiated granulosa cells in preovulatory follicles that have undergone cumulus expansion in preparation for ovulation. Gene expression at this stage is very different to earlier stages when the follicle is still growing and maturing, and *KITL* gene expression may be less profound. Additionally, cumulus cells are obtained from hyperstimulated ovaries which may not reflect basal gene expression levels in unstimulated ovaries, thus confounding treatment effects. The results presented in this chapter may not

recapitulate the effects androgens may have on *KITL* gene expression in granulosa cells *in vivo* because of these limitations. Future study could utilise cultures of intact, isolated human ovarian follicles as an example of a method that may more accurately recapitulate *in vivo* signalling events.

It is possible that regulation of *KITL* by androgen signalling is indirect and the major influence is exerted through interaction with other regulatory factors, and this may result in the abnormal levels present in PCO (Chapter 1, section 1.3.6). For example, AR signalling increases many of the actions of FSH, including induction of genes such as aromatase (Weil *et al.* 1999). In prostate tissues, AR signalling regulates expression of *KGF* in stromal cells to influence growth of the prostate epithelium (Planz *et al.* 1998); a similar paracrine action may also apply to theca and granulosa cells of the human ovary. Finally, AR signalling has been shown to influence the actions of GDF-9 in porcine cumulus cells (Hickey *et al.* 2005), and testosterone was demonstrated to down-regulate *GDF-9* mRNA expression via its receptor in whole mouse ovaries treated with testosterone (Yang *et al.* 2010). Thus, androgens may interact with GDF-9 or other paracrine oocyte growth factors to regulate *KITL* in human granulosa cells.

In conclusion, this chapter found no evidence to support direct regulation of *KITL* gene expression in human GC. There is still the possibility that AR interacts with other factors which could contribute to increased KITL levels in PCO, and this is explored in Chapter 5.

CHAPTER 5

Investigation of Kit Ligand Gene Regulation in Human Granulosa Cells by Endocrine and Intraovarian Factors

Chapter 5: Investigation of Kit Ligand Gene Regulation in Human Granulosa Cells by Endocrine and Intraovarian Factors

5.1 INTRODUCTION

The findings presented in Chapter 4 of this thesis indicate that *KITL* gene expression is not directly regulated by AR signalling in human granulosa cells. Previous studies performed in animal models have shown evidence of *KITL* transcriptional regulation by several other factors, such as oocyte and theca-secreted proteins (Parrott and Skinner 1998b; Elvin *et al.* 1999b; Joyce *et al.* 1999; Otsuka and Shimasaki 2002). Therefore, the objective of this chapter was to examine regulation of *KITL* gene expression by intraovarian factors including oocyte-secreted factors and theca-derived factors, and pituitary-secreted factor follicle stimulating hormone (FSH). Additionally, indirect AR regulation via interaction with GDF-9 was also investigated.

Several factors have been shown to regulate *KITL* gene expression in granulosa cells, both *in vitro* and *in vivo*, such as pituitary-secreted FSH, granulosa-derived activin A, theca-derived keratinocyte growth factor (KGF), GDF-9 and oocyte-derived bone morphogenetic protein 15 (BMP-15) (Laitinen *et al.* 1995; Parrott and Skinner 1998b; Elvin *et al.* 1999b; Joyce *et al.* 1999; Joyce *et al.* 2000; Otsuka and Shimasaki 2002; Thomas *et al.* 2005; Coutts *et al.* 2008). All past studies, with the exception of those utilising FSH, have examined regulation of *KITL* gene expression in non-human models, and the results are yet

to be replicated in the human ovary. Evidence for regulation by these factors is detailed in the following sections.

5.1.1 Follicle Stimulating Hormone

Normal folliculogenesis is dependent on the endocrine signal FSH, a pituitary hormone that signals through its receptor (FSH-R) located on granulosa cells in ovarian follicles (Hillier 2001). The effect of FSH on follicular development is conveyed through regulation of intrafollicular paracrine factors, such as the promotion of preantral follicle differentiation via upregulation of IGF-1 and activin proteins (Adashi *et al.* 1991a; Miro and Hillier 1996). It has been shown that FSH upregulates *KITL* expression in mouse granulosa cells, and there is evidence suggesting this may promote oocyte growth and antral follicle formation (Yang *et al.* 2003). In support of this concept, mice null or haplo-insufficient for the FSH receptor gene (*FSH-R*) exhibited morphological ovarian defects including significantly smaller oocyte diameters, and arrested follicle development at the preantral stage (Yang *et al.* 2003). In addition, KITL protein levels were also decreased in preantral follicles in this study, further supporting a role for FSH regulating KITL actions in promoting occyte growth and folliculogenesis as demonstrated by several studies (Yoshida *et al.* 1997; Hutt *et al.* 2006; Thomas and Vanderhyden 2006; Thomas *et al.* 2008). Furthermore, increased levels of *KITL* mRNA have been observed in co-cultures of murine preantral granulosa cells and oocytes treated with FSH compared to untreated cells (Thomas *et al.* 2005), which was associated with increased oocyte growth (Thomas *et al.* 2005). No effect of FSH treatment was seen in cultured murine mural granulosa cells from

antral follicles, suggesting that FSH induction of KITL occurs only during earlier stages of follicle development.

Evidence indicates that the effect of FSH on *KITL* expression may be species-specific, as FSH treatment of cultured human mural granulosa cells resulted in a time- and dose-dependent decrease in *KITL* mRNA levels (Laitinen *et al.* 1995). No studies have been performed on cultured human preantral granulosa cells due to the difficulty in obtaining and isolating these cell types, making it hard to determine whether FSH may have different effects on *KITL* expression during different stages of follicle development in humans. The granulosa tumour cell line, KGN, is the only human granulosa cell line that expresses functional FSH receptors (Nishi *et al.* 2001). As a model of relatively undifferentiated granulosa cells, it is a valuable system in which to investigate the effect of FSH on *KITL* expression in human preantral granulosa cells.

5.1.2 Theca-Derived Keratinocyte Growth Factor

Cell-cell paracrine communication between theca and granulosa cells is essential for follicular development (Parrott and Skinner 1997; Parrott and Skinner 1998a; Parrott and Skinner 1998b; Kezele *et al.* 2005). Mesenchymal-derived theca cells secrete keratinocyte growth factor (KGF), also known as FGF7, a member of the fibroblast growth factor family (Parrott and Skinner 1998a). KGF signals via its tyrosine kinase receptor KGFR, otherwise known as FGFRII, thereby activating the mitogen-activated protein kinase

(MAPK) pathway to regulate granulosa cell growth (de Giorgi *et al.* 2007). KITL is an important mediator of thecal cell differentiation and proliferation, and communication between granulosa and thecal cells has been shown to involve a positive feedback loop between KGF and KITL in the bovine ovary (Parrott and Skinner 1998b). *KITL* mRNA expression has been shown to be stimulated by recombinant KGF in cultured bovine granulosa cells, and in turn, *KGF* expression was induced in cultured theca cells by recombinant KITL (Parrott and Skinner 1998b). To date, there have been no studies examining *KITL* regulation by KGF in the human ovary.

5.1.3 Oocyte-Secreted Factors

Bidirectional communication between the oocyte and surrounding granulosa cells is critical for ovarian follicle development and for normal oocyte growth and maturation (Kidder and Vanderhyden 2010). KITL has been established to be an essential granulosa-derived factor involved in paracrine signalling interactions with the oocyte (Yoshida *et al.* 1997; Elvin *et al.* 1999a; Elvin *et al.* 1999b; Joyce *et al.* 1999; Joyce *et al.* 2000; Otsuka and Shimasaki 2002; Thomas *et al.* 2005; Thomas and Vanderhyden 2006; Coutts *et al.* 2008; Thomas *et al.* 2008; Kidder and Vanderhyden 2010). Recent studies have provided strong evidence that transforming growth factor-β (TGFβ) superfamily members, including BMP-15 and GDF-9, are important factors mediating the paracrine effects of the oocyte on granulosa cells (Gilchrist *et al.* 2006; Mottershead *et al.* 2011).

BMP-15 is a member of the bone morphogenetic protein family, which forms a subset of the TGF-β superfamily (Dube *et al.* 1998; Laitinen *et al.* 1998). Signalling occurs through activation of the Smad 1/5/8 pathways leading to regulation of gene transcription, following binding of BMP-15 to its cell surface serine/threonine kinase receptors including the type II receptor BMPR2 and type I receptor ALK6, (Vitt *et al.* 2002; Moore *et al.* 2003). *BMP-15* null mice are subfertile and have decreased ovulation and fertilisation rates, suggesting that BMP-15 plays a role in normal folliculogenesis and fertility (Yan *et al.* 2001), while *BMP-15* null sheep were found to be completely infertile and follicle development was arrested at the primary stage (Galloway *et al.* 2000). In humans, *BMP-15* mutations are associated with premature ovarian failure (POF) and hypergonadotropic ovarian failure (HOF) (Di Pasquale *et al.* 2006; Dixit *et al.* 2006; Laissue *et al.* 2006). A negative feedback loop has been shown to exist between *BMP-15* and *KITL* in co-cultured rat oocytes and granulosa cells (Otsuka and Shimasaki 2002). Treatment with recombinant BMP-15 increased *KITL* mRNA expression in early antral granulosa cells, while treatment with recombinant KITL decreased *BMP-15* mRNA levels in oocytes (Otsuka and Shimasaki 2002). This effect has also been demonstrated in mouse oocyte-granulosa cell co-cultures (Thomas *et al.* 2005), but it remains to be shown whether these effects can be elicited in individual cultures of oocytes and granulosa cells without the interference of other paracrine factors.

GDF-9 is also a member of the TGF-β superfamily, and signals via the same type II receptor (BMPR2) as BMP-15, but a different type I TGF-β receptor (TβR1) or ALK5

located on the granulosa cell membrane, thereby activating the SMAD 2/3 pathway leading to regulation of gene transcription (McGrath *et al.* 1995; Vitt *et al.* 2002; Shimasaki *et al.* 2003; Mazerbourg *et al.* 2004). Similarly to BMP-15, *GDF-9* null mice and sheep showed arrested folliculogenesis at the primary follicle stage and were completely infertile (Dong *et al.* 1996; Hanrahan *et al.* 2004). Intriguingly in humans, lower levels of *GDF-9* mRNA have been reported in the oocytes of women with polycystic ovary syndrome (PCOS) (Teixeira Filho *et al.* 2002), and point mutations and deficient GDF-9 levels have also been found in women with premature ovarian failure (POF) (Dixit *et al.* 2005; Laissue *et al.* 2006; Kovanci *et al.* 2007) indicating that GDF-9 also plays an important role in human ovarian function.

GDF-9 negatively regulates *KITL* gene expression in mice. Mutant mice with decreased GDF-9 have increased *KITL-1* and *KITL-2* mRNA levels in granulosa cells of primary follicles, and the presence of enlarged oocytes compared to wildtype littermates is believed to be the result of elevated *KITL-2* levels (Elvin *et al.* 1999b; Thomas and Vanderhyden 2006). Additionally, treatment of mouse preantral granulosa cells with recombinant GDF-9 was shown to inhibit *KITL* mRNA expression (Joyce *et al.* 2000). Interestingly, Hickey *et al*. showed crosstalk exists between GDF-9 and AR signalling, where DHT enhanced the proliferative effect of GDF-9 on porcine granulosa cells (Hickey *et al.* 2005). In prostate cancer cells, AR has been demonstrated to interact with the SMAD 3 pathway (Chipuk *et al.* 2002; Kang *et al.* 2002). While this thesis found no evidence of a direct effect on KITL

gene expression by AR signalling, it remains to be determined whether an indirect effect may be elicited via crosstalk with GDF-9 signalling.

5.1.4 Chapter 5 Aims

The specific aims for this chapter were to determine if KGF or the oocyte-secreted-factors, BMP-15 and GDF-9, regulate *KITL* expression in human granulosa cells using the KGN cell line and human cumulus cells as working models. In addition, regulation by FSH alone, or in combination with OSFs, was examined and crosstalk between AR and GDF-9 signalling was investigated to determine whether indirect AR regulation of *KITL* gene expression may occur in this model of early human granulosa cells. Cumulus cells were not used to examine regulation by FSH treatment or GDF-9 and DHT combination treatment, due to their more limited availability and because they are a model of large preovulatory follicles in which animal models indicate *KITL* regulation is less profound.

5.2 MATERIALS AND METHODS

5.2.1 Cell Culture

KGN cells were maintained in DMEM/Ham's F12 medium (Sigma, St Louis, MO, USA) supplemented with 10% fetal bovine serum (FBS) in an atmosphere of 37°C with 5% CO_2, as described in Chapter 2 (section 2.3.4). For experiments, cells were seeded in DMEM/Ham's F12 medium containing 10% DCC-FBS overnight. For experiments examining protein levels, medium used for seeding was changed to fresh medium prior to treatment. Human cumulus cells were obtained from women undergoing assisted reproductive technology at Flinders Medical Centre following informed consent, and maintained in Opti-MEM medium (Gibco, Carlsbad, CA, USA) supplemented with 2% DCC-FBS in an atmosphere of 37°C with 5% CO_2 as described in Chapter 2 (section 2.3.4). Total time in culture, including treatments, did not exceed 30 hours to limit their time in separation from the oocyte and to avoid possible luteinisation.

5.2.2 Treatments

5.2.2.1 KGF treatments

KGF (R&D Systems, Minneapolis, MN, USA) was obtained as a recombinant human lyophilized protein and reconstituted as a 10 µg/mL stock solution in sterile phosphate-buffered saline (PBS) (Sigma, St Louis, MO, USA) containing 0.1% bovine serum albumin (BSA). Stock was aliquoted to avoid repeated freeze-thaw cycles and stored at -

20°C. Treatments ranging from 10-100ng/mL were prepared using DMEM/Ham's F12 medium as a diluent. Doses were chosen based on the study demonstrating KITL to be regulated by KGF in bovine granulosa cells, in which 50 ng/mL was used (Parrott and Skinner 1998b). This thesis chose to examine a range of doses above and below this concentration.

5.2.2.2 FSH treatments

Recombinant human FSH (Puregon, N.V. Organon, Oss, Netherlands) was obtained as an aqueous solution and diluted in culture medium to deliver treatments at a final concentration of 50 or 100 mIU/mL. Doses were chosen based on a study examining the effects of FSH treatment on cultured human granulosa-luteal cells (Laitinen *et al.* 1995) .

5.2.2.3 BMP-15 and GDF-9 treatments

Recombinant human BMP-15 (R&D Systems) and recombinant mouse GDF-9 (R&D Systems) were obtained as lyophilised proteins. Reconstitution was performed at 100 µg/mL in sterile 4 mM HCl containing 0.1% BSA. Samples were aliquoted to avoid repeated freeze-thaw cycles and stored at -20°C. Treatments ranging from 50-200ng/mL were chosen based on studies examining [^{3}H]-thymidine incorporation in mouse granulosa cells (personal communication, Mottershead D.). Doses were prepared using DMEM/Ham's F12 medium supplemented with 10% DCC-FBS as a diluent.

5.2.2.4 RNA extraction and generation of cDNA

RNA was extracted from KGN cells using an RNeasy Mini Kit (Qiagen, Hilden, Germany), and extracted from cumulus cells using an RNeasy Micro Kit (Qiagen) as described in Chapter 2 (section 2.3.7), and stored at -80°C. Quantitation of RNA is described in Chapter 2 (section 2.3.7), and cDNA was generated from total RNA from cumulus cells (100 ng) and KGN cells (500 ng) respectively as described in Chapter 2 using an iScript cDNA Synthesis Kit (Biorad, Hercules, CA, USA) containing oligo (dT) and random primers.

RNA positive controls were commercially obtained and included Universal Human Prostate, Universal Human Ovary and Universal Human Breast (Agilent Technologies, Santa Clara, CA, USA) which are produced as reference samples and are derived from human tissue samples. cDNA was generated from 1000 ng of RNA as described above.

5.2.2.5 Quantitative Real-Time PCR

Quantitative real-time PCR reactions for *FGFRII* and housekeeping gene *RPL19* were performed using 2x iQ SYBR Green Supermix as outlined by the manufacturer (Biorad). Primers for each gene were commercially obtained (Qiagen) and diluted as per the manufacturer's instructions using Nuclease-Free Water (Ambion). Target cDNA was amplified on an iQ5 iCycler (Biorad) using the following PCR conditions: 1 cycle at 95°C

for 3 min, 40 cycles at 95°C for 15 sec, 60°C for 15 sec, 72°C for 30 sec, 95°C for 1 min, 55°C for 1 min. Melt curve analysis occurred at 60°C for 10 sec.

Quantitative real-time PCR reactions for *KITL-1*, *KITL-2* and *PTX3* were performed using Taqman Gene Expression Assays (Applied Biosystems, Boston, MA, USA) as described in section 2.3.8. The assay specifically detecting *KITL-1* had one primer within exon 6, which is absent in *KITL-2*. A custom assay to specifically detect *KITL-2* was designed by placing one of the primers on the boundary spanning exons 5 and 7, thus enabling specific detection of *KITL-2*. The sequence for the custom-made *KITL-2* assay is as follows: forward primer, TGAGAAAGGGAAGGCCAAAA; reverse primer, AGAAAACAATGCTGGCAATGC; probe, ACTCCAGCCTACACTGG.

5.2.3 Western Blot

Western blotting to specifically detect KITL isoforms, PTX3 and HSP90 was performed on KGN cell lysates electrophoresed on Criterion XT precast gels (Biorad, Hercules, CA, USA) as described in Chapter 2 (section 2.3.9). PTX3 rat monoclonal antibody (Alexis Biochemicals, Exeter, UK) was diluted in TBST containing 1% skim milk powder to a concentration of 1:3000. HSP90α/β rabbit polyclonal antibody (Santa Cruz Biotechnology, Santa Cruz, CA, USA) was diluted in TBST containing 1% skim milk powder to a concentration of 1:1000.

5.2.4 Statistical analysis

GraphPad Prism 5 for Windows (GraphPad Software, Inc.) statistical software was used to analyse all data. Statistical differences were determined by one-way ANOVA with Tukey's post-hoc test, and a p-value of <0.05 was considered statistically significant. Analysis of human cumulus cells treated with or without GDF-9 was performed using a Student's T-test ($P<0.05$).

5.3 RESULTS

5.3.1 Effect of keratinocyte growth factor on *KITL* mRNA levels

The effect of a theca-derived factor, KGF, on *KITL* mRNA levels was examined in KGN cells and human cumulus cells. Treatment of KGN cells with 10, 50 or 100 ng/mL KGF for 6 hours had no effect on *KITL-1* and *KITL-2* mRNA levels (Fig 5.1A). Similarly, *KITL-1* and *2* mRNA levels were unchanged after treatment with 50 ng/mL KGF for 24, 48 or 72 hours (Fig. 5.1B). Similar to KGN cells, *KITL-1* and *KITL-2* mRNA levels were unchanged in human cumulus cells treated with 50 ng/mL KGF for 6 and 24 hours (Fig. 5.1C). Levels of the KGF receptor, *FGFRII*, were then compared in KGN and cumulus cells with cDNA generated from RNA samples obtained commercially (Universal Human Prostate, Universal Human Breast and Universal Human Ovary, Agilent Technologies) which were used as positive controls, and with cDNA generated from human breast cell lines (MDA-MB453, T47D and ZR75-1), used as negative controls. Figure 5.2 shows that universal human prostate and universal human ovary expressed the highest levels of *FGFRII*, followed by much lower levels in universal human breast, MDA-MB453, T47D and ZR75-1 human breast cancer cell lines. In comparison, the ovarian granulosa cell models KGN and cumulus cells also markedly lower levels of the receptor mRNA compared to universal human prostate and ovary similarly to the breast cell lines, which may prevent KGF signalling from being functional in these cells.

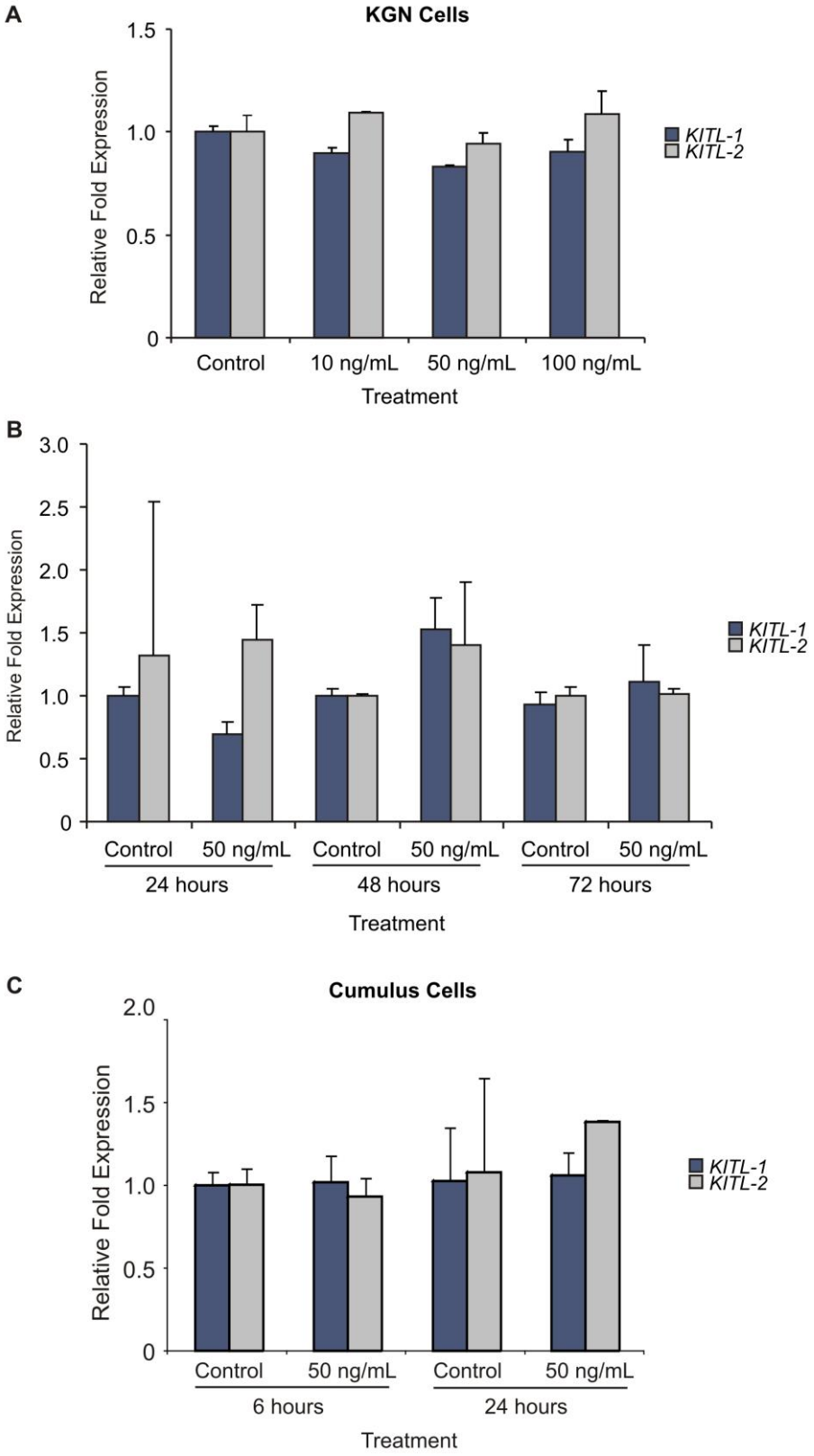

A
KGN Cells
Relative Fold Expression
1.5
1.0
0.5
0
KITL-1
KITL-2
Control
10 ng/mL
50 ng/mL
100 ng/mL
Treatment
B
Relative Fold Expression
3.0
2.5
2.0
1.5
1.0
0.5
0
KITL-1
KITL-2
Control
50 ng/mL
Control
50 ng/mL
Control
50 ng/mL
24 hours
48 hours
72 hours
Treatment
C
Cumulus Cells
Relative Fold Expression
2.0
1.5
1.0
0.5
0
KITL-1
KITL-2
Control
50 ng/mL
Control
50 ng/mL
6 hours
24 hours
Treatment

Figure 5.1: KGF treatment does not alter *KITL* expression in KGN or cumulus cells. (A) *KITL* isoform steady-state mRNA levels in KGN cells treated with or without (Control) 10-100 ng/mL KGF for 6 hours. (B) *KITL* isoform mRNA levels in KGN cells treated with or without 50 ng/mL KGF for 24-72 hours. (C) *KITL-1* and *KITL-1* steady-state mRNA levels in cumulus cells treated with or without 50 ng/mL KGF for 6 and 24 hours. Error bars represent the standard deviation of the mean for 3 replicate experiments. qPCR data for *KITL-1* and *KITL-2* was normalised to reference gene *RPL19* and calibrated to control.

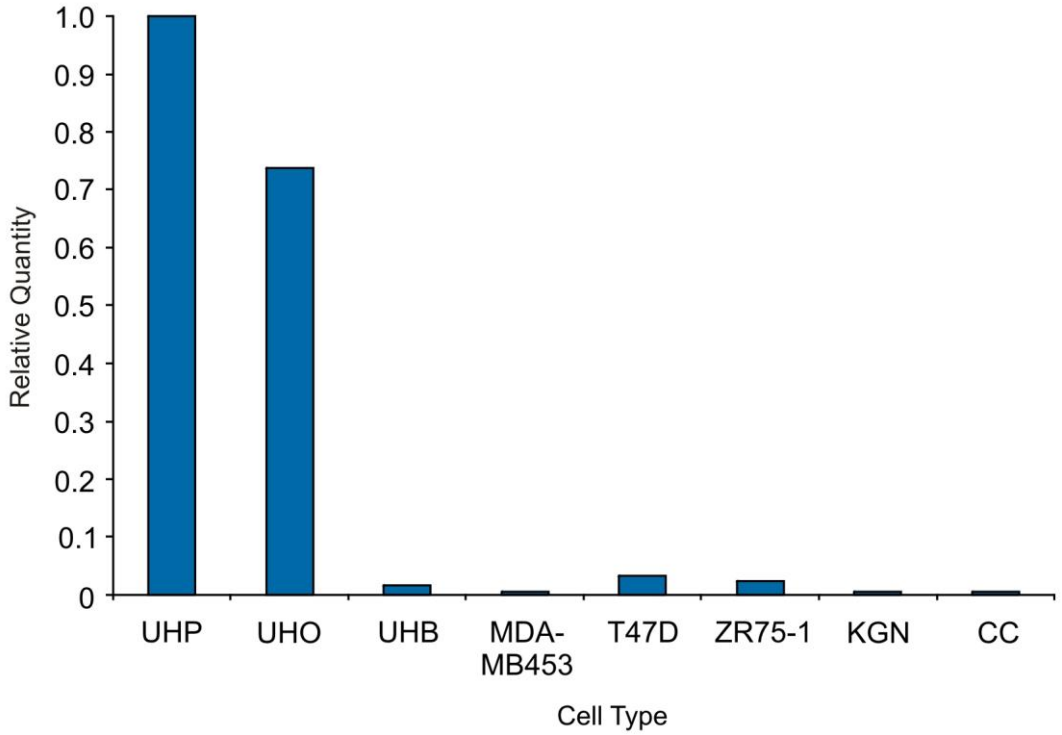


Figure 5.2: Comparative levels of *FGFRII* steady-state mRNA levels in human cell types. UHP: universal human prostate; UHO: universal human ovary; UHB; universal human breast; CC; cumulus cells. Human breast cell lines include MDA-MB453, T47D and ZR75-1. Data is represented as relative quantity and calibrated to UHP which appeared at the earliest Cq cycle.

5.3.2 Effect of follicle stimulating hormone on KITL mRNA levels

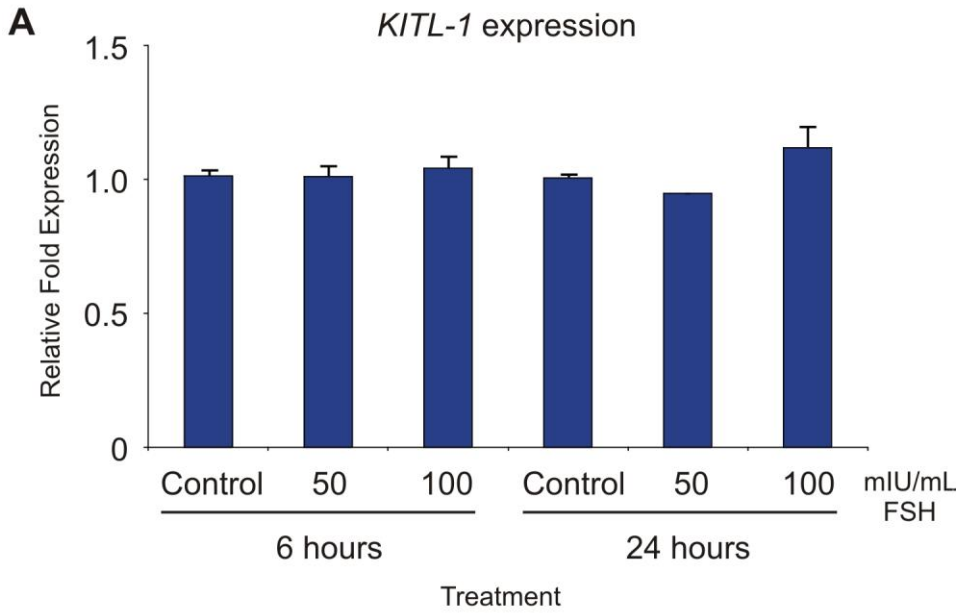
KGN cells treated with 50 or 100 mIU/mL FSH showed no change in *KITL-1* mRNA levels at 6 or 24 hours (Figure 5.3A). *KITL-2* was not examined in this particular experiment as data presented in this thesis so far has shown no evidence of differential changes in the levels of each isoform. Aromatase (*CYP19*) gene expression was induced 3-fold after 50 mIU/mL FSH treatment compared to control, and 6-fold after 100 mIU/mL FSH treatment at 6 hours. At 24 hours, aromatase expression had increased 6-fold after 50 mIU/mL FSH treatment compared to control, and 7-fold after 100 mIU/mL treatment. Collectively, these result indicate that although FSH signalling is functional as shown by the induction of aromatase expression after FSH treatment, KITL gene expression is not regulated by FSH in KGN cells.

5.3.3 Effect of oocyte-secreted factors BMP-15 and GDF-9 on KITL expression

TGFβ oocyte-secreted-factors were then examined to determine whether they regulate *KITL* expression in human granulosa cells. The effects of BMP-15 and GDF-9 were examined on KITL gene expression firstly in a model of relatively undifferentiated (preantral) granulosa cells, the KGN cell line. Cells were also treated with BMP-15 and GDF-9 in combination with FSH, based on evidence that FSH may exert its effects via intraovarian paracrine factors (Adashi *et al.* 1991b; Miro and Hillier 1996). Treatment with 50 or 100 ng/mL BMP-15 resulted in no change in *KITL-1* mRNA levels after 6 or 24 hours, and levels also remained unchanged after treatment with BMP-15 in combination

with 50 mIU/mL FSH (Fig. 5.4A). As expected, no change in *KITL-1* mRNA levels was observed after FSH treatment alone (Fig. 5.4). In contrast, treatment with 50 and 100ng/mL GDF-9 resulted in a significant decrease in *KITL-1* mRNA levels at both 6 and 24 hours compared to each timepoint's respective control ($P<0.05$). Additionally, 100 ng/mL GDF-9 treatment caused a significantly lower reduction in *KITL* levels compared to 50 ng/mL treatment ($P<0.05$), with or without FSH. Co-treatment with FSH did not affect the inhibitory effect of GDF-9 on *KITL-1* expression (Figure 5.4B). Collectively, this data suggests that BMP-15, alone and in combination with FSH, has no effect on *KITL-1* expression in KGN cells, but that GDF-9 acts as an inhibitory factor to decrease *KITL-1* expression for up to 24 hours, independent of FSH.

Regulation of *KITL* gene expression by GDF-9 was then further explored, by examining dose-dependent effects and including *KITL-2* gene expression to determine if any difference exists between regulation of the two isoforms. In addition, *pentraxin 3* (*Ptx3*) was included as a positive control, as GDF-9 is known to regulate *Ptx3* mRNA levels in mouse cumulus cells (Varani *et al.* 2002). Treatment of KGN cells with 100 and 200 ng/mL GDF-9 for 6 and 24 hours resulted in a significant decrease in *KITL-1* and *KITL-2* mRNA levels compared to control (Fig. 5.5) ($P<0.05$). The decreased in KITL expression was supported by a 2-fold reduction in levels of the positive control gene, *Ptx3*, after treatment with each concentration of GDF-9 ($P<0.05$) at 6 and 24 hours (Fig. 5.5 C,D). No dose-dependent effects of GDF-9 were observed on either *KITL* or *Ptx3*, indicating that a maximal effect is achieved with both 100 and 200 ng/mL treatments. Therefore, future


A
KITL-1 expression
Relative Fold Expression
1.5
1.0
0.5
0
Control
50
100
Control
50
100
mIU/mL FSH
6 hours
24 hours
Treatment

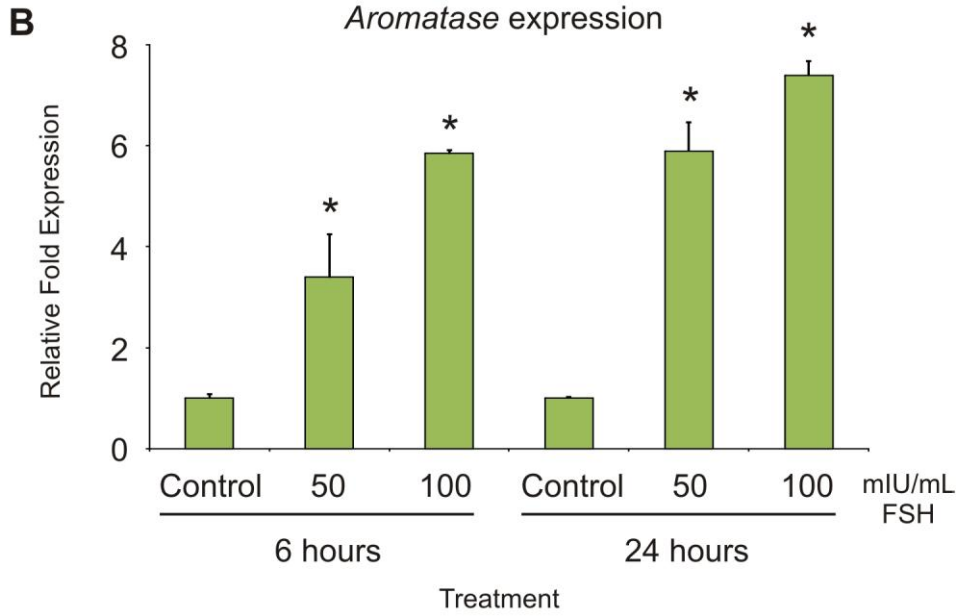

B
Aromatase expression
Relative Fold Expression
8
6
4
2
0
*
*
*
*
Control
50
100
Control
50
100
mIU/mL FSH
6 hours
24 hours
Treatment

Figure 5.3: FSH treatment has no effect on *KITL* mRNA levels in KGN cells. (A) *KITL-1* steady-state mRNA levels after 6 and 24 hours of treatment. (B) Aromatase steady-state mRNA levels used to demonstrate functional FSH signalling. qPCR data was normalised to reference gene *RPL19*. Error bars represent the standard deviation of the mean for 3 replicate experiments. Treatment indicated with an * is significantly different to their respective control for each timepoint, $P<0.05$ (One-Way ANOVA).

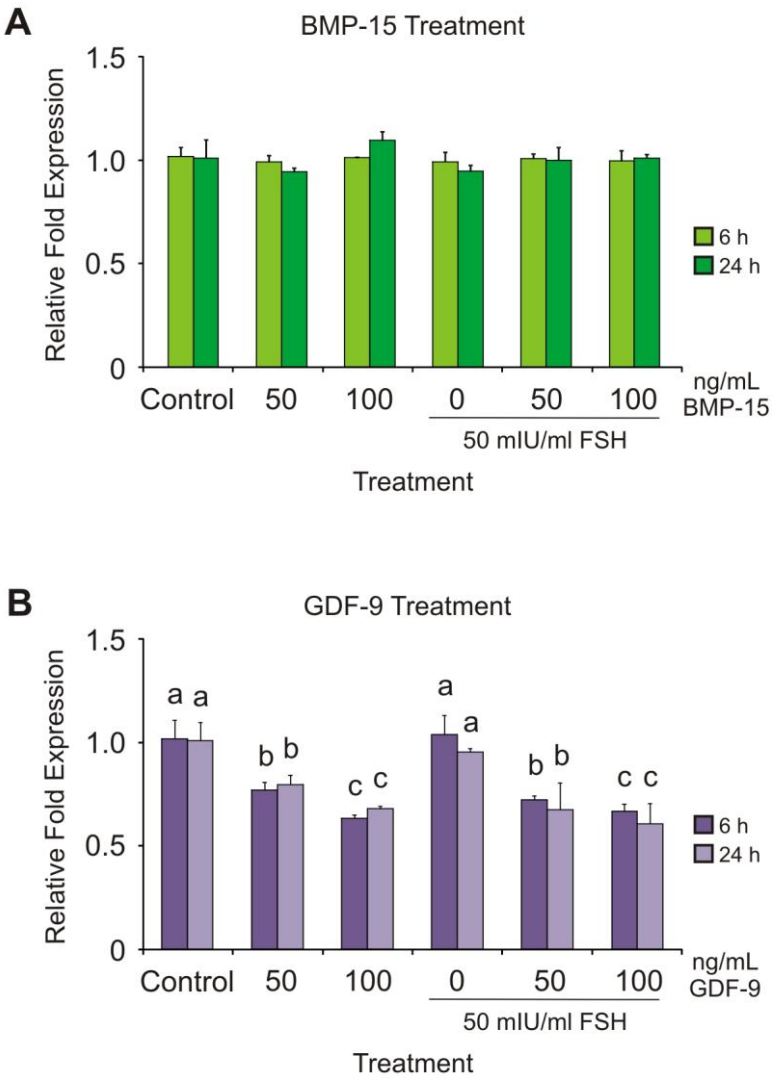


Figure 5.4: *KITL* mRNA levels in KGN cells remain unchanged after BMP-15 treatment but are decreased after GDF-9 treatment. (A) BMP-15 treatment with or without FSH. (B) GDF-9 treatment with or without FSH. qPCR data was normalised to reference gene *RPL19*. Error bars represent the standard deviation of the mean for 3 replicate experiments. Groups indicated with different letters are significantly different, $P<0.05$ (One-Way ANOVA).

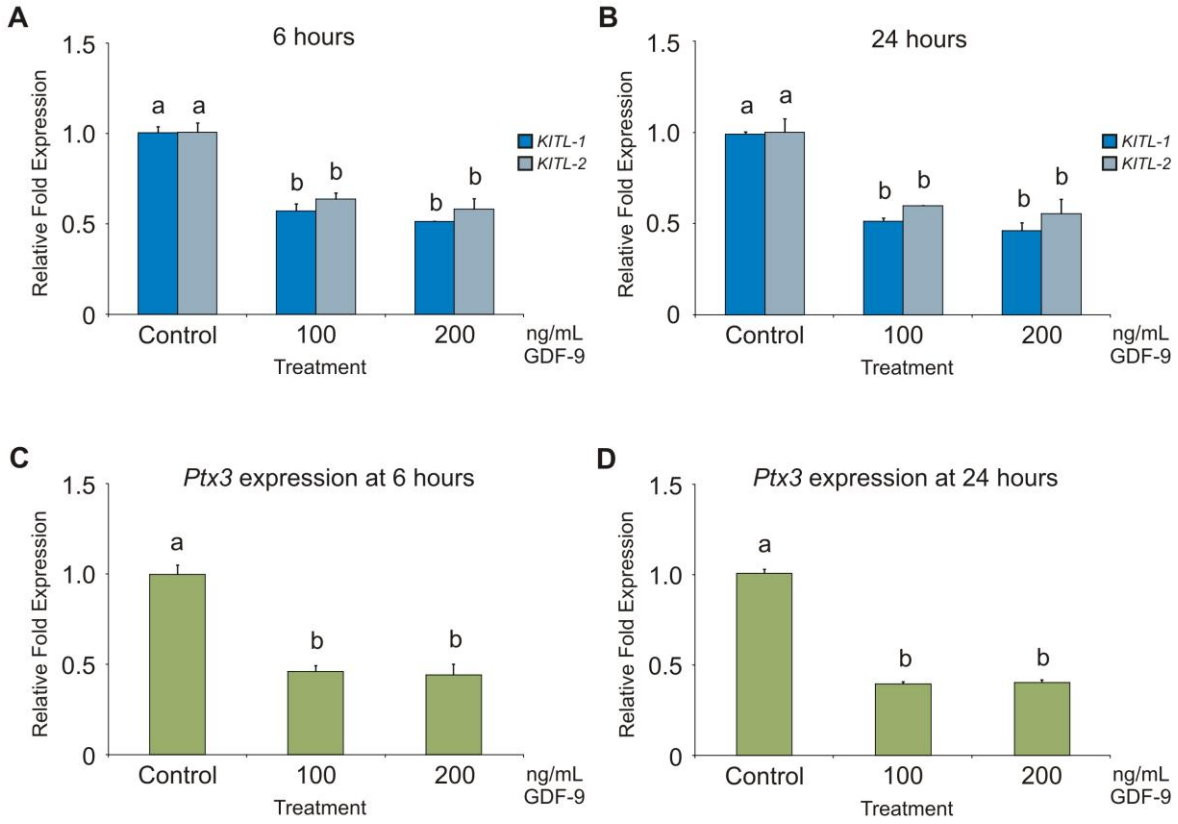


Figure 5.5: GDF-9 treatment decreases *KITL* and *Ptx3* mRNA levels in KGN cells after 6 and 24 hours. (A, B) *KITL-1* and *KITL-2* steady-state mRNA levels. (C, D) *Ptx3* steady-state mRNA levels. qPCR data was normalised to reference gene *RPL19* and calibrated to control. Error bars represent the standard deviation of the mean for 3 replicate experiments. Groups indicated with different letters are significantly different, $P<0.05$ (One-Way ANOVA).

experiments utilised 100ng/mL GDF-9 as it was equally effective as the higher concentration. There was no difference in levels between *KITL-1* and *KITL-2* mRNA, so subsequent experiments examined KITL-1 only as a representative of the two isoforms.

To attribute these transcriptional effects to the direct actions of the GDF-9 signalling pathway, KGN cells were treated with SBP431542, a potent inhibitor of the TGFβ superfamily type I ALK 4/5/7 receptors (Inman *et al.* 2002), through which GDF-9 is known to signal (McGrath *et al.* 1995; Vitt *et al.* 2002; Mazerbourg *et al.* 2004). Cells were firstly treated for 6 hours with increasing doses of SBP431542 (0.5, 1, 2 and 5 μM) in combination with a constant 100 ng/mL GDF-9, or with 5 μM SBP431542 alone. Treatment with 100 ng/mL GDF-9 resulted in a 2-fold reduction of *KITL-1* mRNA levels compared to control ($P<0.05$), and this effect was abolished by the addition of 0.5-5 μM SBP431542 (Fig. 5.6A). A 0.5-fold induction of *KITL-1* mRNA was caused by treatment with 5 μM SBP431542 alone compared to control ($P<0.05$), and a significant increase in mRNA levels was seen with 2 and 5 μM SBP431542 (1-5 μM) plus 100 ng/mL GDF-9 ($P<0.05$).

Ptx3 showed a similar pattern of response to all treatments as KITL, with a 2-fold decrease in mRNA levels after 100 ng/mL GDF-9 treatment compared to control (Fig. 5.6B) ($P<0.05$). A marked induction of *Ptx3* mRNA was observed in response to treatment with 5 μM SBP431542 alone or in combination with GDF-9 treatment compared to control

(P<0.05). A dose-dependent increase in *Ptx3* levels compared to control resulted after treatment with increasing levels of SBP431542 in combination with 100 ng/mL GDF-9 (P<0.05).

In order to determine the sensitivity of SBP431542 on inhibition of GDF-9 signalling, cells were treated with decreasing concentrations of SBP431542 for 6 hours. Treatment with 100 ng/mL GDF-9 resulted in a 2-fold reduction in *KITL-1* mRNA levels compared to control (P<0.05), and this effect was significantly inhibited by 0.5 µM SBP431542 (P<0.05) (Fig. 5.6C). Treatment with 0.5 µM SBP431542 alone caused a significant increase in *KITL-1* mRNA levels compared to control (P<0.05). No reversal of the GDF-9 effect was seen at concentrations of SBP432542 between 0.1 and 0.01 µM (P<0.05) (Fig. 5.6C). The same pattern of effects in response to all treatments were observed for *Ptx3* mRNA levels (Fig. 5.6D). Both 0.5 and 1 µM SBP431542 were able to reverse the effects of GDF-9 on *KITL-1* gene expression back to control levels as shown in Figure 5.6, and from these 1 µM was used in future experiments. Collectively, these results indicate that GDF-9 activates ALK 4/5/7 receptors to regulate *KITL* and *Ptx3* gene expression in KGN cells.

The effect of GDF-9 was then examined in human cumulus cells, which represent granulosa cells from preovulatory follicles. Due to the limited availability of these cells, a

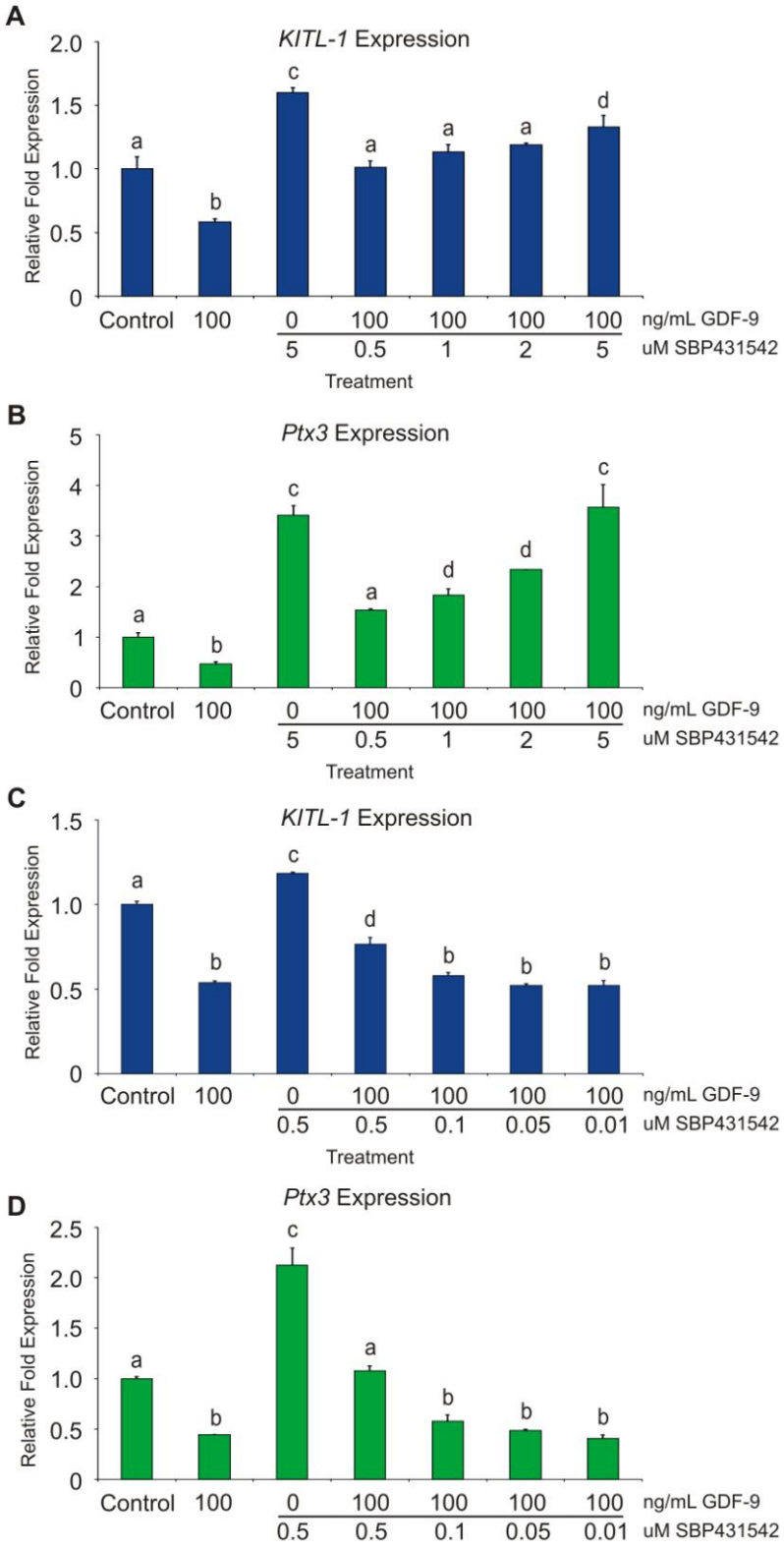

A
KITL-1 Expression
Relative Fold Expression
2.0
1.5
1.0
0.5
0
a
b
c
a
a
a
d
Control
100
0
100
100
100
100
ng/mL GDF-9
5
0.5
1
2
5
uM SBP431542
Treatment
B
Ptx3 Expression
Relative Fold Expression
5
4
3
2
1
0
a
b
c
a
d
d
c
Control
100
0
100
100
100
100
ng/mL GDF-9
5
0.5
1
2
5
uM SBP431542
Treatment
C
KITL-1 Expression
Relative Fold Expression
1.5
1.0
0.5
0
a
b
c
d
b
b
b
Control
100
0
100
100
100
100
ng/mL GDF-9
0.5
0.5
0.1
0.05
0.01
uM SBP431542
Treatment
D
Ptx3 Expression
Relative Fold Expression
2.5
2.0
1.5
1.0
0.5
0
a
b
c
a
b
b
b
Control
100
0
100
100
100
100
ng/mL GDF-9
0.5
0.5
0.1
0.05
0.01
uM SBP431542

Figure 5.6: ALK4/5/7 inhibitor SBP431542 reverses the effect of GDF-9 treatment in KGN cells after 6 hours. (A) Steady-state *KITL-1* expression in cells treated with 100 ng/mL GDF-9 alone or with increasing doses of SBP431542 (0.5-5 μM). (B) *Ptx3* was used as a positive control for functional GDF-9 signalling. (C) Steady-state *KITL-1* expression in cells treated with 100 ng/mL GDF-9 alone or with decreasing doses of SBP431542 (0.5-0.01 μM). (D) Positive control gene *Ptx3*. qPCR data was normalised to reference gene *RPL19*. Error bars represent the standard deviation of the mean for 3 replicate experiments. Groups indicated with different letters are significantly different, $P<0.05$ (One-Way ANOVA).

single dose of 100 ng/mL GDF-9 was examined and SBP431542 was not included. This allowed each patient's cells to represent an individual experiment, increasing sample size and enabling patient variability to be shown. Similarly to KGN cells, 6 hour treatment with 100 ng/mL GDF-9 caused a significant reduction in *KITL-1* mRNA levels in human cumulus cells (Fig. 5.7A) compared to control ($P<0.05$), and this effect was more subtle compared to that seen in KGN cells. Levels of *Ptx3* mRNA were also significantly reduced after treatment with 100 ng/mL GDF-9 compared to control (Fig. 5.7B) ($P<0.05$), and once again the decrease was more subtle than the decrease observed in *Ptx3* levels in KGN cells.

The dramatic induction of *KITL* mRNA by SBP431542 was unexpected and suggests that trophic factors may be negatively regulating KITL expression. To determine if growth factors present in FBS were influencing KITL gene expression during culture, resulting in an increase in expression when inhibited by treatment with SBP431542 alone, KGN cells were cultured in media containing decreasing concentrations of FBS and treated with or without SBP431542 for 24 hours. A concentration of 5 µM SBP431542 was chosen as it had produced the biggest increase in *KITL-1* mRNA levels alone or in combination with GDF-9 compared to lower concentrations (Fig. 5.6). KGN cells were also cultured Opti-MEM medium supplemented with 2% FBS, which is a medium that allows for reduced FBS supplementation with no effect on growth rate or morphology. *KITL-1* levels were no different in cells cultured in DMEM/Ham's F12 medium supplemented with 10, 5 or 1% FBS, or in Opti-MEM medium supplemented with 2% FBS (Fig. 5.8A). Basal *KITL-1* levels were significantly higher in cells cultured in DMEM/Ham's F12 medium

supplemented with only 0.1% FBS (Fig. 5.8A). As expected, treatment with SBP431542 resulted in a significant increase in *KITL-1* mRNA levels compared to untreated cells in media with 10% FBS, and this occurred at all reduced concentrations of FBS. KITL-1 and KITL-2 protein levels did not change after culture in any concentration of FBS with or without SBP431542 treatment after 24 hours (Fig. 5.8B). These results suggest that a growth factor present in FBS is not having an effect on *KITL* gene or protein expression via ALK 4/5/7 receptors, and it remains unknown why SBP431542 is able to increase basal levels of *KITL-1* mRNA in KGN cells.

To examine the effects of GDF-9 on KITL expression at the protein level, KGN cells were treated with different concentrations of GDF-9 for 24 or 48 hours and KITL protein isoform levels were examined by Western blot. Surprisingly, treatment with 50, 100 or 200ng/mL GDF-9 did not appear to have any effect on KITL-1 or KITL-2 protein levels at 24 or 48 hours compared to control (Fig. 5.9A). Similarly, protein levels of PTX3 did not seem to change after treatment at any timepoint (Fig. 5.9B).

Treatments were then performed for 8, 24, 72 and 96 hours to allow a large window of time in which an effect might occur. There appeared to be no change in KITL-1 or KITL-2 protein levels with 100 ng/mL GDF-9 treatment or with 1 or 5 µM SBP431542 alone compared to controls at any timepoint (Fig. 5.9C). In addition, treatment with 1 µM SBP431542 plus 100 ng/mL GDF-9 seemed to cause no change in KITL protein isoform

levels at any timepoint compared to control (Fig. 5.9C). This was confirmed by densitometry, which was performed on bands from five independent experiments comparing KITL-1 and KITL-2 levels in GDF-9-treated cells to control, and was then normalised to HSP90. A value of 1.0 was obtained for the mean of both KITL isoforms in control and GDF-9 treatment groups, confirming there was no effect on KITL protein levels by GDF-9 treatment (data not shown). Similarly, PTX3 levels appeared to be no different after any treatment at each timepoint compared to control (Fig. 5.9D). Collectively, these results suggest that GDF-9 or SBP431542, alone or in combination, appear not regulate KITL or PTX3 expression at the protein level.

5.3.4 Effect of 5α-dihydrotestosterone on GDF-9 regulation of KITL mRNA and protein levels

To investigate potential crosstalk between AR and GDF-9 signalling, KGN cells were treated with 100 ng/mL GDF-9 with or without 1 or 10nM DHT for 6 hours. As expected from previous experiments, *KITL-1* mRNA levels were decreased by 100 ng/mL GDF-9 treatment compared to control ($P<0.05$), and treatment with 1 or 10 nM DHT alone had no effect on *KITL-1* mRNA levels compared to control (Fig. 5.10). Treatment with 100ng/mL GDF-9 in combination with 1 or 10 nM DHT caused a small decrease in *KITL-1* mRNA levels compared to control and 1 and 10 nM DHT treatment, but levels were higher than GDF-9 treatment alone (Fig. 5.10). These results suggest that androgen may have a subtle, indirect effect on KITL-1 gene expression via crosstalk with the GDF-9 signalling pathway in KGN cells.

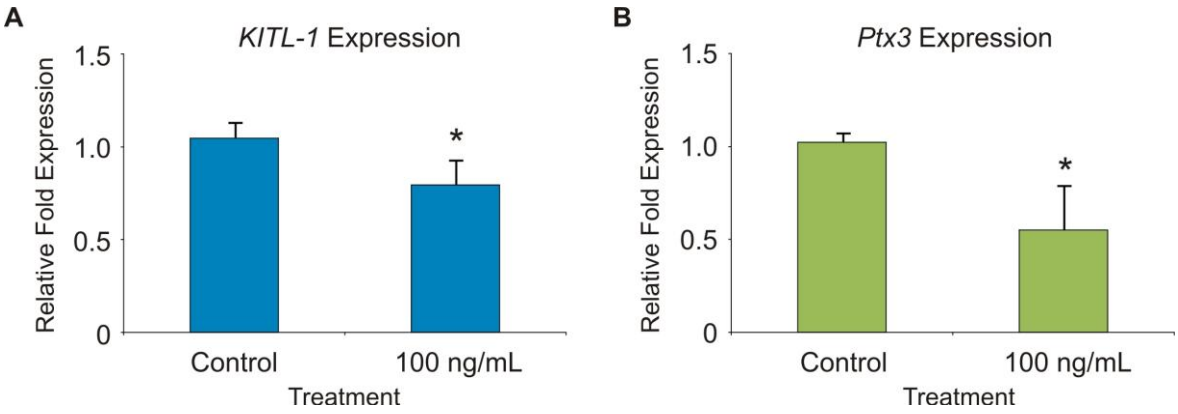


Figure 5.7: *KITL-1* and *Ptx3* mRNA levels in human cumulus cells are decreased by GDF-9 treatment at 6 hours. qPCR data was normalised to reference gene *RPL19* and calibrated to control. Error bars represent the standard deviation of the mean for 7 individual patient samples. Treatment indicated with an * is significantly different to control, $P<0.05$ (Student's T-test).

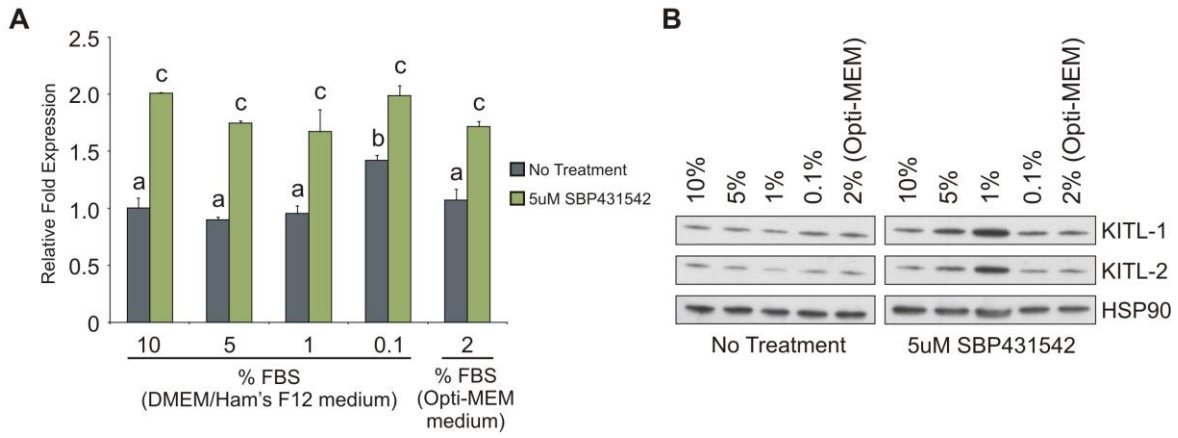


Figure 5.8: KGN cells cultured in DMEM/Ham's F12 medium supplemented with diminishing concentrations of FBS (10-0.1%) or Opti-MEM medium (containing 2% FBS) for 24 hours and treated with or without SBP431542 (5 µM). (A) *KITL-1* steady-state mRNA expression. qPCR data was normalised to reference gene RPL19 and expressed relative to cells in media containing 10% FBS. Error bars represent the standard deviation of the mean for 3 replicate experiments. Treatments with different letters are significantly different, $P<0.05$ (One-Way ANOVA). (B) KITL isoform protein levels after 24 hours. HSP90 was used as a loading control.

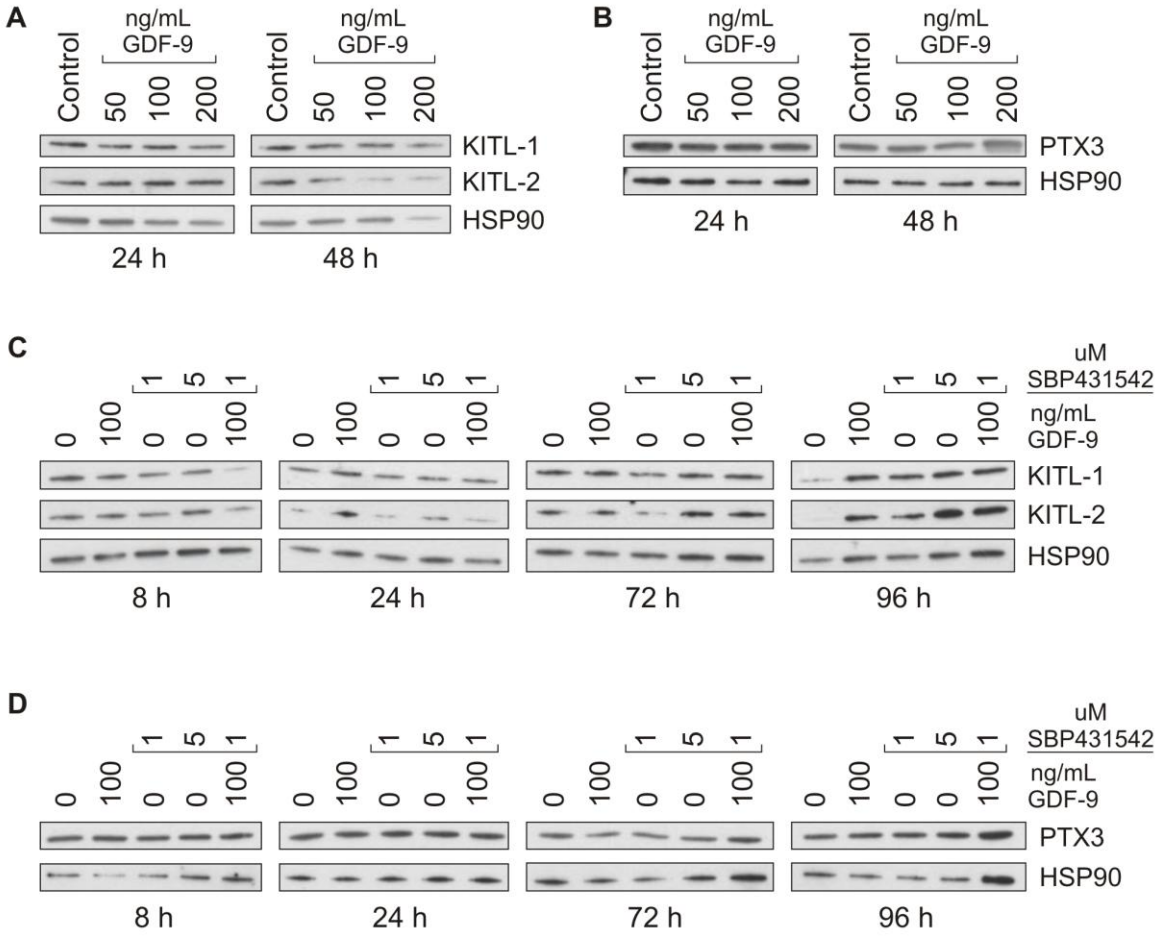


Figure 5.9: KITL and PTX3 protein levels are unchanged in KGN cells treated with GDF-9 and SBP431542. (A) KITL isoform levels in cells treated with or without GDF-9 (50-200 ng/mL) for 24 and 48 hours. (B) Levels of PTX3 protein used as a positive control for (A). (C) KITL isoform levels in cells treated with or without GDF-9 (100 ng/mL) and/or SBP431542 (1 or 5 µM) for 8-96 hours. (D) Levels of PTX3 protein used as a positive control for (C). HSP90 was used as a loading control in (A-D).

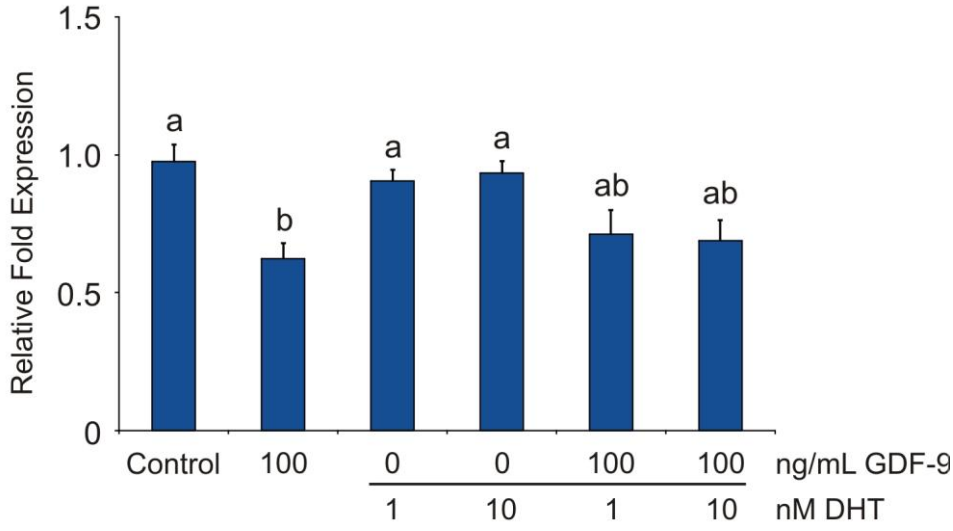


Figure 5.10: *KITL-1* steady-state mRNA levels in KGN cells treated with GDF-9 and/or DHT for 6 hours. Cells were treated with either vehicle, 100ng/mL GDF-9 and/or 1 or 10 nM DHT. qPCR data was normalised to reference gene RPL19. Error bars represent the standard deviation of the mean for 4 replicate experiments. Treatments with different letters are significantly different, $P<0.05$.

5.4 DISCUSSION

Several endocrine and intraovarian factors have been shown to regulate *KITL* in animal models, and this chapter examined the best candidates including FSH, KGF, BMP-15 and GDF-9. The results presented in this chapter suggest that of all the tested candidate factors, GDF-9 down-regulated *KITL* gene expression in two models of human granulosa cells. The inconsistency of results between animal models may reflect key differences in the molecular biology of ovarian function between animals and humans, due perhaps to humans being a mono-ovulatory species versus poly-ovular rodents. *In vivo* experiments performed in animal models may also provide different results that may better represent ovarian biology than the *in vitro* experiments performed in this thesis, a limitation that is insurmountable in human ovarian molecular biology studies.

Optimal communication between the oocyte and surrounding granulosa cells via paracrine GDF-9 signalling is a critical component of normal oocyte and granulosa cell growth and function (Moley and Schreiber 1995). This chapter showed that treatment with GDF-9 caused a significant reduction in *KITL* mRNA levels in KGN cells, and builds on previous studies demonstrating GDF-9 regulation of *KITL* in mouse granulosa cells (Elvin *et al.* 1999b; Joyce *et al.* 1999; Joyce *et al.* 2000; Thomas and Vanderhyden 2006). The GDF-9-mediated decrease in *KITL* in KGN cells was supported by a similar decrease in *Ptx3* levels, which demonstrates the presence of functional GDF-9 signalling in KGN cells. Although GDF-9 has been demonstrated to induce *Ptx3* gene expression in mouse

granulosa cells (Varani *et al.* 2002), the decrease shown in KGN cells may be due to their representation of undifferentiated granulosa cells from preantral follicles, in which *Ptx3*, a cumulus cell-derived factor required for cumulus expansion, would not be normally induced. The data presented in this chapter provides the first evidence that paracrine signalling between the oocyte and granulosa cells in the human ovary may involve GDF-9 regulation of *KITL*. Human cumulus cells showed a much more varied and subtle response to GDF-9 treatment, with a trend towards decreased *KITL* mRNA levels. There is little evidence to suggest that *KITL* plays an important role in cumulus expansion or ovulation, and therefore the *KITL* gene may be less responsive to regulation by GDF-9 in expanded cumulus cells. *Ptx3* showed a two-fold decrease in response to GDF-9 treatment in most cases, which was unexpected as GDF-9 is known to increase *Ptx3* expression in mouse cumulus cells during preovulation as part of the GDF-9 signalling cascade (Varani *et al.* 2002). Since the cells used in this chapter were expanded cumulus cells, GDF-9 may instead cause a decrease in gene expression as *Ptx3* is no longer required to induce expansion. Collectively, these results indicate that *KITL* is a GDF-9-regulated gene in human granulosa cells both in early and late stages of follicle development.

The significance of KITL regulation by GDF-9 in human granulosa cells may be important for understanding the mechanisms behind abnormalities of the PCOS ovary. It has been shown that *GDF-9* mRNA levels are lower in the oocytes of women with PCOS compared to normal oocytes (Teixeira Filho *et al.* 2002; Wei *et al.* 2011), and we previously showed increased KITL protein levels in PCO compared to non-PCO (Tuck *et al.* 2007; Tuck *et al.*

2010a). Lower *GDF-9* levels may result in abnormally increased KITL levels in PCO, which could then lead to abnormal ovarian features such as hyperthecosis, enlarged oocytes and increased follicle numbers (Parrott and Skinner 1997; Yoshida *et al.* 1997; Parrott and Skinner 2000; Reynaud *et al.* 2000; Hutt *et al.* 2006; Thomas and Vanderhyden 2006; Thomas *et al.* 2008). Identification of these molecular mechanisms may potentially lead to the development of new therapeutic targets for PCOS treatment.

Understanding the interactions between oocyte- and granulosa-secreted paracrine factors is important for understanding human female fertility and developing assisted reproductive technologies. Successful pregnancies have been correlated with increased soluble KITL levels in follicular fluid of patients undergoing *in vitro* fertilisation (IVF) (Smikle *et al.* 1998). Furthermore, a study examining the effect of coculture of KGN cells with mouse embryos reported significantly increased embryo development via upregulation of KITL gene expression in KGN cells, which was abolished by the addition of the c-kit blocking antibody ACK2 (Taniguchi *et al.* 2004). It was proposed that KGN cells may have potential for future application in human IVF by acting as an effective "feeder layer" for embryos by promoting embryo development for transfer to the patient, biopsy of the embryo for genetic analysis and cryopreservation. The studies utilising KGN cells to examine *KITL* expression in this chapter may also be of relevance for *in vitro* maturation (IVM), an assisted reproductive technique currently under development, which aims to mature human preantral follicles *in vivo* from cryopreserved or fresh ovarian tissue. This technique is critically dependent on understanding the right balance of paracrine factors to

include in growth medium to ensure success in promoting normal, healthy oocyte maturation in conjunction with timely somatic cell growth and differentiation (Gilchrist 2011). This chapter has shown evidence of the involvement of KITL and GDF-9 in paracrine signalling of preantral granulosa cells via the KGN cell model, which may be of use in developing effective IVM culture environments. Evidence in this thesis indicating that KITL is part of the important oocyte-granulosa cell intercommunication in humans is a step towards making new advances in human infertility treatment.

Regulation of both *KITL* and *Ptx3* by GDF-9 in this study was shown to be specific to the SMAD 2/3 signalling pathway. SBP431542 prevents phosphorylation of SMAD 2/3 by inhibition of the closely related ALK receptors 4, 5 and 7, and is most effective as an inhibitor of ALK5 (Inman *et al.* 2002) which forms half of the GDF-9 receptor heterodimer with type II receptor BMPR2 (Vitt *et al.* 2002; Kaivo-Oja *et al.* 2003; Mazerbourg *et al.* 2004; Kaivo-Oja *et al.* 2005; Kaivo-oja *et al.* 2006). Treatment with SBP431542 abolished the effect of GDF-9 on *KITL* and *Ptx3* mRNA levels in KGN cells, indicating that the SMAD 2/3 pathway is the mechanism through which GDF-9 influences *KITL* and *Ptx3* gene expression. Future study would examine the effect of SBP431542 treatment in human cumulus cells to determine whether GDF-9 is also signalling through ALK5 and SMAD 2/3 in these cells. The effect of SBP431542 in this chapter is consistent with active GDF-9 signalling in KGN cells and activation of SMAD2/3 as shown in animal studies (Inman *et al.* 2002; Mottershead *et al.* 2011).

Unexpectedly, treatment with SBP431542 caused an induction of *KITL* and particularly *PTX3* mRNA levels, and it remains unclear why the inhibitor would have such a marked effect alone. It was speculated that a growth factor present in FBS may constitutively suppress *KITL* expression, and treatment with SBP431542 thus allowed levels to return to normal. However, *KITL* mRNA levels were similarly increased in cells cultured in different concentrations of FBS with SBP431542 treatment compared to untreated cells. The increase in *KITL-1* mRNA levels present in cells cultured in 0.1% FBS without treatment can likely be attributed to the sickly, sparse appearance of cells after over 24 hours in culture with very little FBS as part of a survival response to stress, as has been demonstrated in granulosa cells of animal models (Dolci *et al.* 1991; Pesce *et al.* 1993; Felici *et al.* 1999; Morita *et al.* 1999; Jin *et al.* 2005a; Mahakali Zama *et al.* 2005; Lobascio *et al.* 2007; Moniruzzaman *et al.* 2007). Future study would examine the levels of phospho-SMAD 2 in cells treated with or without SBP431542 to determine if activation of the SMAD 2/3 pathway is occurring without the addition of exogenous factors, such as via an autocrine mechanism. Recently, a study showed evidence suggesting that GDF-9 may not be acting through ALK5 alone in mouse granulosa cells. Conditional knockout of ALK5 gene *Tgfbr1* in the female reproductive tract of mice showed no ovarian phenotype, thus indicating that ALK5 signalling is dispensable for GDF-9 signalling *in vivo (Li et al. 2011)*. It is possible that SBP431542 may also target another receptor, which would explain the induction of *KITL* and *PTX3*. Further study is required in human granulosa cells to determine whether GDF-9 and SBP431542 signal via a receptor other than ALK5.

Although GDF-9 regulated *KITL* at the transcriptional levels, regulation of KITL by GDF-9 at the protein level in KGN cells was not evident in this study. Extensive investigation using different GDF-9 concentrations, a range of timepoints and co-treatment with the ALK 4/5/7 inhibitor SBP431542, showed no effect on KITL isoform or PTX3 protein levels. To date, no previous studies have examined the effect of GDF-9 on PTX3 protein levels, so in this case it may not be effective as a positive control for protein translation. There may be several explanations for the absence of any changes in KITL or PTX3 levels, despite marked effects on mRNA levels. A limitation of the model used is that KGN cells are a cancer cell line, which may mean that some functions are absent or modified compared to normal granulosa cells. Thus, while GDF-9 may affect KITL protein levels in vivo, this effect may be lost or non-functional in KGN cells. Another explanation is that while GDF-9 may be sufficient to affect *KITL* mRNA levels, the effect at the protein level may require crosstalk from other intraovarian factors to achieve a significant and observable change in levels. For example, there is strong evidence for a synergistic interaction between GDF-9 and BMP-15 clearly indicating that each factor is unlikely to act alone *in vivo* (Mottershead *et al.* 2011). Crossing heterozygote *GDF-9* +/- and *BMP-15* +/- animals, including mice (Yan *et al.* 2001; Su *et al.* 2004) and sheep (Hanrahan *et al.* 2004; McNatty *et al.* 2006), gave the first indications of a convergence of the signalling pathway and this has since been shown in several biochemical studies (McNatty *et al.* 2005b; McNatty *et al.* 2005a; Edwards *et al.* 2008; McIntosh *et al.* 2008). Most recently, a study examining the interaction between the same purified recombinant proteins used in this study showed a clear synergism that was specific only for GDF-9 and BMP-15

(Mottershead *et al.* 2011). Specifically, cultured mouse granulosa cells showed a marked increase in DNA synthesis and activation of the SMAD 3 pathway in response to treatment with GDF-9 and BMP-15 in combination, compared to treatment with each factor alone. No synergism occurred between any other member of the TGFβ superfamily with GDF-9 or BMP-15, despite utilising the same signalling pathways. This effect was mediated specifically by the SMAD 2/3 pathway, and not SMAD 1/5/8, as shown by treatment with SBP431542 (Mottershead *et al.* 2011). It is more than likely that an interaction of this nature, or one similar, is required for GDF-9 to have a visible effect on KITL at the protein level. While protein studies were not feasible in human cumulus cells due to their limited numbers, it is doubtful that a change in KITL or PTX3 levels would be observed after GDF-9 treatment since decreases in mRNA levels were quite variable and tended towards a more subtle nature.

Despite evidence from previous studies showing FSH regulation of *KITL* expression in mouse preantral granulosa cells (Yang *et al.* 2003; Thomas *et al.* 2005), this study showed no effect of FSH on *KITL* mRNA levels alone or in combination with OSFs. In one study, cultured human mural granulosa cells demonstrated decreased *KITL* mRNA expression in a time and concentration-dependent manner in response to FSH treatment (Laitinen *et al.* 1995). Whether the stage or phenotype of granulosa cells is an important factor that determines FSH regulation is unclear; a study examining mural granulosa cells from mice showed no effect of FSH on *KITL* regulation, whereas preantral granulosa cells did show an effect (Joyce *et al.* 1999). This chapter utilised a model of undifferentiated preantral

granulosa cells, KGN, to examine the effects of FSH and this may account for the differences in results compared to Laitinen et al. (1995) who used primary, highly differentiated mural granulosa cells. Induction of aromatase expression by FSH in this study indicates a present and functional FSH signalling pathway in KGN cells as per the original characterisation study (Nishi *et al.* 2001), but as results suggest, regulation of *KITL* expression by this endocrine factor does not occur in these cells.

Crosstalk between the androgen and the GDF-9 signalling pathway has been demonstrated in previous studies (Chipuk *et al.* 2002; Kang *et al.* 2002; Hickey *et al.* 2005). Co-treatment of KGN cells with GDF-9 and DHT in this study caused a small but significant increase in *KITL* mRNA levels compared to GDF-9 treatment alone, but did not restore levels to those of control and DHT-treated cells. The addition of testosterone or DHT to porcine granulosa cells treated with GDF-9 enhanced the proliferative effect of GDF-9 (Hickey *et al.* 2005), and it is interesting that the subtle change in *KITL* levels observed in this chapter suggests a slight reversal of the GDF-9 effect. Whether a more potent response would be seen with different combinations of ligand concentrations at different timepoints is unknown. The effect observed using the optimal concentration of GDF-9 at 6 hours with physiological doses of DHT can suggest that any interaction between androgen and GDF-9 signalling may be small and biologically inconsequential. Further study is required for a greater understanding of indirect androgen regulation of KITL via interaction with the GDF-9 signalling pathway.

In summary, this chapter has provided evidence that *KITL* is regulated by the oocyte-secreted factor GDF-9 in human granulosa cells. This suggests that KITL is involved in paracrine communication between the oocyte and granulosa cells in the human ovary, which is further supported by the observation of c-kit protein present in adult human oocytes as shown in Chapter 3. Cumulatively the results provide a deeper understanding of the molecular mechanisms involved in regulation of KITL in human granulosa cells and may be of significance for the development of assisted reproductive technologies.

CHAPTER 6

General Discussion

Chapter 6: General Discussion

6.1 OVERVIEW

While much effort has been made to understand the ovarian factors controlling fertility at the molecular and cellular level, particularly in animal models, the key factors in humans remain to be fully elucidated. The studies described in this thesis focus on the cytokine growth factor KITL and its receptor c-kit, known to be vital for fertility in mice but their role in the human ovary is not well established. A previous study demonstrating a correlation between soluble KITL levels in human follicular fluid and successful IVF pregnancy rates suggested that KITL may also be important for fertility and reproductive success in women (Smikle *et al.* 1998). Difficulties in obtaining human adult ovarian tissues have limited most of the studies to date examining KITL and c-kit in human ovarian tissues to investigations of fetal ovaries obtained from aborted pregnancies. While this experimental system remains valuable for investigations into early follicle formation and oogenesis, it does not provide an understanding of the follicular events that occur post-puberty leading up to ovulation and the cyclical establishment and remodelling of the corpus luteum. The studies described in this thesis have utilised fresh and archival adult human ovarian tissues to provide novel information about KITL and c-kit expression in the normal and PCOS ovary, and to examine potential endocrine and intraovarian factors that may regulate KITL gene expression in human granulosa cells.

This is the first study to describe KITL protein isoform expression in mammalian granulosa cells representing different stages of follicle development. The evidence presented in this thesis suggests markedly different expression levels of both isoforms in preantral human granulosa cells compared to preantral murine and bovine granulosa cells (Parrott and Skinner 1997; Thomas *et al.* 2005). Furthermore, protein localisation experiments described in this thesis and the results of my previous study (Tuck *et al.* 2007) examining KITL in adult human ovarian tissues demonstrate distinct differences in expression patterns of both KITL and c-kit throughout human follicle development compared to the patterns described for other mammalian species (Driancourt *et al.* 2000). Differential expression of each isoform across species may explain the variations in some KITL functions that have been described in animal models that are poly-ovulatory, such as the mouse, or mono-ovulatory, such as the cow. The human studies described in this thesis, showing distinct differences in KITL and c-kit protein expression compared to animal models, demonstrate the importance of examining the KITL/c-kit system in the human ovary as opposed to animal models to fully understand its function and importance.

The finding that c-kit expression patterns differed in the oocyte and granulosa cells of PCO compared to non-PCO is supportive of an involvement of KITL and c-kit in abnormalities of the PCOS ovary. Previously, I showed increased levels of KITL protein in granulosa cells, theca cells and the oocyte of PCO compared to non-PCO (Tuck *et al.* 2007; Tuck *et al.* 2010a), and the c-kit observations presented in Chapter 3 add further evidence to support dysregulation and involvement of this ligand-receptor signalling pathway in PCO

pathology. The findings presented in Chapter 3 showed no difference in *KITL* mRNA levels in cumulus cells from women with or without PCOS and as discussed, these cells were from women who had been hyperstimulated with gonadotrophins and therefore gene expression levels may not be representative of cumulus cells prior to the stimulus. Further study is required and is discussed in more detail in the next section (6.2 Future Directions). The roles of KITL throughout folliculogenesis that have been ascribed from studies of animal models would indicate that the KITL/c-kit system is an ideal candidate to explain several abnormalities characteristic of PCOS such as increased numbers of growing follicles, abnormal oocyte growth, thickened thecal layers and increased thecal androgen biosynthesis. However, until specific actions of KITL in human ovarian cells are elucidated, the consequences of perturbed KITL/c-kit signalling in PCO remain speculative.

In vitro studies were performed to examine potential endocrine and intraovarian factors that may regulate KITL gene expression in human granulosa cells. This is the first study to show evidence of GDF-9 mediated down-regulation of *KITL* gene expression in human granulosa cells, shown to be dependent upon the ALK5 receptor. Evidence presented herein also suggests that androgens may influence *KITL* expression indirectly in human granulosa cells via crosstalk with GDF-9 signalling, acting to reverse the inhibitory effect of GDF-9. This finding is supported by a recent study examining cultured neonatal mouse ovaries, demonstrating that testosterone treatment decreased *GDF-9* gene expression in whole ovaries (Yang *et al.* 2010). Co-localisation of GDF-9 and AR in mouse oocytes

suggested the effect may be AR-dependent, which was confirmed by the transfection of a mouse testes cell line, TM4, with a *GDF-9*-luciferase reporter construct and treatment with an AR-antagonist. *GDF-9* promoter activity was decreased after testosterone treatment compared to control, and the addition of an AR antagonist flutamide attenuated the effect of testosterone (Yang *et al.* 2010). The data presented in Chapter 5 not only suggest that GDF-9 paracrine communication with human granulosa cells involves regulation of *KITL* gene expression, but may also provide a mechanism for the abnormal KITL levels in PCO observed in my previous study. *GDF-9* mRNA levels have previously been examined in oocytes of ovarian tissue from women with or without PCOS by *in situ* hybridisation, and *GDF-9* levels were found to be lower in the oocytes of women with PCOS compared to oocytes from women without PCOS (Teixeira Filho *et al.* 2002). Futhermore, a recent study examining *GDF-9* mRNA levels in oocytes obtained from women undergoing hyperstimulation for assisted reproductive techonology reported lower *GDF-9* levels in the oocytes of women with PCOS, and the abnormally low levels were unable to increase to those of control oocytes despite the gonadotrophin stimulus (Wei *et al.* 2011). The findings of this thesis suggests a model whereby lower levels of GDF-9 in PCOS oocytes result in increased KITL levels, and that this effect may be further exacerbated by the reversal of GDF-9 inhibition by excess androgens (Fig. 6.1).

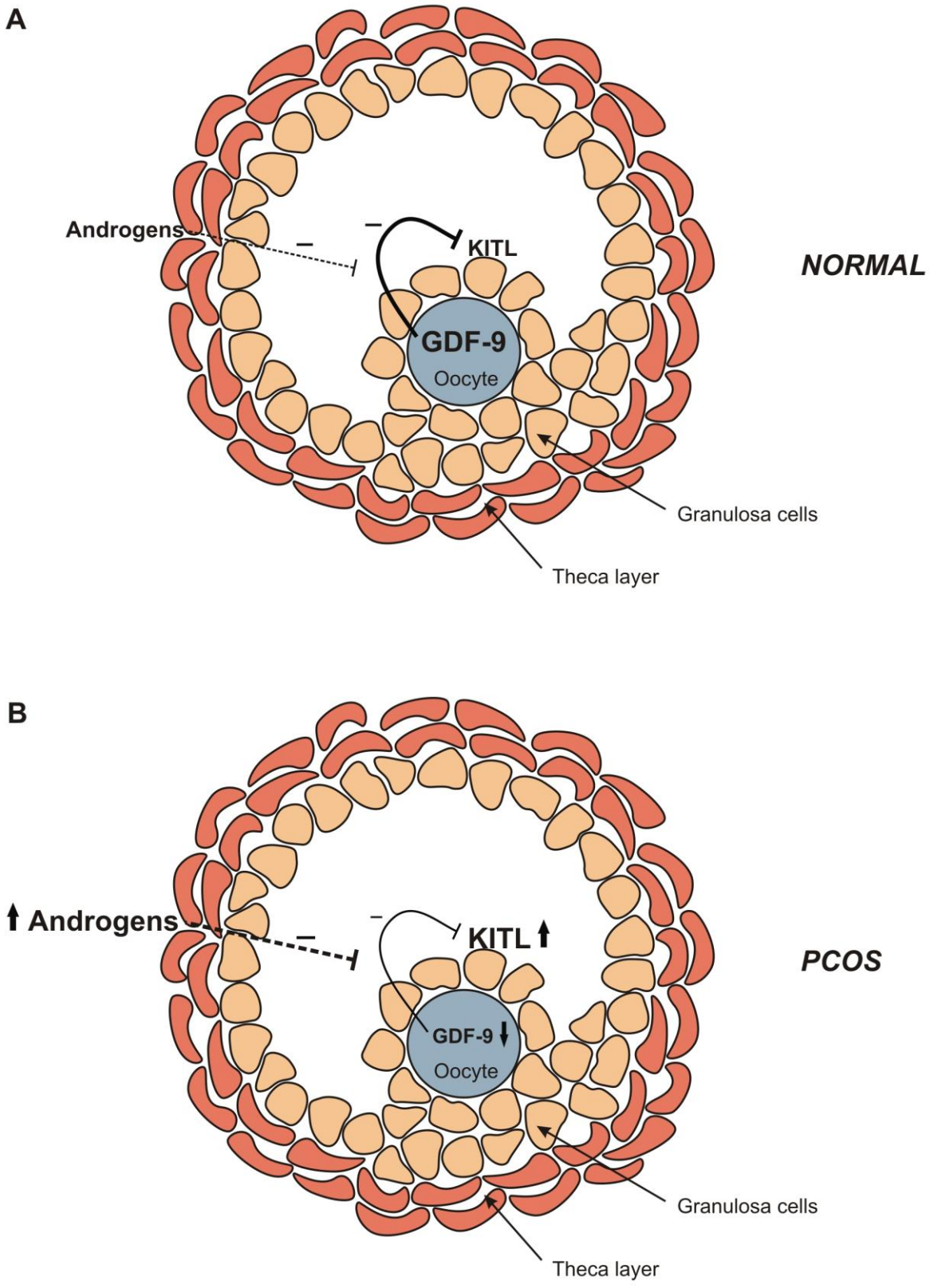

A
Androgens
KITL
GDF-9
Oocyte
NORMAL
Granulosa cells
Theca layer
B
Androgens
KITL
GDF-9
Oocyte
PCOS
Granulosa cells
Theca layer

Figure 6.1. Schematic representation of the working model proposed for perturbed KITL expression in the normal and PCOS ovary. (A) In the normal ovary, *KITL* expression is inhibited by oocyte-secreted factor GDF-9, while theca-derived androgens may partially reverse this inhibition via interaction with the GDF-9 signalling pathway. (B) In the PCOS ovary, *GDF-9* mRNA levels within oocytes are lower than in non-PCOS oocytes (Teixeira Filho *et al.* 2002; Wei *et al.* 2011), possibly resulting in less inhibition of *KITL* gene expression. Excess androgens may act to further reduce the inhibitory effect of GDF-9, thus resulting in abnormally increased KITL protein levels.

6.2 FUTURE DIRECTIONS

While this study has demonstrated that the human ovary expresses multiple KITL and c-kit isoforms, the specific cell types which express each isoform throughout follicle development are yet to be characterised. As discussed earlier in this thesis, each KITL and c-kit isoform has been shown to display markedly different activation, down-stream signalling and receptor-ligand complex downregulation characteristics (Caruana *et al.* 1999). Identifying specific localisation of the isoforms throughout follicle development is important to begin to understand the functions of KITL and c-kit in the human ovary. This could be achieved by the use of laser capture microdissection, which enables selective isolation of specific cell types from follicles of frozen tissue sections that would yield samples of RNA with minimal contamination (Sakurada *et al.* 2006). In addition, identification of the third KITL antibody-immunoreactive protein band, shown in Chapter 3, is important for determining whether a third KITL isoform exists in the human ovary, unlike other mammalian species. Immunoprecipitation of ovarian tissue lysates could be performed using a specific KITL antibody to isolate KITL protein, followed by protein identification using liquid chromatography-electrospray ionisation ion-trap (LC-eSI-IT) mass spectrometry (MS). Characterisation of both KITL and c-kit isoform expression in individual cell types of the ovary has not been performed in any species, and would be valuable in the elucidation of their function and importance in folliculogenesis and ovarian disorders.

One such ovarian disorder is PCOS, a complex fertility disorder with an unknown aetiology. A much larger patient population is required to confirm the preliminary observations my studies have shown of abnormal KITL and c-kit protein expression in PCO compared to non-PCO, in which a major limitation has been the small number of archival PCO and non-PCO tissues available. Furthermore, detailed patient information was absent in most cases, including details such as clinical PCOS diagnosis, serum androgen and hormone levels and body mass index (BMI). Assembling a larger prospective cohort of tissues from clinically diagnosed PCOS patients would enable a more powerful study to be performed, which could include both qualitative and quantitative observations via immunohistochemical staining analysis by an appropriate software program.

The KITL/c-kit system may be utilised as a potential therapeutic target in the treatment of PCOS. Currently, c-kit is a very effective target in the treatment of gastrointestinal stromal tumours (GIST), in which c-kit is known to be a primary "driver" of the disease (Ashman and Griffith 2013). Small molecule kinase inhibitors (SMIs), such as imatinib, have shown striking responses in patients with metastatic disease including increased relapse-free or progression-free survival (Corless *et al.* 2011; Adekola and Agulnik 2012). Success has also been observed in patients with primary disease following treatment with imatinib as an adjuvant to surgery (Dematteo *et al.* 2009; Adekola and Agulnik 2012). A similar approach may be trialled for the treatment of women with PCOS. Inhibitors of KIT kinase activity by drugs such as imatinib may help to alleviate ovarian phenotypes such as

increased numbers of growing follicles, hyperthecosis and excess androgen production, thereby potentially relieving symptoms such as hirsutism. Additionally, it may be possible to specifically target downstream signalling pathways of c-kit that may be involved in specific PCOS pathologies. For example, haematopoetic tumourigenicity was blocked in mice in vivo following pharmacological inhibition of phosphoinositide 3-kinase (PI3K) (Chian *et al.* 2001). Evaluation of agents targeting other downstream effectors, such as AKT and mammalian target of rapamycin (mTOR), are currently underway (Chian *et al.* 2001; Kim and Zalupski 2011). Further investigation into the specific downstream signalling pathways of c-kit in the normal and PCOS ovary may provide specific therapeutic targets for the certain phenotypes, as well as the use of KIT kinase inhibitors as a broader treatment.

To elucidate some of the key factors controlling *KITL* gene expression in the normal ovary, which may lead to abnormal levels of KITL in PCOS, several candidate factors were tested. Oocyte-secreted factor GDF-9 was found to negatively regulate *KITL* expression in human granulosa cells. To specifically determine which SMAD is required for GDF-9 regulation of *KITL*, two approaches could be used. SIS3 is a specific SMAD3 inhibitor, believed to inhibit SMAD3 action via the SXSS motif, which is phosphorylated by TGFβR1 kinase after association of SMADs with the type I TGF-β receptor (TβR1) (Jinnin *et al.* 2006). Both SMAD2 and 3 contain the SXSS motif, but the inhibitor has been proposed to specifically target SMAD3 by interaction with unknown nuclear receptors. Co-treatment of KGN or cumulus cells with GDF-9 and SIS3, as well as treatment with

each factor alone, would determine if SMAD3 is required for GDF-9 to decrease *KITL* gene expression. To support these experiments, levels of activated SMAD2 could be examined in KGN cells treated with or without GDF-9, using a specific phospho-SMAD2 polyclonal antibody. Currently, no antibody is commercially available to specifically detect activated SMAD3 protein. These approaches may also be used for the co-treatment of granulosa cells with GDF-9 and factors such as DHT or BMP-15.

As discussed in Chapter 5, GDF-9 may have a more potent effect on *KITL* gene expression via crosstalk with other factors such as androgens or other oocyte-secreted factors, resulting in an effect at the protein level that may be observable by Western blot. Pooling cumulus cells from several patients with similar characteristics, such as fertility factor and the presence or absence of PCO, would provide enough cells to examine KITL protein levels by Western blot. Co-culture of KGN and human cumulus cells with or without denuded mouse oocytes would be an interesting follow-up experiment to those performed in this thesis. This would enable paracrine communication to occur between the oocyte and granulosa cells, in addition to examining the effect of a non-recombinant GDF-9 on *KITL* gene expression in the presence of other oocyte-secreted factors. In order to investigate potential synergism specifically between GDF-9 and BMP-15 on *KITL* gene expression, co-treatment of these factors would need to be performed in human cumulus cells or the human antral granulosa cell line COV434, as KGN cells do not respond to BMP-15 signalling as discussed in Chapter 5. Furthermore, the evidence shown in Chapter 5 for potential crosstalk between GDF-9 and DHT signalling should be further investigated by

the co-treatment of KGN, COV434 and cumulus cells with GDF-9 and DHT to examine both mRNA and protein levels, as well as including ALK 4/5/7 inhibitor SMP431542 and AR antagonist bicalutamide to confirm the specificity of the effects.

Following on from these studies, validation of the PCOS model proposed in Figure 6.1, would require development of an *in vitro* human follicle experimental system to attempt to recapitulate the events occurring *in vivo*. Isolation of human follicles of varying stages from fresh human ovarian tissue for culture has been performed by a number of groups, and while the technique is still undergoing optimisation for long-term culture and oocyte maturation, follicles have successfully been maintained in culture for up to four weeks (Roy and Greenwald 1996; Abir *et al.* 1997; Abir *et al.* 1999; Abir *et al.* 2001; Rice *et al.* 2007; Barrett *et al.* 2010). To observe the effects of decreased GDF-9 expression in a follicle, injection of small interfering RNA (siRNA) can be used to knockdown *GDF-9* gene expression in oocytes, as previously demonstrated in a study examining bovine oocytes aiming to knockdown (IGF-R1) (Wang *et al.* 2011). siRNA is a synthetic, double-stranded RNA approximately 20-25 nucleotides in length, which enables the transient knockdown of a specific gene target relatively quickly by leading to degradation of the target mRNA. Follicles which had oocytes injected with *GDF-9* siRNA would then have their granulosa cells collected in order to compare KITL gene expression with granulosa cells from non-injected follicles. In addition to examining *KITL* expression, a comprehensive genome analysis could also be performed. Exogenous androgen treatment could be used to simulate hyperandrogenism in order to examine the effects of androgen

on *KITL* gene expression with or without GDF-9 knockdown. Access to fresh human premenopausal ovarian tissue is difficult and is a major limitation to performing these experiments, including the necessary optimisation and inclusion of biological replicates. Such a system could initially be optimised using a mammalian species such as the sheep, which is mono-ovulating and has ovaries of a comparable size and density to the human ovary, minimising potential wastage of human tissues on initial experimental set-up and optimisation. Development of such an experimental system would be a valuable tool for the examination of *KITL* gene expression in human granulosa cells, cultured in a context that more accurately represents an *in vivo* environment.

6.3 SUMMARY AND FINAL CONCLUSIONS

To advance our knowledge of the molecular and cellular biology of the human ovary, leading to better management and treatment of ovarian disease and infertility disorders, more studies examining adult human ovarian tissue are required. Determining how new knowledge gained from animal models may be applied to the human ovary is essential for the development of clinical applications from these findings. The work completed for this thesis examined a growth factor, KITL, and its receptor c-kit, which are well-characterised in the mouse as being essential for fertility, but their role is poorly understood in the human ovary. The findings of this thesis have shown KITL and c-kit expression and localisation throughout follicle development, demonstrating distinct differences in expression patterns to previously studied animal models. Importantly, this thesis has also shown evidence of different c-kit immunostaining patterns in PCO compared to non-PCO, suggesting that abnormal receptor expression may be involved in PCOS pathology in addition to increased KITL levels. Furthermore, this thesis has demonstrated that oocyte-secreted factor GDF-9 regulates *KITL* gene expression in human granulosa cells, warranting further characterisation of its regulatory mechanisms in addition to the effects of other paracrine factors and the implications in PCOS.

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