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Investigation of the P13K/AKT/FOXO and the Ras/Raf/MEK/ERK Signalling Pathways in Ovarian Development

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INVESTIGATION OF THE PI3K/AKT/FOXO

AND THE Ras/Raf/MEK/ERK SIGNALLING

PATHWAYS IN OVARIAN DEVELOPMENT

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ABSTRACT

The formation and development of ovarian follicles is regulated by the interactions of germ cells and somatic cells, intraovarian growth regulatory factors and the pituitary gonadotrophins, FSH and LH. Despite the fact that granulosa cells of large follicles are highly proliferative, granulosa cell tumours are rare suggesting that potent regulatory mechanisms, including apoptotic factors, control proliferation and differentiation of these cells. Two critical pathways that intercommunicate to influence ovarian cell proliferation and differentiation are the PI3K/AKT/FOXO1 pathway and the Ras/Raf/MEK/ERK cascade. As FSH can stimulate both pathways, a super-ovulatory regimen of hormones PMSG/hCG (FSH/LH analogues) administered to immature wildtype mice allowed the investigation of key components in these pathways at different stages of follicular development.

In this project cell cycle regulators and apoptotic markers were investigated to determine the role they play in ovarian development. Specific PI3K and Ras pathway components were studied to decipher how they behaved throughout follicular growth. Results indicated that the exact phosphorylation and expression pattern of proteins from these pathways happens in a very transient, coordinated and timely way. Granulosa cell culture work displayed that extracellular ligands as well as FSH have the ability to stimulate both the PI3K pathway and the Ras pathway in quite a time-dependent manner. The transcription factor CEBPβ is present from once hCG is administered to 5 days post hCG treatment suggesting it plays a role at many stages of follicular development as well as at the corpora lutea regression stage.

Some mutant mouse models were investigated to determine the importance of key components in the PI3K pathway and the Ras pathway. This novel insight into normal ovarian development suggests that it is the specific time dependent expression and cross talk of these pathway components that ultimately results in the coordinated and synchronised growth of immature follicles to the corpora lutea stage.

DECLARATION

I certify that this thesis, which I now submit for examination for the award of MPhil, is entirely my own work and has not been taken from the work of others save and to the extent that such work has been cited and acknowledged within the text of my work.

This thesis was prepared according to the regulations for postgraduate study by research of the Dublin Institute of Technology and has not been submitted in whole or in part for an award in any other Institute of University.

The work reported on in this thesis conforms to the principles and requirements of the Institute's guidelines for ethics in research.

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Candidate

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ABBREVIATIONS

ABL1: Abelson murine leukaemia viral oncogene homolog 1

AC: Adenylate cyclise

ACTH: Adrenocorticotrophic hormone

ADAMTS1: A disintegrin-like and metallopeptidase with thrombospondin motifs-1

AKAP: A kinase anchoring protein

AKT: Protein Kinase B

AP 1: Activator Protein 1

AREG: Amphiregulin

ARKO: Aromatase knock-out

ATP: Adenosine Triphosphate

BrdU: Bromodeoxyuridine

BSA: Bovine Serum Albumin

CAK: cdk-activating kinase

cAMP: cyclic adenosine monophosphate

CBP: CREB binding protein

cDNA: clonal DNA

CDK: Cyclin Dependent Kinase

CDKI: Cyclin Dependent Kinase Inhibitor

C/EBPβ: CAAT enhancer binding protein beta

CKO: Conditional knock out

COC: Cumulus oocyte complex

Cox 2: Cyclo-oxygenase enzyme 2

CRE: cAMP response elements

CREB: cAMP response element binding

CNGC: Cyclic nucleotide-gated channels

CYP19: Aromatase

dNTP: Deoxyribonucleotide triphosphate

eCG: equine chorionic gonadotrophin

E: Estradiol

ECL: Electro-chemiluminescence

EDTA: Ethylenediaminetetraacetic acid

EGF: Epidermal growth factor

EGFR: Epidermal growth factor receptor

Egr1: Early growth regulator-1

EPAC: Exchange protein activated by cAMP

EP2: Prostaglandin receptor

ER: Estrogen receptor

ERK1/2: Extracellular signal related kinase 1/2

ERKO: Extracellular signal related kinase knock-out

ES: Embryonic stem cells

Fo: Forskolin

FOXO1: Forkhead transcription factor 1

Fra2: Fos-related AP1 transcription factor

FSH: Follicle stimulating hormone

FSHR: Follicle stimulating hormone receptor

GAP: GTPase activating protein

GDP: Guanosine diphosphate

GEF: Guanine nucleotide exchange factor

GnRH: Gonadotrophin releasing hormone

GPCR: G protein coupled receptor

GSK-3β: Glycogen synthase kinase 3 beta

GTP: Guanosine triphosphate

HA: Hyaluronic acid

HAS-2: Hyaluronic acid synthase 2

hCG: Human chorionic gonadotrophin (LH analogue)

HCL: Hydrochloric Acid

HRP: Horse radish peroxidise

IAP: Inhibitor of Apoptosis protein

IGF-1: Insulin like growth factor 1

IGF-1R: Insulin like growth factor 1 receptor

IgG: Immunoglobulin G

IL6: Interleukin 6

JAK: Janus kinase

JNK: Jun-amino-terminal kinase

KCL: Potassium Chloride

LAP: Liver enriched transcriptional activator protein

LH: Luteinising hormone

LIP: Liver enriched transcriptional inhibitory protein

MAPK: Mitogen activated protein kinases

MEK1: MAPK/ERK kinase-1

MEK2: MAPK/ERK kinase-2

MEKK: MAPK/ERK kinase kinase

$MgCL_2$: Magnesium Chloride

MMP 19: Matrix metallopeptidase 19

mRNA: messenger RNA

MST1: Mammalian Ste20-like kinase

mTOR: Mammalian target of rapamycin

mTORC: Mammalian target of rapamycin complex

NaCl: Sodium Chloride

NFkB: Nuclear Factor kappa B

NT: Not Treated

PBS: Phosphate Buffered Saline

PCNA: Proliferating Cell Nuclear Antigen

PCR: Polymerase Chain Reaction

PDK 1: Phosphinositide Dependant protein Kinase 1

PFA: Paraformaldehyde

PH domain: Pleckstrin Homology domain

PIAS: Protein Inhibitors of Activated STATs

PI3K: Phosphoinositide-3 kinase

PIP_2: Phosphatidylinositol (4,5) bisphosphate

PIP_3: Phosphatidylinositol (3,4,5) triphosphate

PKA: Protein Kinase A

PMSG: Pregnant mare serum gonadotrophin (FSH analogue)

POS: Polycystic Ovary Syndrome

PR: Progesterone Receptor

Pten: Phosphate and tensin homologue (gene)

PTEN: Phosphate and tensin homologue (Protein)

p70S6k: p70S6 kinase

ras: rat sarcoma homologue gene (gene)

RAS/Ras: rat sarcoma homologue (protein)

RGS2: Regulator of G-protein coupled 2

RTK: Receptor Tyrosine Kinase

RT-PCR: Reverse transcriptase-PCR

SAPK: Stress-Activated Protein Kinase

SDS: Sodium Dodecyl Sulfate

sFRP4: secreted frizzled related protein 4

SGK: Serum Glucocorticoid Kinase

SH: Src homology domain

SOCS: Suppressors of cytokines Signalling

SOS: Son Of Sevenless

SRC: Rous sarcoma oncogene

StAR: Steroidogenic acute regulatory protein

STAT: Signal Transducers and Activators of Transcription

T: Testosterone

TBST: Tris Buffer Saline with Tween

TIMP 1: Tissue inhibitor of matrix metalloprotease 1

TSG6: Tumour necrosis factor induced gene-6

Wnt 4: Wingless-related MMTV integration site 4

3αHSD: 3α-hydroxysteroid dehydrogenase

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1.0 INTRODUCTION

1.1 The Ovary

An ovary is an ovum-producing reproductive organ found in female organisms. Ovaries are oval shaped, measuring approximately 3cm x 1.5cm x 1.5cm in the human. The ovary is located in the lateral wall of the pelvis in a region called the ovarian fossa. The fossa usually lies beneath the external iliac artery and in front of the ureter and the internal iliac artery. Each ovary is attached to the fimbre of the fallopian tube. Usually each ovary takes turns releasing eggs every month, however if there was a case where one ovary was absent or dysfunctional then the other ovary would continue providing eggs to be released. In the human the paired ovaries lie within the pelvic cavity, on either side of the uterus, to which they are attached via a fibrous cord called the ovarian ligament. There are two extremities to the ovary. The end to which the uterine tube attaches is called the tubal extremity. The other extremity is called the uterine extremity which points downwards and is attached to the uterus via the ovarian ligament. The outermost layer is called the germinal epithelium. The tunica albuginea covers the cortex. The ovarian cortex consists of ovarian follicles with stroma in between them. Included in the follicles are the cumulus oophorus, membrane granulose with granulosa cells inside it, corona radiate, zona pellucida and primary oocyte. The zona pellucida, theca of follicle, antrum and liquor folliculi are also contained in the follicle. Also in the cortex is the corpus luteum derived from the follicles. The innermost layer is the ovarian medulla. It can be hard to distinguish between the cortex and medulla, but follicles are usually not found in the medulla. If the egg fails to be released from the ovarian follicle an ovarian cyst may form.

Figure 1.1: Location of ovaries within the uterus
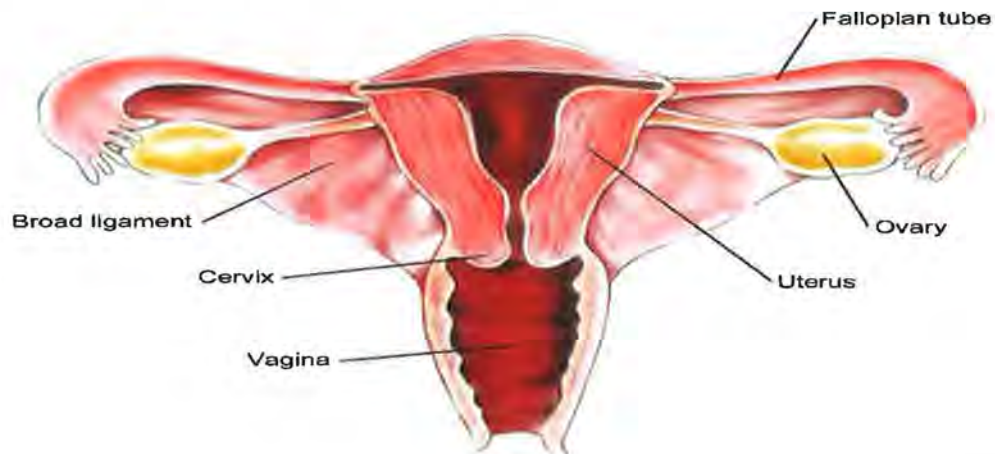
Fig 1.1: Showing where the paired ovaries lie within the pelvic cavity. One on either side of the uterus, to which they are attached via the ovarian ligament (http://ovariancancerosw.org/images.ovary.gif)

1.2 Ovarian Follicular Structure and Development

The follicle is the basic structural and functional unit of the mammalian ovary. Its major function is to produce sexual hormones and support the development of oocytes. According to the stages of development, follicles can be classified into primary follicles, secondary follicles and antral follicles. A follicle is composed of an oocyte in the centre which is surrounded by granulosa cells and theca cells which form the boundary of the follicle. In the antral follicle, the granulosa cells further differentiate into the mural granulosa cells, which line the antrum and basement membrane and the cumulus cells which are found directly adjacent to the oocyte *(Fig 1.2b)*.

Figure 1.2a: Cross section of ovary
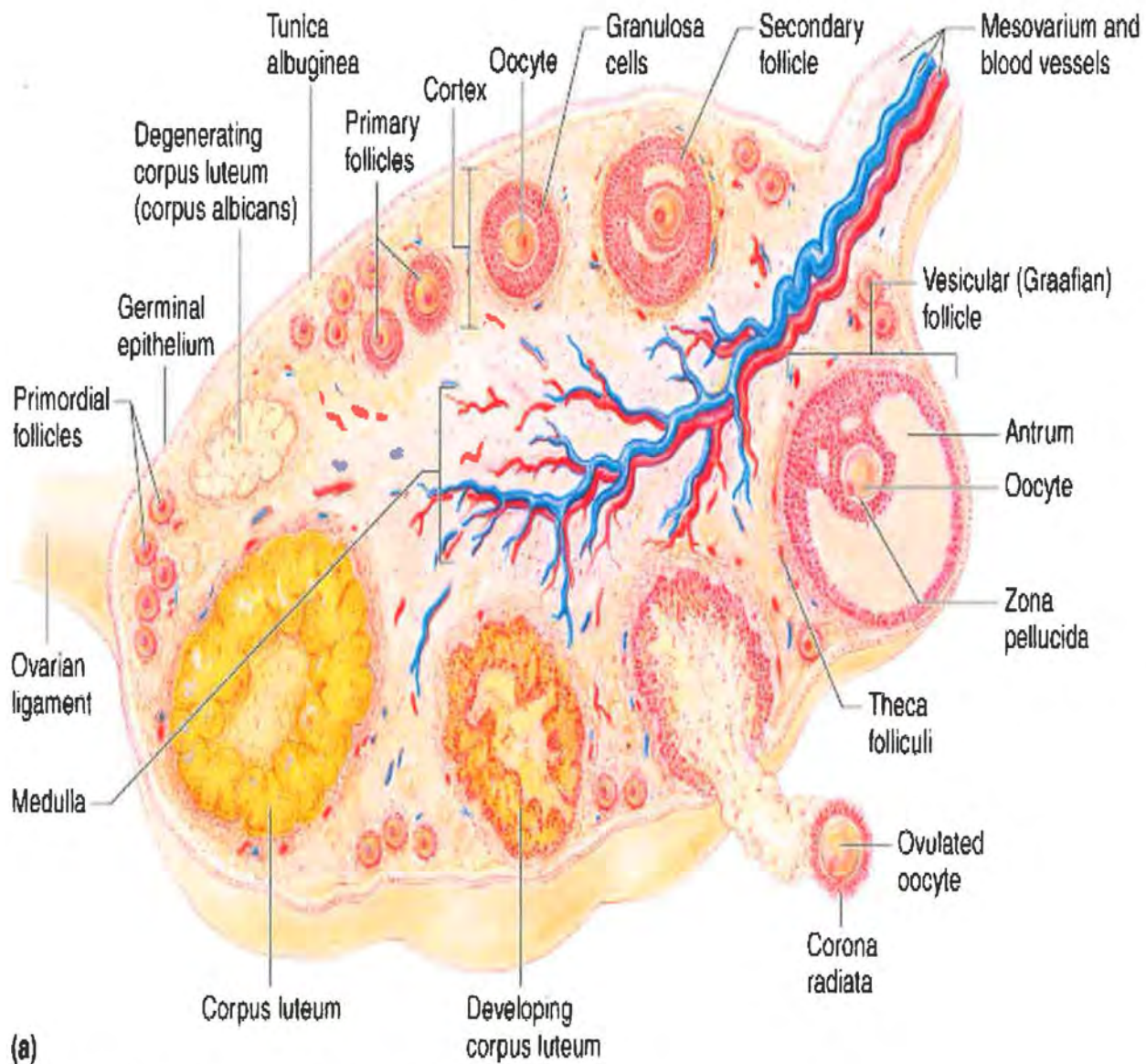
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Fig 1.2a: Showing in a clockwise manner, ovarian development from primordial follicle to the antral follicle which ovulates the oocyte. Development of the corpus luteum is also shown. The ovary has a rich blood supply which spreads out from the medulla of the ovary to the surrounding outer edges. The ovary is attached to the uterus by the ovarian ligament.

Fig 1.2b: Ovarian Histology

Primary follicle
oocyte
cumulus cells
Granulosa cells
Theca cells
Secondary follicle
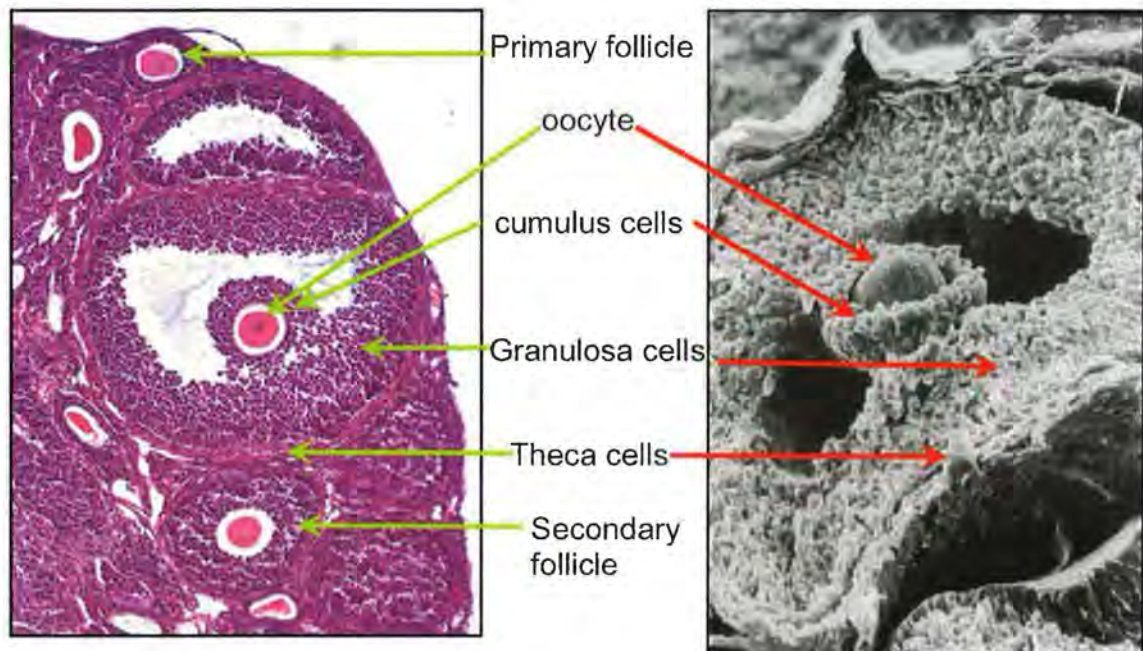
Fig 1.2b: The image on the left is an ovarian section stained with haematoxylin and eosin. The image on the right is a follicle viewed under a scanning electron microscope. According to the stages of development, follicles can be classified into primary follicles, secondary follicles and antral follicles. A follicle is composed of an oocyte in the centre, surrounded by granulosa cells and theca cells which form the boundary of the follicle. In the more mature follicles, outer theca cells are evident. In these follicles the oocyte is surrounded by the Cumulus-Oocyte Complex made up of hyaluronic acid and granulosa cell matrix which provides a structural support and an optimal communicative/signalling microenvironment for the ovarian cells to communicate.

The development of ovarian follicles involves specific stages: initiation, growth, selection, ovulation and luteinisation (Richards JS 1994). At birth the mammalian ovary contains all the oocytes which it will produce in a lifetime. These oocytes remain in a quiescent state until they are individually stimulated to grow by transcription factors (Richards JS 2001a). Preantral follicles are primordial and primary follicles in which the antrum had not yet formed as shown in *Fig 1.2a.* These follicles are classified by the expression of follicle stimulating hormone (FSH) receptors on the surface of granulosa cells and the expression of luteinising hormone (LH) receptors on theca cells. Selection of the follicle destined to ovulate occurs when subtle increases in FSH and LH secretion from the pituitary signals the granulosa cells and theca cells to proliferate and ultimately form an antral or

preovulatory follicle. Preovulatory follicles are characterised histologically by the formation of fluid filled space or antrum. The granulosa cells continue to differentiate during this stage and begin to express LH receptors in

addition to P450 aromatase which are responsible for the conversion of androgens from the theca cells into estrogens. Increasing amounts of estradiol from the preovulatory follicle triggers the pituitary to release surge levels of LH (Downie *et al* 2005). The LH surge induces two key events in the ovary, ovulation and luteinisation.

1.3 Cumulus-Oocyte-Complex Expansion

The cumulus-oocyte-complex (COC) is an important microdomain within the follicle. It consists of the oocyte and 3-4 surrounding layers of specialised cumulus granulosa cells which are separated from the mural granulosa cells by the antrum. Communication between the cumulus cells and the oocyte is critical for COC expansion and ovulation to occur. This can be demonstrated by the infertility of mice null for the gap junction protein connexin 37 which links the oocyte to the adjacent cumulus cells (Salustri A *et al* 1999). COC expansion is initiated by the LH surge and involves the formation of a highly complex extracellular matrix by cumulus cells. The main structural component of this matrix is the glycosaminoglycan hyaluronan. Hyaluronic acid (HA) is a polymer of glucuronic acid and N-acdetylglucosamine and can exceed 5000kDa in size (Chen L *et al* 1990). It has been reported that mice null for the prostaglandin synthesis enzyme called cyclo-oxygenase enzyme 2 (COX-2) and prostaglandin receptor (EP2) exhibit abnormal COC expansion which is associated with decreased tumour necrosis factor induced gene-6 (TSG-6) mRNA levels in cumulus cells, indicating that TSG-6 contributes to the proper formation of the cumulus-derived extracellular matrix (Mukhopadhyay D *et al* 2001), (Ochsner SA *et al*

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2003). Experiments have shown that COC expansion is a critical event which must occur for ovulation to proceed (Russell DL *et al* 2003), (Hizaki H *et al* 1999), (Kennedy CR *et al* 1999). In addition to protecting the oocyte from the proteolytic and mechanical stresses during ovulation, the COC matrix has been shown to play a role in fertilisation by facilitating egg sperm interactions and the subsequent acrosomal reaction (Chen L *et al* 1990), (Hizaki H *et al* 1999).

Figure 1.3: Cumulus Oocyte Complex expansion in vitro and in vivo

LH or hCG

In vivo

FSH/AREG/PGE

In vitro
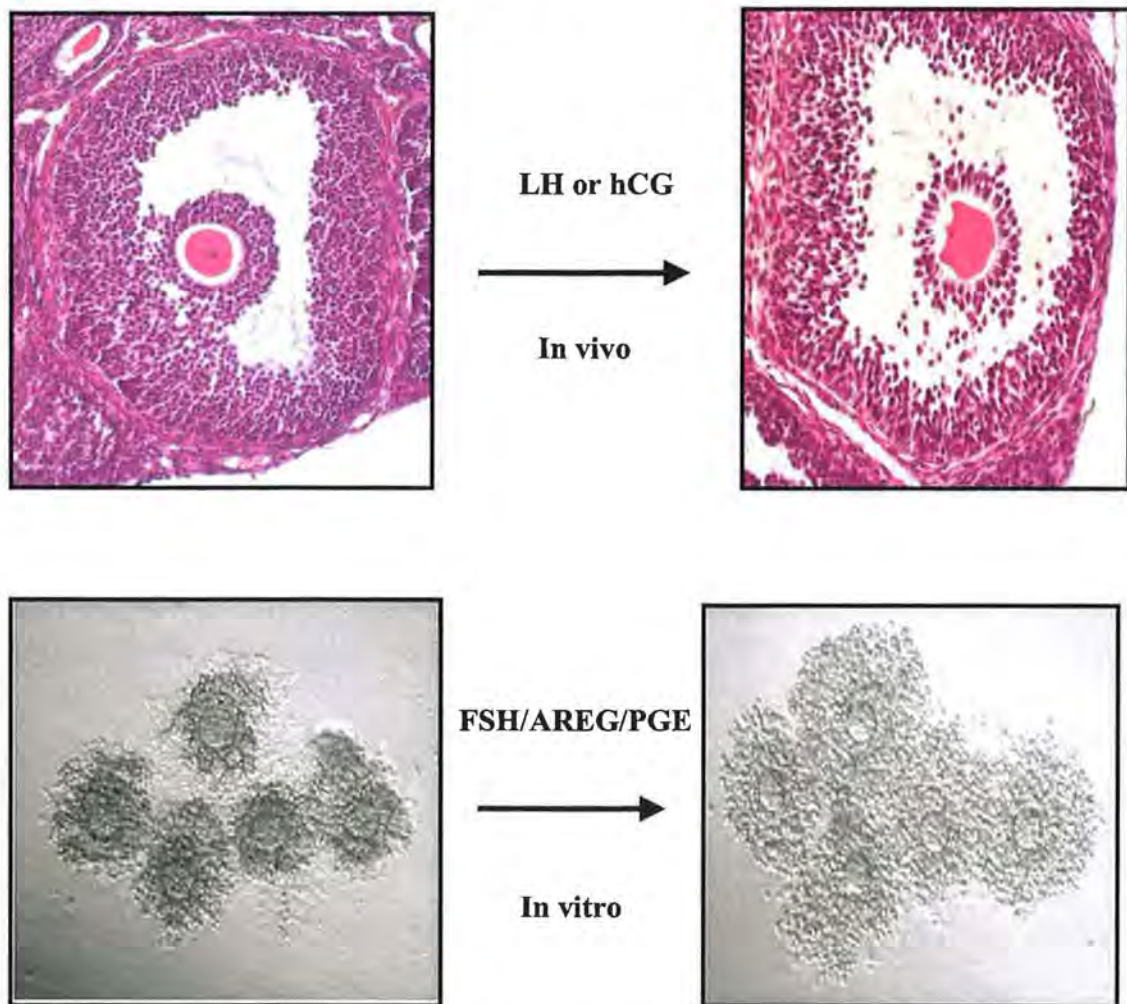
Fig 1.3: Cumulus expansion is another unique feature of ovulation in mammals. With the stimulation of LH or hCG, the cumulus cells produce large amount of matrix molecules such as hyaluronan. The formation of this matrix is critical for successful ovulation and fertilisation. In cultured cumulus-oocyte complexes, cumulus expansion can also be induced by adding FSH, amphiregulin or prostaglandin E2 to the media (Heng-Yu Fan Richards lab).

1.4 The Human Menstrual Cycle

The sequence of events in the menstrual cycle is determined by the relative hormone levels at each stage. The follicular phase of the menstrual cycle spans the first day of menstruation until ovulation. The primary goal during the follicular phase is to develop a viable follicle capable of undergoing ovulation. The early events of the follicular phase are initiated by a rise in FSH levels at the first day of the cycle. The rise in FSH levels can be attributed to a decrease in progesterone and estrogen levels at the end of the previous cycle and the subsequent removal of inhibition of FSH by these ovarian hormones. FSH stimulates the development of 15-20 follicles each month and stimulates follicular secretion of estradiol by upregulating secretion of androgens by the theca externa and by inducing the aromatase enzyme receptor on granulosa cells. FSH further induces expression of FSH receptors on follicles. As estradiol levels increase under the influence of FSH, estradiol inhibits the secretion of FSH and FSH levels decrease (JS Richards 2001a).

Figure 1.4a: The human menstrual cycle
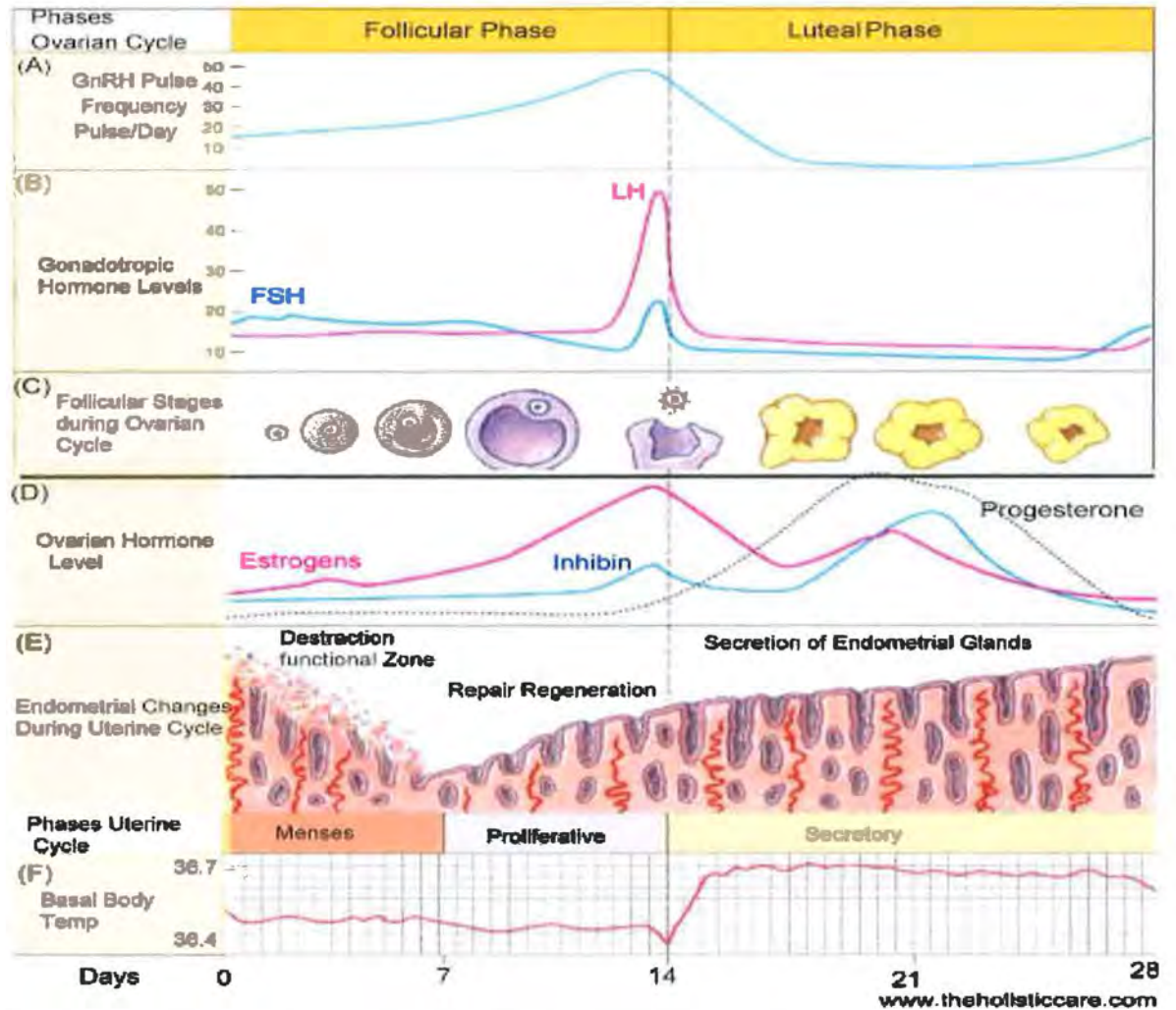
Fig 1.4a: Figure showing gonadotrophin releasing hormone (GnRH) pulse frequency, gonadotrophic hormone levels, follicular development stages, hormone levels, endometrial changes and basal body temperature during the follicular and luteal phase of the menstrual cycle.

FSH functions mainly during follicular growth from the primordial stage to the secondary follicle stage. Once the antrum is formed, a LH surge triggers the oocyte to be released. This ovulated follicle will then develop into a corpus luteum and eventually undergo apoptosis.

Figure 1.4b: Hormonal control of folliculogenesis and ovulation
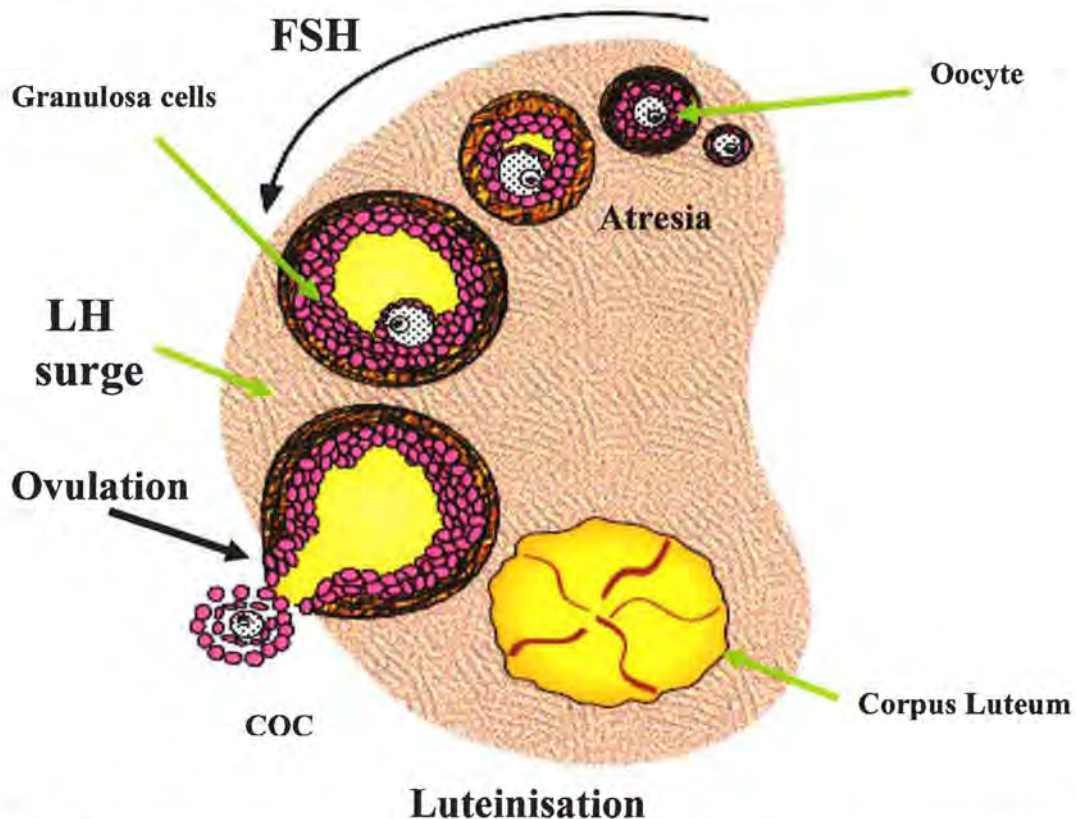
Fig 1.4b: Follicle development in the rodent ovary: Follicles consist of an oocyte surrounded by granulosa and theca cells. FSH binds to the FSH receptor on granulosa cells and together with growth factors induces proliferation of granulosa and theca cells. LH binds its receptor on granulosa cells of preovulatory follicles to induce ovulation and luteinisation. Ovulation is the process by which the cumulus-oocyte-complex (COC) is released from the ovary. Luteinisation results in the formation of the corpus luteum, which will eventually regress. Some follicles will not develop completely and will instead undergo programmed cell death or atresia.

Under normal circumstances, one follicle evolves into the dominant follicle, destined for ovulation, while the remaining follicles undergo atresia. It is currently not known how the dominant follicle is selected, yet it has been observed that the dominant follicle always expresses an abundance of FSH receptors (JS Richards 1994). As FSH levels decrease towards the end of the follicular phase, the developing follicles must compete for relatively small amounts of FSH. The dominant follicle, with its high concentration of FSH receptors, continues to acquire more FSH even as FSH levels decrease. The dominant follicle can continue to synthesise estradiol, which is essential for its complete maturation.

The remaining, poorly FSH receptor-endowed follicles can not produce the requisite amount of estradiol. These follicles cease to develop and ultimately undergo atresia. The dominant follicle matures and secretes increasing amounts of estrogen. Estrogen levels peak towards the end of the follicular phase of the menstrual cycle. At this critical moment, estrogen exerts positive feedback on LH, generating a dramatic preovulatory LH surge. Estrogen can only exert positive feedback on LH at this precise stage in the menstrual cycle, if estrogen is artificially provided earlier in the cycle, ovulation will not be induced. The LH surge is required for ovulation. Under the influence of LH, the primary oocyte enters the final stage of the first meiotic division and divides into a secondary oocyte and the first Barr body. The LH surge induces release of proteolytic enzymes, which degrade the cells at the surface of the follicle, and stimulates angiogenesis in the follicular wall and prostaglandin secretion. These effects of LH cause the follicle to swell and rupture. At ovulation, the oocyte and corona radiata are expelled into the peritoneal cavity. The oocyte adheres to the ovary and muscular contractions of the fallopian tube bring the oocyte into contact with the tubal epithelium to initiate migration through the oviduct. The luteal phase is defined by the luteinisation of the components of the follicle which were not ovulated and is initiated by the LH surge. The granulosa cells, theca cells, and some surrounding connective tissue are all converted into the corpus luteum, which eventually undergoes atresia. The major effects of the LH surge are the conversion of granulosa cells from predominantly androgen-converting cells to predominantly progesterone-synthesising cells, the expression of new LH receptors which fosters increased progesterone synthesis, and reduced affinity of granulosa cells for estrogen and FSH. Combined, these changes promote increased progesterone secretion with some estrogen secretion. Progesterone secretion by the corpus luteum peaks between five and seven days post-ovulation. High progesterone levels exert negative feedback on GnRH and subsequently GnRH pulse frequency decreases. As GnRH pulse frequency decreases, FSH

and LH secretion also decreases. The corpus luteum further loses its FSH and LH receptors. Lacking stimulation by FSH and LH, after 14 days the corpus luteum undergoes atresia and begins evolving into the corpus albicans. With the decline of both estrogen and progesterone levels, an important negative feedback control on FSH is removed and FSH levels rise once again to initiate the next menstrual cycle (Schomberg DW *et al* 1999).

1.5 Hypothalamic-Pituitary-Ovary Axis

The hypothalamus is a region of the brain that controls an immense number of bodily functions. It is located in the middle of the base of the brain and encapsulates the ventral portion of the third ventricle. The pituitary gland lies immediately beneath the hypothalamus, resting in a depression of the base of the skull called the sella turcica (http://www.vivo.colostate.edu/hbooks/pathphys/endocrine/ hypopit/anatomy.html). In the presence of a GnRH pulse, the pituitary and ovarian hormones exert mutual control over the circulating levels of one another. This complex interaction between pituitary and ovarian hormones involves forward control, positive feed-back, and negative feed-back mechanisms which serve to sustain a self-perpetuating monthly endocrine cycle. The levels of circulating hormones are regulated by a feed-back mechanism. Increased levels of circulating estrogen and progesterone have an inhibitory effect on the hypothalamic neurons, which through GnRH decreases the secretion of FSH and LH.

Figure 1.5: Negative feed-back control of gonadotrophin secretion from pituitary gland
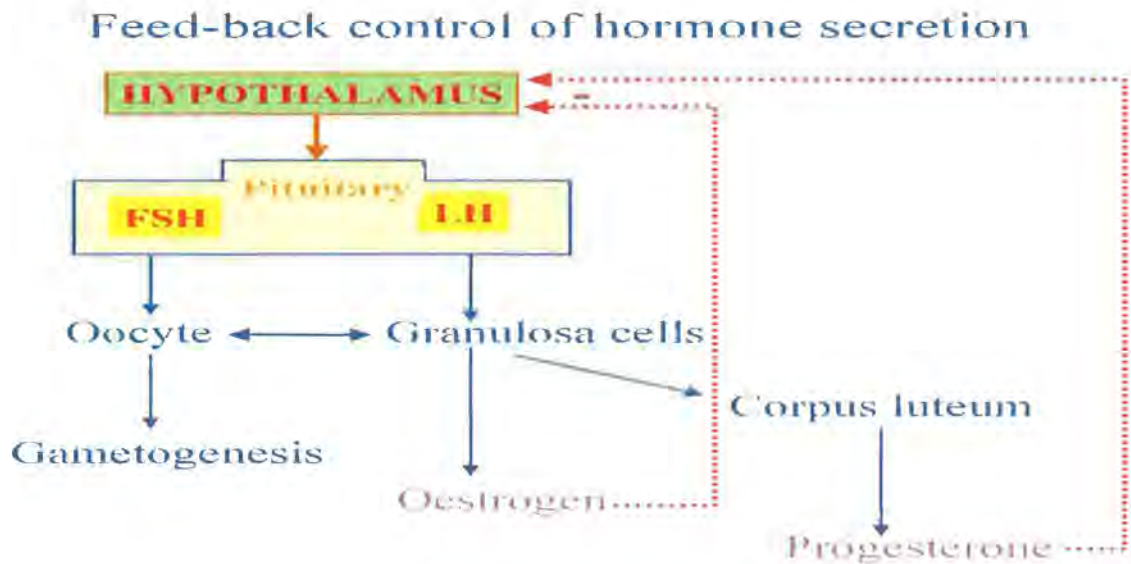
Fig 1.5: As estrogen is produced from the granulosa cells a negative feed-back mechanism back to the hypothalamus results in a decrease in gonadotrophin secretion. Once progesterone is produced from the corpus luteum, a negative feed-back mechanism results in a decrease in gonadotrophin secretion from the pituitary gland (http://staff.um.edu.mt/acus1/CyclicalChanges.htm)

1.6 The Rodent Oestrus Cycle

The normal mouse and rat cycle is 4-5 days, with the ovulatory process requiring 12-14 hours from the LH surge to the release of the oocyte. Typically oestrus cycles continue until death. One difference between the menstrual cycle and the oestrus cycle is that animals have the ability to reabsorb the endometruim if conception does not occur during that cycle. In rodent ovaries, similar to human ovaries, only one or a few follicles can be successfully ovulated in every oestrus cycle. More than 90% of the recruited follicles stop growth at various stages of development and are eliminated by apoptosis. This phenomenon is called follicle atresia. The pattern of follicular development is highly similar in all mammals with the main difference being the rate of growth, the number of follicles which are selected and ovulated within each cycle and the cycle length (Richards JS *et al* 1975).

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The four phases of the oestrus cycle

Proestrus	One or several follicles of the ovary are starting to grow. Typically this phase can last as little as one day or as long as 3 weeks, depending on the species. Under the influence of estrogen the endometrium starts to develop. Some animals may experience vaginal secretions that could be bloody. The female is not yet sexually receptive
Oestrus	Oestrus refers to the phase when the female is sexually receptive. Under regulation by gonadotrophic hormones, ovarian follicles are maturing and estrogen secretions exert their biggest influence. The animal exhibits a sexually receptive behavior, a situation that may be signalled by visible physiologic changes. A signal trait of oestrus is the lordosis reflex, in which the animal spontaneously elevates her hindquarters.
Metestrus	During this phase, the signs of estrogen stimulation subside and the corpus luteum starts to form. The uterine lining begins to secrete small amounts of progesterone. This phase typically is brief and may last 1 to 5 days. In some animals bleeding may be noted due to declining estrogen levels.
Diestrus	Diestrus is characterised by the activity of the corpus luteum that produces progesterone. In the absence of pregnancy the diestrus phase terminates with the regression of the corpus luteum. The lining in the uterus is not shed, but will be reorganised for the next cycle.

Rats typically have rapid cycle times of 4 to 5 days. Although they ovulate spontaneously, they do not develop a fully functioning corpus luteum unless they receive coital stimulation. Fertile mating leads to pregnancy in this way, but infertile mating leads to a state of pseudopregnancy which lasts about 10 days. Mice and hamsters have similar behaviour. The events of the cycle are strongly influenced by lighting periodicity. A set of follicles start to develop near the end of proestrus and grow at a nearly constant rate until the beginning of the subsequent oestrus when the growth rates accelerate eightfold. They then ovulate about 109 hours after starting growth. Estrogen peaks at about 11am on the day of proestrus. Between then and midnight there is a surge in progesterone, LH and FSH,

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and ovulation occurs at about 4am on the next, oestrus day. The following day, metestrus, is called early diestrus. During this day the corpora lutea grow to a maximal volume, achieved within 24 hours of ovulation. They remain at that size for 3 days, halve in size before the metestrus of the next cycle and then shrink abruptly before oestrus of the cycle after that. Thus the ovaries of cycling rats contain three different sets of corpora lutea at different phases of development. (www.embrology.med.unsw.edu.au/otherEmb/mouse2. htm).

1.7 Gonadotrophins

Follicle Stimulating Hormone

FSH is a heterodimer glycoprotein which is comprised of an a- and ß-subunit. The a-subunit is common to both FSH and LH while the ß-subunit is unique to FSH alone. The FSH dimer is synthesised and secreted into the circulation in response to hypothalamic GnRH. FSH exerts its effects on granulosa cells by binding to its receptor and initiating downstream signalling cascades. The FSH receptor is a seven transmembrane G-protein coupled receptor (GPCR). Upon binding ligand the trimeric G protein exchanges guanosine 5'-diphosphate (GDP) for guanosine 5'triphosphatee (GTP) causing the Ga subunit to dissociate from the G protein complex and bind to the membrane bound effector adenylyl cyclase (AC). Activated AC hydrolyses ATP to cAMP to increase intracellular cAMP levels. cAMP can in turn activate three classes of intracellular signalling effectors: the well characterised cyclic AMP dependent protein, protein kinase A (PKA), cyclic nucleotide-gated channels (CNGC), and a class of cAMP-activated guanine nucleotide exchange factors (Exchange protein activated by cAMP-EPAC) (Kawasaki H *et al* 1998), (de Rooij J *et al* 1998), (Richards JS 2001b).

PKA is the best characterised effector in granulosa cells. PKA is activated when cAMP binds to the regulatory subunit of PKA, which causes the dissociation and activation of the C subunit. The C subunit may then either phosphorylate cytoplasmic substrates or translocate to the nucleus to phosphorylate transcription factors and thereby affect gene transcription. FSH primarily effects granulosa cell differentiation by inducing the expression of specific genes that promote the growth of the follicle such as aromatase, the estrogen receptors (ERa/ERß) (Couse JF *et al* 1990), (Dupont S *et al* 2000), (Couse JF *et al* 1999a), (Couse JF *et al* 1999b), IGF-1, IGF-1 receptor (Monget P *et al* 2000), (Zhou J et al 1997) and cyclinD2 (Sicinski P *et al* 1996). These genes have been extensively studied in mutant mouse models lacking the respective genes; all of these models display abnormal ovarian phenotypes which underline the importance they play in the gonad.

Luteinising Hormone

LH, like FSH is a heterodimeric glycoprotein and shares a common a-subunit with FSH, while the ß-subunit is unique to LH. The presence of the LH receptor allows the granulosa cells of preovulatory follicles to be responsive to the surge of LH which initiates both ovulation and luteinisation. LH binds to its cognate receptor which like the FSH-R is a seven pass transmembrane G-protein coupled receptor and primarily activates the PKA pathway. LH induces genes necessary for ovulation in a rapid but transient manner. These genes include: the rate limiting enzyme in prostaglandin biosynthesis *COX-2* (Sirius J *et al* 1992a), (Sirius J *et al* 1992b) and two transcription factors the progesterone receptor *(PR)* (Lydon JP *et al* 1995) and the CAAT enhancer binding protein beta *(C/EBPß)* (Sirios J *et al* 1993). Mice null for these genes fail to ovulate, indicating a clear role for these factors in the process of ovulation. Other genes and their protein products have been shown to be induced by the LH surge include two enzymes which metabolise steroids: prostanoids

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carbonyl reductase (Espey LL *et al* 2000a) and 3a-hydroxysteroid dehydrogenase (3aHSD) (Espey LL *et al* 2001). 3aHSD is a regulator of G-protein coupled signalling (RGS-2) (Ujioka T et al 2000). The cumulus expansion associated gene tumour necrosis factor induced gene-6 *(TSG-6)* (Yoshioka S *et al* 2000) and the transcription factor early growth regulator-1 *(Egr1)* (Espey LL *et al* 2000b) are also induced by the LH surge. Additionally, the LH surge initiates cumulus-oocyte-complex expansion as part of the ovulatory program. This process involves the production of a highly organised matrix by the cumulus cells. While many of the LH-regulated genes listed above *(PR, C/EBPβ, TSG-2 and COX-2)* are expressed rapidly and transiently after the LH surge (4-8hrs), other genes are induced rapidly and sustained over longer periods of time, including *Wnt 4* and *Fz-4* (Hsich M *et al* 2002). Additionally, some LH induced genes are expressed at maximal levels around the time of ovulation, such as *ADAMTS-1* (Espey LL *et al* 2000c), *MMP-19* (Hagglund AC *et al* 1999), *Fz-1* (Hsich M *et al* 2002) *and TIMP-1* (Hagglund AC *et al* 1999), and still yet others are induced later during luteinisation.

Luteinisation occurs after the mature oocyte has been ovulated. The LH surge terminates follicular growth by down regulating genes that were induced by FSH; i.e. *cyclin D2, cyclin E* (Robker RL *et al* 1998a), *aromatase* (Fitzpatrick SL *et al* 1997), LH and FSH receptors (Richards JS 1994). Both the mural granulosa cells and theca cells cease to divide, undergo hypertrophy, and become highly steroidogenic. Genes induced by LH in luteal cells include steriodogenic enzymes, P450scc, *StAR* (steroidogenic acute regulatory protein) (Richards JS 1994), kinases, serum/glucocorticoid induced kinase *(Sgk)* (Alliston TN *et al* 2000), the cell cycle inhibitor $p21^{Cip}$ and $p27^{Kip}$ (Robker RL *et al* 1998b), transcription factors, Activator protein 1(AP1) factors Fra2 (Herdegen T *et al* 1998) and JunD (Sharma SC *et al* 2000), and secreted frizzled related protein *(sFRP4)* (Hsieh M *et al* 2003). All these genes are induced either directly or indirectly by LH and how this occurs is not yet understood.

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How do the FSH and LH receptors work?

Since the induction of LH-responsive genes does not follow one set pattern, multiple mechanisms of LH regulation must exist. One well characterised mechanism of LH-mediated gene regulation involves a principal target of cAMP dependent phosphorylation and activation by PKA, cAMP response element binding protein (CREB). Many LH regulated genes contain cAMP response elements (CRE sites) to which CREB binds, including aromatase (Carlone DL *et al* 1997), inhibin a (Pei L *et al* 1991) and the AP1 factors, c-fos (Mukherjee A *et al* 1996) and fra2 (Herdegen T *et al* 1998). Both FSH and LH activate similar signalling pathways, so how do they elicit differential responses? One theory on how

FSH receptor and LH receptor mediate differential signals is that they are coupled to different cellular signal transducers (Richards JS 1994). There are numerous isoforms of adenylyl cyclase (Sunhara et al 1996) which may transduce differential signals. The FSH and LH receptors may couple to different isoforms of adenylyl cyclase and therefore initiate separate signalling pathways. *Carr et al* has reported that the difference between FSH and LH signalling is not due to differential expression of PKA isoforms in granulosa cells (Carr DW *et al* 1999). Cells of preantral follicles which respond to the LH surge or high levels of cAMP express equivalent amounts of the haloenzymes PKAI and PKAII. However, they did report a difference in interactions between PKA and A-kinase-anchoring proteins (AKAPs) in cells of follicles which respond to FSH and those that primarily respond to LH. AKAPs are a family of 25 individual proteins that bind to RII dimers of PKAII with nanomolar affinity (Carr DW *et al* 1999). These complexes are anchored through the targeting substrate phosphorylation in a specific location. The differential binding of AKAPs to R subunits suggests a redistribution of PKA to a different subcellular fraction and therefore possibly the phosphorylation of a specific set of genes.

Additionally, FSH has been shown to phosphorylate protein kinase B (Akt) and Sgk through the activation of the PI3 kinase signalling pathway (Gonzalez-Robayna IJ *et al* 2000). Richards *et al* propose that the activation of the PI3 kinase pathway by cAMP may occur through the activation of cAMP-guanine nucleotide exchange factor (cAMP-GEF) or exchange proteins activated by cAMP (Epac) (Richards JS 2001b). These proteins may activate Ras which in turn can activate the PI3 kinase signalling pathway (Pham N *et al* 2000). This mechanism appears to be independent of PKA, suggesting that FSH activates alternative signalling pathways.

1.8 Steroidogenesis

Figure 1.8: Production of estradiol from cholesterol
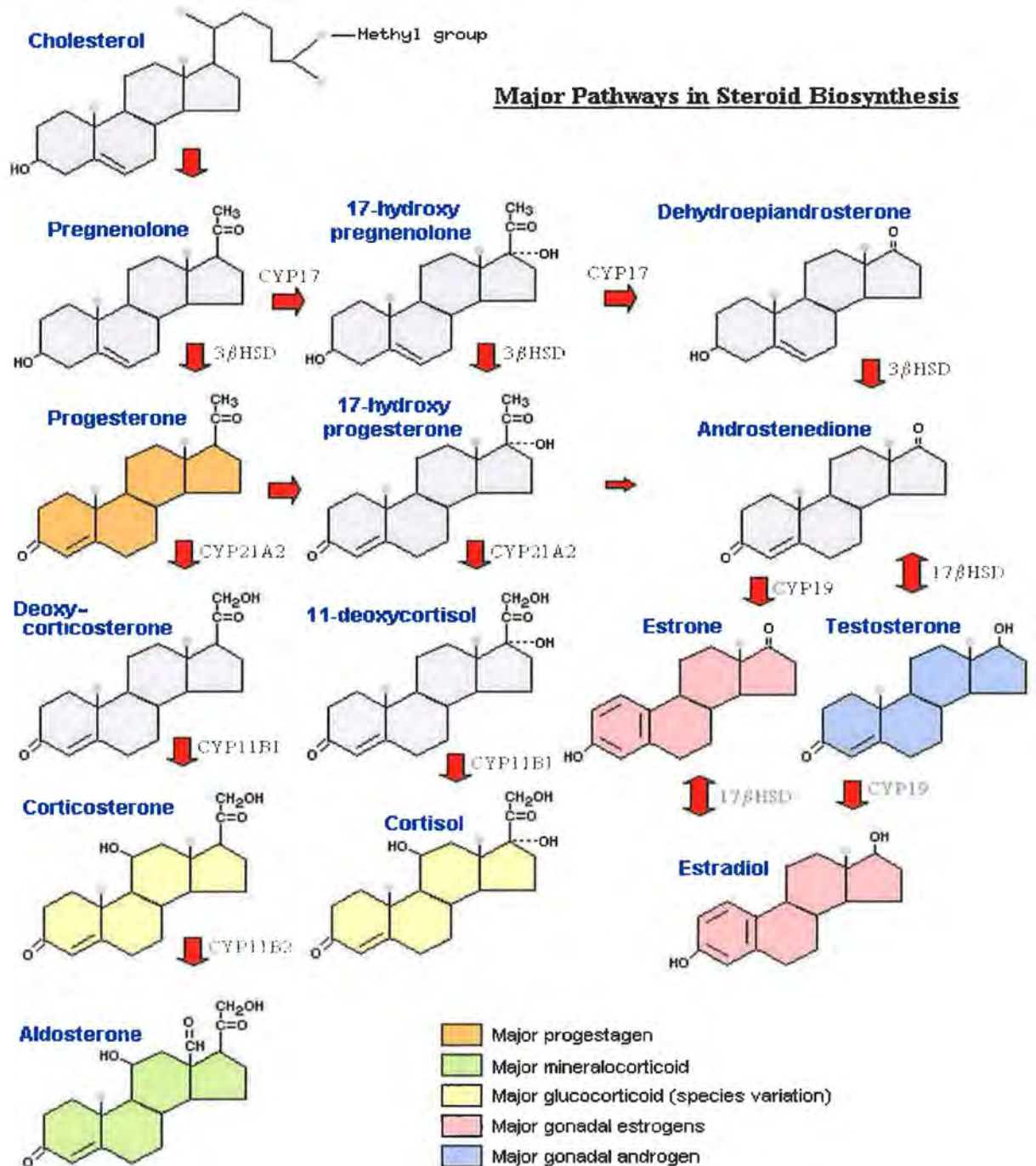
Fig 1.8: Showing the production of estradiol from cholesterol and the key enzymes involved in this steroid biosynthetic pathway (www.vivo.colostate.edu/hbooks/pathway/endocrine/basics/steroiogensis/htm).

FSH and LH are steroid hormones which are derivatives of cholesterol that are synthesised by a variety of tissues, most prominently the adrenal gland and gonads. The cholesterol precursor comes from

cholesterol synthesised within the cell from acetate, from cholesterol ester stores in intracellular lipid droplets or from uptake of cholesterol-containing low density lipoproteins. Lipoproteins taken up from plasma are most important when steroidogenic cells are chronically stimulated. Biosynthesis of steroid hormones requires a battery of oxidative enzymes located in both mitochondria and endoplasmic reticulum. The rate-limiting step in this process is the transport of free cholesterol from the cytoplasm into mitochondria. Within mitochondria, cholesterol is converted to pregnenolone by an enzyme in the inner membrane called CYP11A1 (*Fig 1.8*). Pregnenolone itself is not a hormone, but is the immediate precursor for the synthesis of all of the steroid hormones. Aromatase serves to convert androgens from the theca cells to estrogen. The importance of aromatase to follicular development is illustrated by mice lacking this gene, aromatase knock out mice (ARKO). ARKO mice have elevated levels of both LH and FSH and folliculogenesis is arrested at the antral stage (Fisher CR *et al* 1998). Aromatase activity increases estrogen which in turn acts via the ERa and ERß receptors. Ablation of each ER isoform individually (aERKO and ßERKO) as well as together (aßERKO) has revealed that both isoforms are important for follicular development and ovulation. aERKO mice exhibit increased levels of serum LH which leads to accelerated follicle growth (Schomberg DW *et al* 1999). These mice do not ovulate unless stimulated with exogenous hormones (superovulation) at a young age, however they ovulate fewer oocytes and have more oocytes that become entrapped in corpora lutea than wild-type mice, indicating an important role for ERa in ovulation. While ERß is the prominent isoform in granulosa cells (Sharma SC *et al* 1999), ßERKO mice still remain fertile, however their ovulatory capacity is reduced from that of wild-type mice (Krege JH *et al* 1998). aßERKO mice have

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a much more severe ovarian phenotype than the individual knockout models. These mice do not ovulate and have even higher levels of serum LH than the aERKO mice, indicating that both isoforms are needed for estradiol-mediated regulation of LH secretion from the pituitary.

Role of Estrogen

One primary role of estrogen in the ovary is to enhance the effects of FSH by maintaining FSH receptor levels (Tonetta SA *et al* 1989) and inducing the expression of specific genes including the LH receptor (Wang XN *et al* 1993), cyclin D2 (Robker RL et al 1998a), the transcription factor Forkhead (FOXO), components of the insulin growth factor (IGF-1) pathway, IGF-IRß subunit and the glucose transporter Glut-1 (Richard JS 2001a). IGF-1 and its receptor also serve to enhance FSH action in the growing follicle by presumably increasing energy utilisation and cell survival in granulosa cells (Richard JS 2001a). Cyclin D2 is a cell cycle associated factor that promotes cellular proliferation by positively affecting the kinase cascades that regulate transitions through the checkpoints of the cell cycle (Inaba T *et al* 1992). In addition to the regulation of the above genes, the importance of FSH to follicular growth is demonstrated by the fact that mice lacking the FSHß subunit of the FSH receptor (Kumar TR *et al* 1997), (Dierich A *et al* 1998), (Abel MH Wootton AN *et al* 2000) present with impaired follicular growth at the preantral stage.

1.9 Cell cycle regulators

Regulation of the cell cycle within any cell type is complex, involves the balance of many regulatory molecules, and can be altered by numerous external signals acting at multiple steps in the cycle. In the ovary, estradiol, FSH, and LH are essential signals for the growth of preovulatory follicles and their subsequent terminal differentiation as corpora lutea.

Each hormone acts via specific receptors and intracellular signalling pathways. Additionally, FSH and LH act by controlling distinct levels of cAMP (Jonassen JA *et al* 1980). The growth of ovarian follicles, ovulation, and the formation of the corpus luteum involves dramatic changes in granulosa cell function. The changes are sequential and are dictated by specific, tightly regulated responses to gonadotrophins, steroids, and growth factors (Hirshfield AN *et al* 1991), (Pederson T 1970), (Richards JS 1978). One of the most dramatic changes in granulosa cell function is the rapid switch from the highly proliferative stage characterising granulosa cells of preovulatory follicles to the nonproliferative, terminally differentiated phase of luteal cells. In primordial follicles, the oocyte is surrounded by a single layer of nondividing granulosa cells arrested in Go phase of the cell cycle. Primordial follicles leave this quiescent state and initiate a phase of slow growth in which the granulosa cells have entered the cell cycle but proliferation is exceedingly slow (Hirshfield AN *et al* 1991). However, as these slowly dividing granulosa cells acquire enhanced responsiveness to FSH and LH and begin producing estradiol, exposure to these hormones triggers a rapid burst of proliferation that results in the formation of large preovulatory follicles (Rao MC *et al* 1978). Granulosa cells of these preovulatory follicles not only are highly proliferative but are also differentiating and acquire LH receptors (Richards JS 1994). The LH surge then triggers dramatic changes in both follicular structure and function. LH terminates follicular growth by causing granulosa cells of preovulatory follicles to exit the cell cycle (Hirshfield AN *et al* 1991), (Rao MC et al 1978) and rapidly initiates a program of terminal differentiation (luteinisation) in which the cells cease to divide (Richards JS 1994).

This cell cycle progression and proliferation is controlled by a balance of positive and negative regulators converging on cell cycle kinase cascades. The pivotal roles of Cyclin D2 and p27^{Kip1} in ovarian follicular growth and differentiation are indicated by their selective expression and regulation in the ovary and their critical and opposing effects on

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cyclin dependent kinase (CDK) activity that controls entry and progression through G1 of the cell cycle. Members of the CDK family play a central role in controlling the normal orderly progression of the cell through the division cycle *(Fig 1.9)*. Early in G1 phase, the presence of D-type Cyclins activates CDK4 or CDK6. The binding of Cyclin D and CDK4/6 results in the formation of a complex that is then phosphorylated by CDK-activating kinase (CAK). The active Cyclin-CDK complex in turn phosphorylates cellular substrates that regulate DNA synthesis. The Cip/Kip family of CDK inhibitors, which includes $p27^{KIP1}$, acts to block cell cycle progression by binding and inhibiting the activity of CDKs. The Cip/Kips ($p21^{Cip1}$, $p27^{Kip1}$, $p57^{Kip2}$) have relatively broad specificity and are able to bind not only CDK4/6, but also CDK2 and CDC2, enabling them to inhibit the activity of several kinase cascades and thereby block cell cycle progression at multiple points (Robker RL et al 1998b).

In the ovary, Cyclin D2 is highly expressed, specifically in granulosa cells, as is CDK4 (Rhee K *et al* 1995) while Cyclin D1 and Cyclin D3 are barely detectable (Robker RL *et al* 1998a) and mice lacking Cyclin D1 exhibit normal ovarian function (Sicinski P *et al* 1995). In contrast, Cyclin D1 and Cyclin D3 are expressed in an overlapping pattern, with both expressed at the highest levels in theca cells (Robker RL *et al* 1998a). Cyclin D2 acts as a positive regulator of cell cycle progression by its ability to bind CDK 4 or 6 and thereby activate a cascade of events that permits progression through G1 phase of the cell cycle (Xiong Y *et al* 1992). In mice null for Cyclin D2, granulosa cell proliferation is impaired, the ovarian follicles remain small, and ovulation fails to occur (Sicinski P *et al* 1996). In contrast, $p27^{Kip1}$ blocks cell cycle progression by inactivating these same CDK cascades, and cells remain in G1 phase (Polyak K *et al* 1994). Both $p27^{Kip1}$ and a related family member, $p21^{Cip1}$, are highly expressed in the corpus luteum with only slightly different patterns of induction (Robker RL *et al* 1998a). However, they appear to play unique roles in this tissue. Mice lacking $p27^{Kip1}$ exhibit an altered ovarian phenotype,

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follicular growth is not compromised but granulosa cells do not luteinise properly in response to LH (Nakayama K *et al* 1996), (Kiyokawa H *et al* 1996), (Fero ML *et al* 1996).

Figure 1.9 CDK Inhibition during the cell cycle
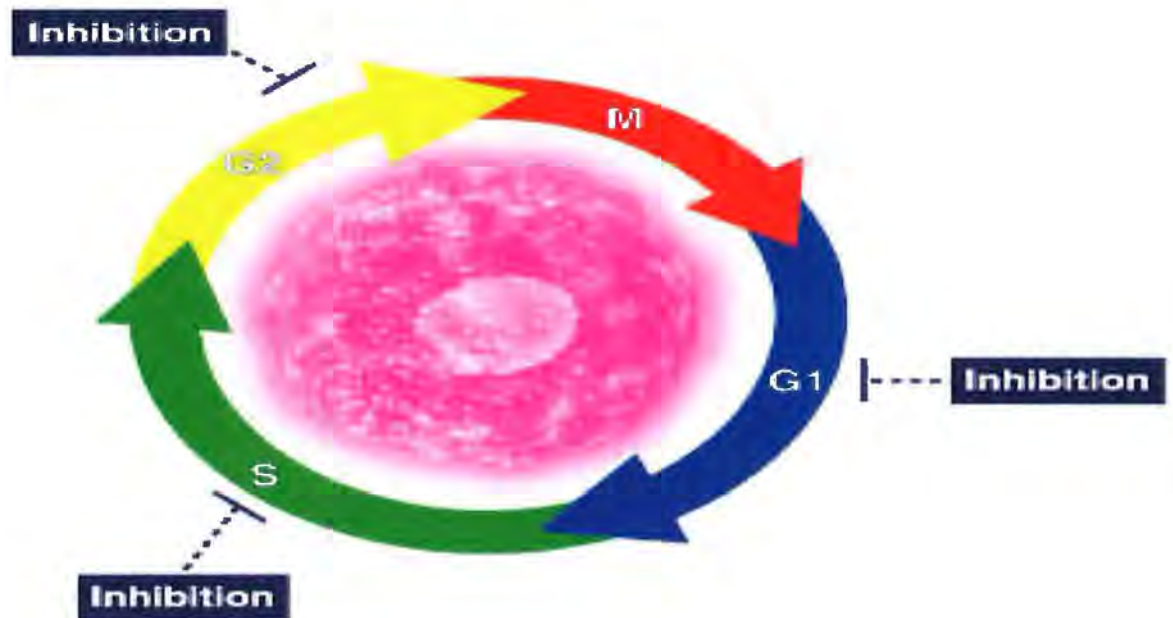
Fig 1.9: CDK can inhibit at various stages of the cell cycle (http://www.cancerline.com/CancerLineHCP/15602_18505_9_5_0.aspx)

Many cancers exhibit at least one alteration in the complex mechanisms controlling CDK activity, such as over-expression of Cyclins or loss of CDK inhibitor expression, which can lead to uncontrolled cell division. Therefore targeting CDKs in tumour cells offers an attractive therapeutic approach for a wide range of tumour types.

In order to better understand the control of cellular proliferation in the ovary, we analysed the expression of these cell cycle regulatory molecules and their regulation by hormones during follicular development, specifically when granulosa cell proliferation is rapid and during luteinisation when cell division has terminated. The results presented highlight the ability of the LH surge to acutely regulate Cyclin D2 and p27^{Kip1} in an inverse manner.

1.10 Study Design

Mice were used for western blot procedures using whole ovary lysates, immunofloursescence staining and immunohistochemistry staining. Rats were used for granulosa cell isolation and cell culture work. (Note: In the case of figure 4.13 mice granulosa cells were cultured to use for the longer hCG series for comparison purposes with the mouse hCG series in figure 4.12. Also in figure 4.17 demonstrating a comparison of wildtype ovary with Pten CKO ovary, granulosa cells were isolated).

Figure 1.10: Study Design showing how mice and rat ovaries were treated

Mice work

Mice were injected with PMSG and 48 hours later with hCG at 2, 4, 8, 12 and 16 hour time points. A non-treated (NT) mouse was used as a negative

Sacrificing mice after various stages of hCG treatment allowed the investigation of key pathway components at specific stages of follicular development

Western Blot: Ovaries were removed, lysed, sonicated and quantified by Bradford assay. 5 ovaries were pooled and used for each time point

Immunofluorescence staining: Ovaries were snap frozen once removed. Haematoxylin and Eosin staining: Ovaries were paraffin wax embedded once removed

Rat work

Rats were injected with estradiol for 3 consecutive days

Ovaries were now at the preovulatory stage and so allowed us to investigate key pathway components in immature granulosa cells

Rats were sacrified and ovaries removed. Granulosa cells were isolated from rat ovaries by poking the ovaries using syringes

Granulosa cells were cultured overnight and treated with various ligands. Cells were then lysed and used for western blot analysis/mRNA extraction

1.11 Cell Signalling Pathways in the Ovary

The epidermal growth factor receptor (EGFR) family plays an important role in cell lineage determination, the morphogenesis of many organs and in cell survival in the adult (Yarden 2001, Jorrissen *et al*, 2003; Nair 2005, Henson and Gibson 2006). Moreover, activating mutants and over-expression of these family members contribute to oncogenesis by inducing cells to proliferate and to resist apoptosis. ErbB3, a member of the EGFR family, has an inactive kinase domain therefore these receptors are thought to serve as co-receptors. Amphiregulin is a ligand for the ErbB family which binds to EGFR. Upon ligand-binding, receptors homo-dimerise or hetero-dimerise triggering tyrosine trans-phosphorylation of the receptor sub-units. Intracellular tyrosine kinases of the Src family and Abl family are also able to tyrosine phosphorylate ErbB receptors. These tyrosine phosphorylated sites allow proteins to bind through their Src homology 2 (SH2) domains leading to the activation of downstream signalling cascades including the RAS/extracellular signal regulated kinase (ERK) pathway, the PI3K pathway and the JAK/STAT pathway. Differences in the C-terminal domains of the ErbB receptors govern the exact second messenger cascades that are elicited conferring signalling specificity. The EGF signal is terminated primarily through endocytosis of the receptor-ligand complex. The contents of the endosomes are then either degraded or recycled to the cell surface.

Figure 1.11: An overview of signalling pathways investigated in this study
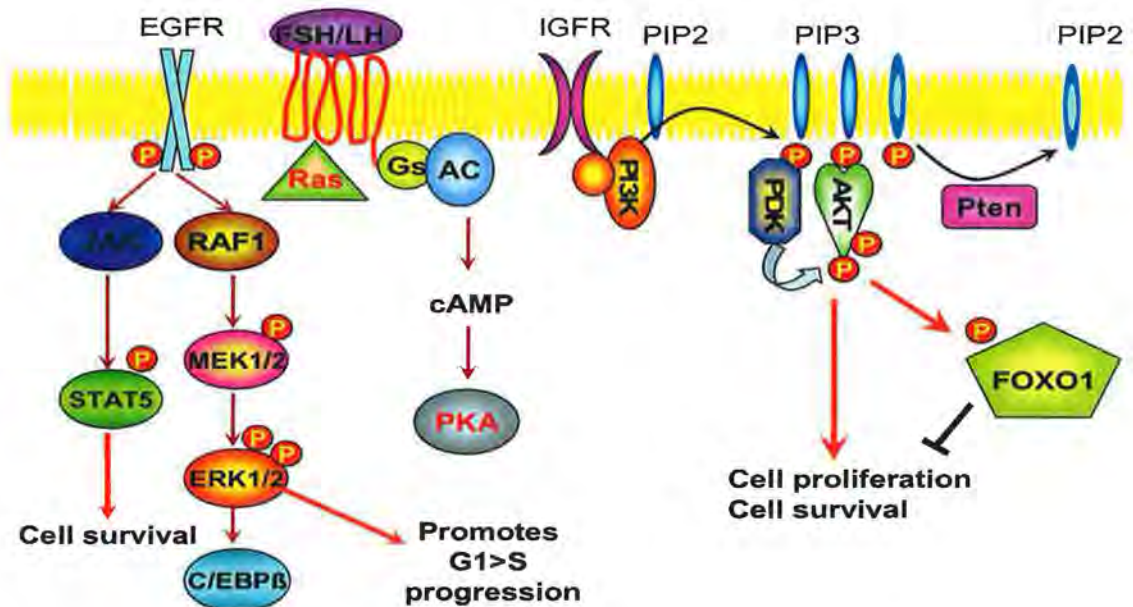
Fig 1.11: Once FSH/LH binds its 7-transmembrane G-protein coupled receptor the main pathway activated is the PKA pathway. FSH/LH binding can also induce the activation of the JAK/STAT pathway and the Ras/Raf/MEK/ERK pathway through EGFR receptor activation. It is though that once FSH/LH binds its receptor, Ras is activated and binds and activates the EGF receptor. Activation of the EGF receptor results in autophosphorylation of key tyrosine residues. These tyrosine phosphorylated sites allow proteins to bind through their Src homology 2 (SH2) domains and leads to the activation of downstream signalling cascades. Ras can also bind the IGF receptor resulting in PI3K activation and the resulting activation of the PI3K/AKT/FOXO pathway. These pathways act in a coordinated manner to promote cell survival.

1.11 (i) PI3 kinase pathway

Epidermal Growth Factor (EGF) promotes cell survival through the activation of PI3K signalling (Henson and Gibson 2006). PI3K are a family of related enzymes that are capable of phosphorylating the 3 position hydroxyl group of the inositol ring of phosphatidylinositol. EGF triggers the recruitment of PI3K to activated ErbB receptors, which is mediated by the binding of SH2 domains in PI3K to phosphorylated tyrosine residues. The catalytic subunit of PI3K in turn phosphorylates phosphatidylinositol (4,5) bisphosphate (PIP_2) leading to the formation of phosphatidylinositol (3,4,5) triphosphate (PIP_3). The PI3K family is composed of Class I, II and III, with Class I kinases being the

only type with the ability to convert PIP_2 to PIP_3 on the inner leaflet of the plasma membrane. PI3K can also activate *ras* (rat sarcoma homologue gene), resulting in the activation of ERK signalling, thereby facilitating cross-talk between survival pathways. A key downstream effector of PIP_3 is Protein Kinase B (AKT). AKT requires the formation of the PIP_3 molecule in order to be translocated to the cell membrane. AKT promotes cell survival through the transcription of anti-apoptotic proteins. A downstream target of AKT is glycogen synthase kinase 3 (GSK3). Under basal conditions the constitutive activity of GSK3 leads to the phosphorylation and inhibition of a guanine nucleotide exchange factor eIF2B, which regulates the initiation of protein translation. Therefore, upon inactivation of GSK3 by AKT, eIF2B is dephosphorylated resulting in the promotion of protein synthesis and the storage of amino acids (Lizcano and Alessi 2002). AKT also activates mammalian target of rapamycin (mTOR), which promotes protein synthesis through p70 ribosomal S6 kinase (p70s6k) and inhibition of eIF-4E binding protein (4E-BP1) (Asnaghi *et al* 2004). Collectively, these processes all promote cell growth and survival in response to EGF.

FSH can activate the PI3K pathway by associating with its G-protein coupled receptor (FSHR) which binds the p85 subunit of PI3K and activates the p110 catalytic subunit of PI3K. This mediates the phosphorylation of PIP_3, a key signalling intermediate that recruits and activates downstream molecules. AKT (originally identified as the oncogene in the transforming retrovirus, AKT8 by Dr. Philip TSichlis at Fox Chase Cancer Centre in the 1990's) is one key downstream target of PI3K, activated by serine and threonine phosphorylation. AKT possesses a protein domain known as Pleckstrin Homology (PH) domain, named after Pleckstrin, the protein in which it was first discovered. This domain binds to PIP_2 or PIP_3 with high affinity. Once correctly positioned in the membrane via binding of PIP_3, AKT can then be phosphorylated by its activating kinases, phosphoinositide dependent kinase 1 (PDK1 at threonine 308) and

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mTORC2 (mammalian target of rapamycin complex 2) at serine 473. Phosphorylation of mTORC2 stimulates the subsequent phosphorylation of AKT by PDK1. Activated AKT can then go on to activate or deactivate its myriad substrates via its kinase activity. In addition to AKT and PDK1, one other related serine threonine kinase is bound at the PIP_3 molecule created as a result of PI3K activity, SGK. SGK and AKT can phosphorylate the forkhead transcription factor 1 (FOXO1) rendering it inactive and excluding it from the nucleus *(Fig 1.11(i))*. FOXO factors impact a diverse number of cellular processes including apoptosis, cell cycle progression, DNA repair, responses to oxidative stress as well as longevity. Recent studies indicate that these diverse effects of FOXO1 may be regulated by a diverse set of kinases that phosphorylate specific residues in the FOXO molecule. For example, FOXO1 can be phosphorylated by kinases that are linked to cell cycle progression (CDK2), oxidative stress (AMPK) and apoptosis (MST1 and JNK). Interestingly phosphorylation of FOXO1 by CDK2 promotes nuclear exclusion whereas phosphorylation by stress-related kinases promotes nuclear retention and presumably increased transcriptional activity of FOXO factors (Huang *et al* 2006). Thus the cellular localisation of FOXO factors is regulated by more complex mechanisms than originally thought. $p27^{Kip}$ has been identified as a FOXO1 regulated gene in liver endothelial cells. FOXO factors appear to work in a cell context specific manner. In general, growth factors activate the PI3K/AKT pathway leading to FOXO phoshorylation and inactivation and often cell differentiation (Stahl *et al* 2002).

Apart from being a downstream effector of PI3K, AKT may also be activated in a PI3K-independent manner. Studies have suggested than cAMP-elevating agents could activate AKT through protein kinase A (PKA), although the mechanism of action is unclear. PI3K dependent AKT activation can be regulated through the tumour suppressor phosphate and tensin homologue (PTEN) which works essentially as the

opposite of PI3K. PTEN acts as a phosphatase to dephosphorylate PIP_3 back to PIP_2. This removes the membrane-localisation factor from the AKT signalling pathway. Without this localisation, the rate of AKT activation decreases significantly, as do all the downstream pathways that depend on AKT for activation. PTEN is absent in many tumours, hence PI3K activity contributes significantly to cellular transformation and the development of cancer.

Figure 1.11 (i): FSH regulation of the PI3K/AKT/FOXO pathway in granulosa cells
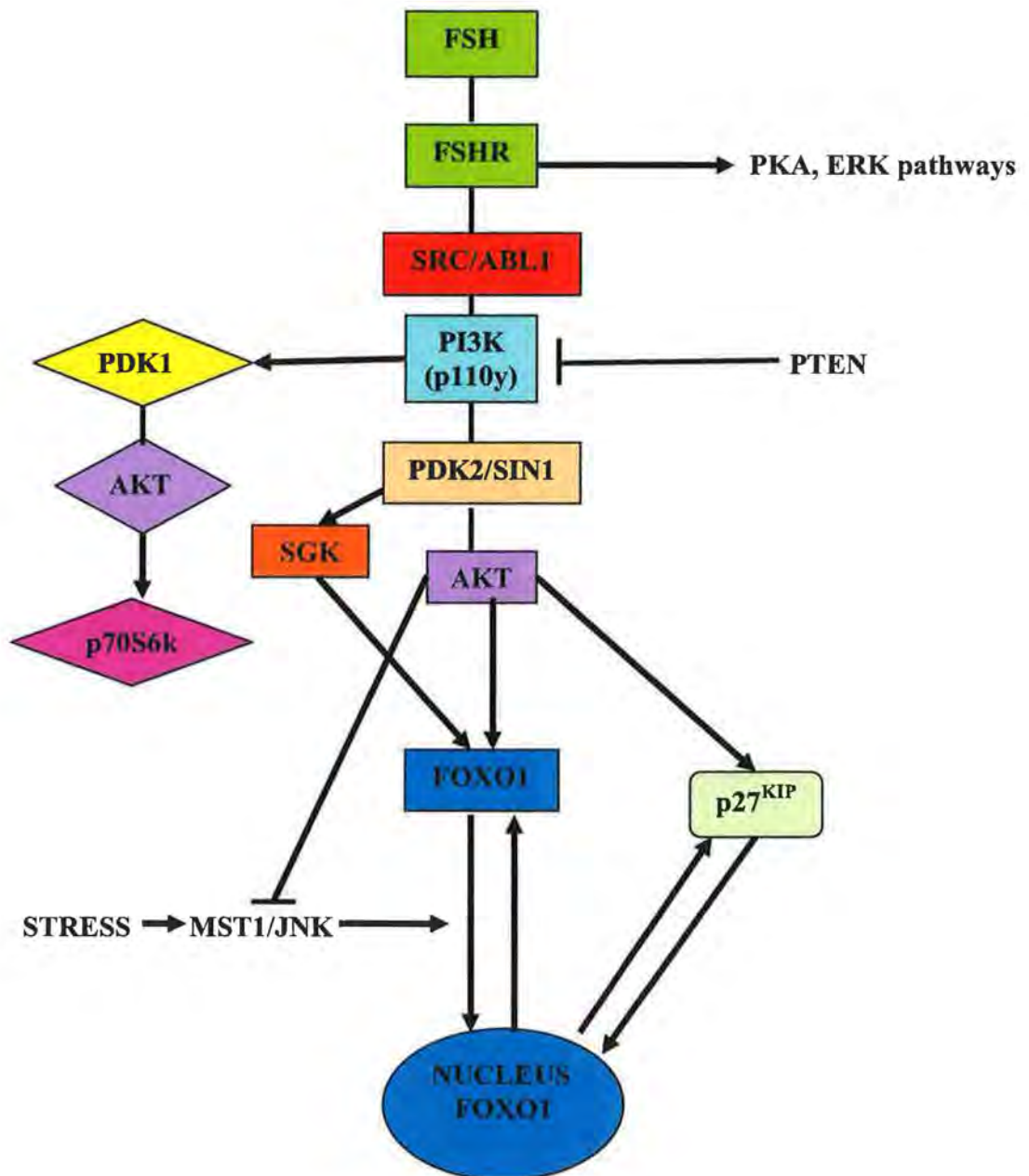
Fig 1.11 (i): This diagram shows how the balance of proliferation, apoptosis and differentiation is maintained within granulosa cells. FSH binds and activates its receptor FSHR. PI3K is activated through SRC/ABL1. PI3K can then result in activation of the PDK1/AKT/p70S6k pathway or the PDK2/AKT/FOXO1(PDK2/SGK/FOXO1) pathway. When phosphorylated by AKT/SGK, FOXO factors can be excluded from the nucleus and rendered inactive. PI3K can be inhibited by PTEN. Stress induced MST1 and JNK are thought to play a part in targeting FOXO1 from the cytoplasm to the nucleus. p27[KIP] phosphorylation can affect both cytoplasmic FOXO1 and nuclear FOXO1.

1.11 (ii) Ras/Raf/MEK/ERK pathway

Mitogen-activated protein kinase (MAPK) pathways are major signal transduction routes that transfer and amplify messages from the cell surface to the nucleus, producing a range of cellular effects, eg cell proliferation. There are several distinct MAPK pathways important in the regulation of cell proliferation, differentiation, development, inflammation, survival, and migration. One of these pathways, the Ras-Raf-MEK-ERK pathway *(Fig 1.11(ii))* is activated by a range of growth factor receptors including EGFR, platelet derived growth factor receptor, type-1 insulin-like growth factor receptor, and fibroblast growth factor receptor. The pathway can also be activated by cytokines, hormones such as FSH and several agonists that act via G-protein-coupled receptors. Receptor-linked tyrosine kinases such as the EGFR are activated by extracellular ligands. Binding of epidermal growth factor (EGF) to the EGFR activates the tyrosine kinase activity of the cytoplasmic domain of the receptor. The EGFR becomes phosphorylated on tyrosines. Docking proteins such as GRB2 contain SH2 domains that bind to the phosphotyrosines of the activated receptor. GRB2 binds to the guanine nucleotide exchange factor, Son Of Sevenless (SOS), by way of an Src homology 3 (SH3) domain of growth factor receptor bound protein 2 (GRB2). When the GRB2-SOS complex docks to phosphorylated EGFR, SOS becomes activated. Activated SOS promotes the removal of GDP from rat sarcoma homologue (Ras).

Ras is an oncogene product that is found on chromosome 11. It is found in normal cells, where it helps to relay signals by acting as a switch (http://www.ncbi.nlm.nih.gov/books/ bv.fcgi?call=bv.View.ShowSection&rid=gnd). Ras is a G protein, specifically a small GTPase. Ras serves as a binary molecular switch and its bioactivity is controlled by a regulated GDP/GTP cycle (Campbell SL *et al* 1998). Ras cycles between two conformations, an activated or inactivated form, respectively RAS-GTP and RAS-GDP. When receptors on the cell surface are stimulated (by FSH for

example), Ras is switched on and transduces signals that tell the cell to grow. If the cell-surface receptor is not stimulated, Ras is not activated and so the ERK pathway is not initiated. In about 30% of human cancers, Ras is mutated so that it is permanently switched on, telling the cell to grow regardless of whether receptors on the cell surface are activated or not. Ras can then bind GTP and become active. (Henson and Gibson 2006). SOS then activates RAS leading to the activation of RAF-1. RAF-1 subsequently phosphorylates MAPK/ERK kinase 1 (MEK1) and MAPK/ERK kinase 2 (MEK2) which activate ERK1 and ERK2 respectively. MEK1/2 is a dual-specificity kinase that is essential to the propagation of growth factor signalling and is known to amplify signals to ERK1/2, also known as MAPK1/2, that in turn can phosphorylate and activate a range of proteins, including several transcription factors (Campbell *et al* 1998). MAPK regulates the activities of several transcription factors. MAPK can phosphorylate C-myc. MAPK phosphorylates and activates MNK which in turn phosphorylates CREB. MAPK also regulates the transcription of the Fos gene. By altering the levels and activities of transcription factors, MAPK leads to altered transcription of genes that are important for the cell cycle (Johnson *et al* 2001).

Figure 1.11 (ii): The Ras-Raf-MEK-ERK signalling pathway

Fig 1.11 (ii): In the case of granulosa cells FSH binds its G-protein coupled receptor and activates the membrane bound GTPase Ras. Ras then activates Raf when subsequently phosphorylates MEK1/2. Activation of ERK1/2 follows which results in cell proliferation, differentiation and survival.

Constitutive activation of the Ras-Raf-MEK-ERK pathway has been demonstrated in several cancer types, pancreas (90%), colon (50%), lung (30%), thyroid (50%), bladder (6%), ovarian (15%), breast, skin, liver, kidney, and some leukemias (http://www.cancerquest.org/index.cfm?page=553). Inhibition of this pathway via MEK1/2 is an attractive strategy for therapeutic intervention in cancer because it has the potential to block inappropriate signal transduction regardless of the upstream position of the oncogenic aberration. Furthermore, ERK1/2 are the only known substrates for MEK1/2. This pathway results in cell proliferation and in the increased transcription of Bcl-2 family members and inhibitor of apoptosis proteins (IAPs), thereby promoting cell survival (Saltiel & Kahn 2002, Saltiel & Pessin 2002). The ERKs are proline-directed protein kinases, phosphorylating pro-neighbouring serine or threonine residues. Docking

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sites present on physiological substrates confer additional specificity. These docking interactions, through non-catalytic regions on ERK, team with scaffolding proteins to ensure signalling fidelity and enzymatic efficiency both to and from the MAPK. Downstream, activated ERK regulates growth factor-responsive targets in the cytosol and also translocates to the nucleus where it phosphorylates a number of transcription factors regulating gene expression. In this project a mouse model is generated to investigate the effects of constitutively active Ras on granulosa cell development. Constitutively active Ras is achieved by a mutation that prevents GTP hydrolysis, thus locking Ras in a permanently 'On' state

1.11 (iii) JAK/STAT pathway

Another signalling cascade initiated by EGF is the Janus kinase/Signal Transducers and Activators of Transcription (JAK/STAT) pathway which is also implicated in cell survival responses (Henson and Gibson 2006). JAK phosphorylates STAT proteins localised at the plasma membrane. This leads to the translocation of STAT proteins to the nucleus where they activate the transcription of genes associated with cell survival.

Figure 1.11 (iii): The JAK/STAT pathway
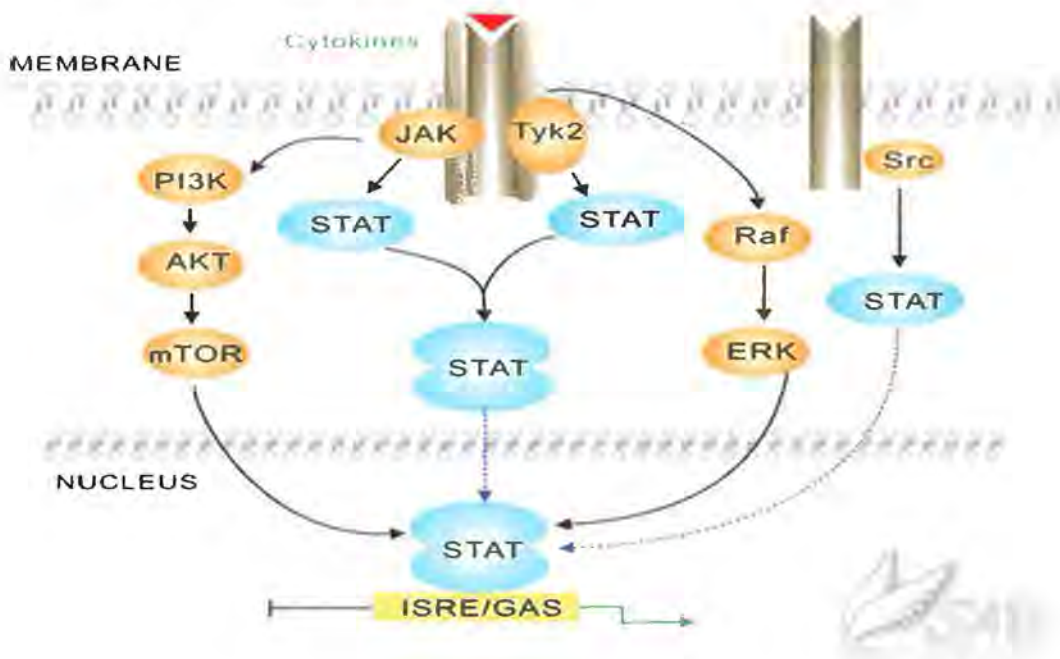
Fig 1.11 (iii): The JAK/STAT pathway can be activated in the same manner as the PI3K pathway shown on the left and the ERK pathway shown on the right of this diagram (http://www.signalwayantibody.com/resurgam/p_img/Jak_Stat_Pathway.gif)

STATs were originally discovered as targets of Janus Kinases, it has now become apparent that certain stimuli can activate them independently of JAKs. The pathway plays a central role in principal cell fate decisions, regulating the processes of cell proliferation, differentiation and apoptosis. JAKs, which have tyrosine kinase activity, bind to some cell surface cytokine receptors *(Fig 1.11(iii)).* The binding of the ligand to the receptor triggers activation of JAKs. With increased kinase activity, they phosphorylate tyrosine residues on the receptor and create sites for interaction with proteins that contain phosphotyrosine-binding SH2 domain. STATs possessing SH2 domains capable of binding these phosphotyrosine residues are recruited to the receptors, and are themselves tyrosine-phosphorylated by JAKs. These phosphotyrosines then act as docking sites for SH2-domains of other STATs, mediating their dimerisation. Different STATs form hetero- as well as homodimers. Activated STAT dimers accumulate in the cell nucleus and activate

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transcription of their target genes. STATs may also be tyrosine-phosphorylated directly by receptor tyrosine kinases, such as the EGFR as well as by non-receptor tyrosine kinases, such as c-src. Stat 5 is of particular interest within the ovary. Stat 5 has previously been identified as a transcription factor activated by prolactin in the mammary gland (Hennighausen *et al* 2001). Prolactin acts in the ovary to promote the maintenance and function of the corpus luteum. However, the cellular signals that induce these responses have not yet been clearly defined. Stat 5 is also activated by prolactin in the ovaries of pseudopregnant rats (Teglund *et al* 1998). Intraperitoneal injection of prolactin into pseudopregnant rats results in the tyrosine phosphorylation and nuclear translocation of Stat 5. This activated Stat 5 possesses DNA-binding activity for a sequence containing the Prolactin-inducible element. It has also been reported that luteinisation of PMSG primed ovaries by the administration of hCG is accompanied by an induction of Stat 5 protein (Ruff *et al* 1996). For this reason, some cell culture work was carried out using PMSG primed, hCG treated ovaries to investigate if cells which have been treated with extracellular ligands such as Amphiregulin, IL6 and FSH can result in the induction of Stat 5 protein.

The JAK/STAT pathway is negatively regulated on multiple levels. Protein tyrosine phosphatases remove phosphates from cytokine receptors as well as activated STATs. More recently identified Suppressors of Cytokines Signalling (SOCS) inhibit STAT phosphorylation by binding and inhibiting JAKs or competing with STATs for phosphotyrosine binding sites on cytokine receptors. STATs are also negatively regulated by Protein Inhibitors of Activated STATs (PIAS), which act in the nucleus through several mechanisms. For example, PIAS1 and PIAS3 inhibit transcriptional activation by STAT1 and STAT3 respectively by binding and blocking access to the DNA sequences they recognise (Ke Shuai 2006).

1.12 CEBP/ß

A large number of intercellular signalling molecules have been identified that orchestrate female reproductive physiology. However, with the exception of steroid hormone receptors, little information exists about the transcriptional regulators that mediate cellular responses to these signals. The transcription factor C/EBPß (CCAAT/enhancer-binding protein beta) is expressed in ovaries and testes, as well as many other tissues of adult mice. C/EBPß is a member of a family of basic helix-loop-helix transcription factors. Mice carrying a targeted deletion of the C/EBPß gene exhibit reproductive defects. Although these animals develop normally and males are fertile, adult females are sterile. C/EBP-deficient ovaries lack corpora lutea and fail to down-regulate expression of the prostaglandin endoperoxidase synthase 2 and P450 aromatase genes in response to gonadotrophins. These findings demonstrate that C/EBPß is essential for periovulatory granulosa cell differentiation in response to LH (Sirios *et al* 1993). Transplantation of normal ovaries into mutant females restored fertility, thus localising the primary reproductive defect to the ovary proper. C/EBPß is thus established as a critical downstream target of G-protein-coupled LH receptor signalling and one of the first transcription factors, other than steroid hormone receptors, known to be required for ovarian follicle development in vivo (Sterneck *et al* 1997). In normal ovaries, C/EBPß mRNA is specifically induced by luteinising hormone (LH/hCG) in the granulosa layer of preovulatory antral follicles. FSH also induces C/EBPß (Pall *et al* 1997). In this project a PMSG primed hCG ovarian series was studied to investigate the time point C/EBPß is induced. Some cell culture work was also carried out to determine if ligands such as Amphiregulin and FSH increase C/EBPß expression in vitro.

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1.13 Apoptotic Markers: MST1 and SAPK/JNK

Mammalian Ste20-like kinase (Mst1) and stress-activated protein kinase/Jun-amino-terminal kinase (SAPK/JNK) are members of the germinal centre kinase family and are thought to be activated and translocated from the cytosol to the nucleus by apoptotic signals as well as other stress conditions. It is thought that activated MST kinases may rely on p38MAPK and JNK pathways to amplify apoptotic signals (Lee, K et al 2001). MST1 is an upstream kinase of the JNK and p38 MAPK pathways whose expression induces apoptotic morphological changes such as nuclear condensation. During apoptosis, caspase cleavage of MST1 removes a C-terminal regulatory domain, increasing the kinase activity of the MST1 N-terminal domain. Downstream pathways of MST1 in the induction of apoptosis remain to be clarified (S Ura et al 2001). SAPK/JNK is potentially and preferentially activated by a variety of environmental stresses, including UV and gamma radiation, ceramides, inflammatory cytokines and in some instances by growth factors. Stress signals are delivered to the cascade by small GTPases of the Rho family. SAPK/JNK, when active as a dimer can translocate to the nucleus where it regulates transcription through its effects on c-Jun, ATF-2 and other transcription factors (Kyriakis et al 2001). The MEKK1 protein kinase is a proximate activator of the SAPK/JNK stress-signalling pathway. A variety of environmental stresses, including inflammatory cytokines, hyperosmotic shock, and UV light stimulate the cascade (Yan et al 1994). MEKK1 is implicated in apoptotic cell death, but surprisingly MEKK1 activates nuclear factor kappa B (NFkB) pathways that induce expression of genes to counteract the apoptotic death response (Cross et al 1999).

MST1 and JNK can be activated by oxidative stress. This stress results in the phosphorylation of FOXO1 at a conserved site within the forkhead domain that promotes FOXO nuclear translocation. This translocation induces cell death (Lehtinen

et al 2006) *(Fig 1.11(i))*. It has been shown that transgenic mouse line expresses a constitutively active p110-alpha subunit in the epithelial cells of the prostate results in an elevated level of MST1. This elevation and the phosphorylation of AKT could be inhibited in vivo in transgenic animals by the PI3K inhibitor LY294002 (Renner *et al* 2007). MST1 is therefore a marker for PI3K activity. To investigate the roles MST1 and SAPK/JNK play in ovarian development and to study how they relate to FOXO1 expression, immunofluorescence staining was carried out on ovarian sections for both MST1 and pSAPK/JNK and an hCG series was also examined for MST1, pSAPK/JNK and FOXO1 protein expression.

1.14 Extracellular ligands

1.14 (i) Interleukin 6

Interleukin 6 (IL6), a broad spectrum cytokine, has previously been demonstrated to be produced by granulosa cells and to directly influence FSH differentiated functions of ovarian granulosa cells (Van der Hoek *et al* 1998). IL6, a B cell differentiation factor, has been shown to exert modulatory influence over ovarian function. IL6 is capable of directly influencing FSH-stimulated progesterone production by granulosa cells in vitro. Moreover, IL6 is produced by the granulosa cell, suggesting a potential role for this cytokine in the autocrine and/or paracrine regulation of ovarian function. IL6 secretion by granulosa cells is an event that may be regulated by a number of cytokines as well as FSH (Van der Hoek *et al* 1998). FSH has been shown to cause significant concentration-dependent increases in IL6 release in granulosa cell release suggesting granulosa cells are a likely source of IL6. cAMP may serve as a second messenger for the stimulated secretion of IL6 by undifferentiated granulosa cells (Gorospe *et al* 1993). As IL6 interacts with its receptor, it

triggers the gp130 and IL-6R proteins to form a complex, thus activating the receptor. These complexes bring together the intracellular regions of gp130 to initiate a signal transduction cascade through the transcription factors JAKs and STATs. IL6 is probably the best studied of the cytokines that use gp130 in their signalling complexes. In addition to the membrane-bound receptor, a soluble form of IL-6R has been purified from human serum and urine. Previous studies indicate that the pleiotropic cytokine IL6 may contribute to the vascularisation of some tumours by disrupting the equilibrium between positive and negative angiogenic regulatory molecules. Human ovarian carcinoma cell lines have been shown to secrete significant levels of the soluble IL6 receptor and elevated serum levels of IL6 has shown to be associated with poor patient survival (Nilsson *et al* 2005). The molecular mechanism(s) by which IL6 can regulate angiogenesis still however remain unclear. Nilsson et al also showed that the addition of IL6 to cultured endothelial cells of the ovary resulted in a significant increase in cell proliferation suggesting that IL6 secreted by ovarian cancer cells can be an endothelial cell mitogen. Recently this group has also shown that stress hormones such as norepinephrine lead to increased expression of IL6 mRNA and protein levels in ovarian carcinoma cells. They also demonstrate that norepinephrine stimulation activates Src tyrosine kinase, and this activation is required for increased IL6 expression (Nilsson *et al* 2007). To test if this ligand has the ability to effect the PI3K/AKT/FOXO and the Ras/Raf/MEK/ERK signalling pathways in ovarian development, granulosa cells were isolated from rat ovaries and treated with IL6 to determine if levels of key components in both these signalling pathways were altered.

1.14 (ii) Amphiregulin

Amphiregulin (AREG) was originally isolated from the conditioned medium of a human breast carcinoma cell line MCF-7 (Shoyab *et al.* 1988). AREG is a member of the EGF family of proteins. AREG binds to the EGF receptor but not as well as EGF does. AREG

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is induced in the rat ovary by gonadotrophin treatment. LH activates a cascade of signalling events that are propagated throughout the ovarian preovulatory follicle to promote ovulation of a mature egg. Critical to LH-induced ovulation is the induction of EGF-like growth factors and transactivation of EGF receptor signalling. The expression of AREG mRNA is inducecd in PMSG treated rats after 24 hours but the expression then decreases 48 hours after treatment. Further treatment with hCG rapidly induces the expression of AREG genes showing maximal levels 4 hours after hCG treatment (Sekiguchi *et al* 2004). To investigate if AREG influences downstream components of the PI3K/AKT/FOXO and the Ras/Raf/MEK/ERK signalling pathways in pre-antral follicles, granulosa cells were isolated from estradiol injected rat ovaries and treated with this ligand.

1.14 (iii) Forskolin

Forskolin is the main active ingredient in the Ayurvedic herb Coleus forskohlii. Coleus is a member of the mint family and grows in subtropical areas in India, Burma, and Thailand. Forskolin is commonly used to raise levels of cyclin AMP (cAMP) in the study and research of cell physiology. Forskolin resensitises cell receptors by activating the enzyme adenylyl cyclase and increasing the intracellular levels of cAMP (Godard *et al* 2005). cAMP appears to have additional actions that are due to its ability to alter a number of membrane transport proteins. cAMP and the chemicals it activates comprise a second messenger system that is responsible for carrying out the complex and powerful effects of hormones in the body. It is required for cell communication in the hypothalamus/pituitary gland axis and for the feed-back control of hormones. It acts by activating protein kinase A (Hedin *et al* 1983). Forskolin has been extensively researched in the medical field for use in the treatment of allergies, respiratory problems, cardiovascular diseases, glaucoma, psoriasis, hypothyroidism, and weight loss. The effects of forskolin on IL6 release by granulosa cells have been examined. The results showed a significant concentration-

dependent increase in IL6 production by granulosa cells in either the absence or presence of FSH (Gorospe *et al* 1993). It has previously been demonstrated that ovulated oocytes treated with LH or forskolin resume meiosis showing that an increase in ovarian cAMP, even if not induced by LH, is sufficient to cause ovulation of preovulatory rat follicles, supporting the involvement of cAMP in the normal ovulatory process (Brannstrom *et al* 1983). The binding of LH to its receptors in the ovary activates adenylate cyclase in the cell membrane, which in turn results in an increase in intracellular cAMP levels. It is also a well-documented fact that cAMP acts as a second messenger for LH in the stimulation of steroidogenesis. For these reasons, granulosa cells from estradiol-treated rats were cultured overnight and treated with forskolin to investigate the effects forskolin treatment has on PI3K pathway and ERK pathway components.

1.15 Mouse Strains

Pten conditional knock out mouse model

PTEN is the negative regulator of the PI3K pathway. In order to investigate the effect of a hyperactive PI3K/AKT/FOXO cascade on ovarian cell function a conditional *Pten* knock out mouse strain was generated. This was done by mating $Pten^{fl/fl}$ mice with transgenic mice expressing CRE recombinase driven by the *Cyp19* (aromatase) promoter. This resulted in *Pten* mRNA and protein being specifically disrupted/reduced in granulosa cells (but not other cells) of growing follicles of the mutant.

LSL-K-ras^{G12D}*-Amhr2-Cre* conditional knock-in mouse model

The small G-protein RAS is critical for FSH-induced signalling events in cultured granulosa cells, but the functions of RAS in granulosa cells during follicular development have not been defined. Therefore, to determine the impact of RAS activation on granulosa cells *in vivo*, a conditional knock-in mouse model in which granulosa cells expressed a constitutively active $K-ras^{G12D}$ was generated, driven by the Anti- Mullerian hormone receptor-2 (*Amhr-2*) promoter. In the (LSL) Lox-Stop-Lox-K-ras^{G12D}-Amhr2-Cre model, Ras was engineered to be constitutively active in granulosa cells. Constitutively active Ras is one which contains mutations that prevent GTP hydrolysis, thus locking Ras in a permanently 'On' state. assay (Soriano P, 1999). These mice displayed a subfertile phenotype with oocytes trapped in corpora lutea 48 hours post hCG injection.

Note: Genotyping as described in Section 3.11 was carried out on both the *Pten* conditional knock out mouse model and the *LSL-K-ras*^{G12D}*-Amhr2-Cre* conditional knock-in mouse model. Immature wild type C57BL/6 mice were also used.

2.0 AIMS

The PI3K/AKT/FOXO and Ras/Raf/MEK/ERK cascades are known vital pathways for sustaining the balance between proliferation, differentiation, cell survival and apoptosis and as a consequence of this affecting folliculogenesis, ovulation and luteinisation.

Considering this, the primary aims of this project were:

1. To study the role that cell cycle regulators Cyclin D2 and $p27^{KIP}$ play in ovarian cell proliferation and differentiation and to investigate the significance of two apoptotic markers MST1 and p-SAPK/JNK in follicle atresia.

2. To study the phosphorylation pattern of some downstream targets in the PI3K/AKT/FOXO pathway and to assess whether extracellular ligands such as Amphiregulin and Interleukin-6 displayed the ability to induce phosphorylation of a key intermediate in the pathway, AKT.

3. To elucidate the role C/EBP β plays in ovarian folliculogenesis and ovulation and to determine the effects Follicle Stimulating Hormone and Amphiregulin have on C/EBP β action.

4. To compare ovarian morphology between wildtype mice and a genetically engineered *Pten* conditional knock out mouse strain, so as to determine the importance of PTEN in maintaining normal phenotype. Also to compare how the phosphorylation pattern of downstream targets in the PI3K cascade such as AKT and FOXO differs between wildtype mice and the *Pten* conditional knock out mouse strain.

5. To investigate the action of the JAK-STAT signalling pathway component pSTAT5 in ovarian granulosa cells and decipher the effects that pituitary hormones and extracellular ligands have on STAT5 phosphorylation.

6. To examine the effects of extracellular ligands and hormones on pERK, a signalling intermediate of the Ras/Raf/MEK/ERK cascade.

7. To study the effect of constitutively active Ras on ovarian histology using the *K-ras^{G12D}-Amhr2-Cre* mouse strain and as a result of this investigate some ovarian signalling genes associated with senescence so as to determine the extent to which Ras activation impinges on vital upstream receptor genes that relay the FSH and LH message allowing successful folliculogenesis and ovulation.

This study was carried out on mice of the required genotype that had been treated with gonadotrophin representing the normal physiological state to induce ovarian folliculogenesis, ovulation and luteinisation as required. Pregnant mare serum gonadotrophin (PMSG) an FSH analogue was used in place of FSH and human chorionic gonadotrophin (hCG) an LH analogue was used in place of LH.

3.0 MATERIALS AND METHODS

MATERIALS

3.1 Materials Used for Animal Treatment and Collection of Ovarian Material

- VMR International Gonadotrophin Pregnant Mare Serum (PMSG) 4IU Cat No. 80056-608

- Human Chorionic Gonadotrophin (hCG) 5IU (Gestyl; Diosynth, Oss, The Netherlands)

- Saline

- Estradiol

- Propylene glycol

- Decapitation guillotine

- Forceps

- Small sharp scissors

- Magnifying glass

- RIPA buffer (20mM Tris [pH 7.5], 150mM NaCl, 1% Nonidet P-40, 0.5% Sodium Deoxycholate, 1mM EDTA, 0.1% SDS)

- Roche complete mini protease inhibitor

- Sonicator

- Gibco Dulbecco's Modified Eagle Medium, Nutrient Mixture F-12 Ham 1X (DMEM/F12 1:1) Lot number 352081

- Gibco Invitrogen Corporation Penicillin-Streptomycin Cat No: 15140-122 Lot No: 1409377

- Small petri-dishes

- 26.5 gauge needles

- Trypsin

- Trypsin inhibitor

- DNase

- 12-well culture dish

- Fresh bovine serum

- Microscope

- Testosterone National Hormone and Pituitary Program, Baltimore, Maryland 21201

- Follicle Stimulating Hormone (FSH) National Hormone and Pituitary Program, Baltimore, Maryland 21201 (100ng/ml)

- Interleukin 6 (IL6) Research and Development (100ng/ml)

- Amphiregulin (Areg) Research and Development (100ng/ml)

- Forskolin (Fo) Calbiochem (100ng/ml)

3.2 Western Blot Materials and Solutions

Materials

- Protein samples.

- BioRad Precision Plus Protein^{TM} Dual Color Standards Marker Cat No. 161-0374

- BioRad glass plates, rack and electrophoresis tank Biorad, 2000 Alfred Nobel Drive, Hercules, CA 94547, USA.

- BioRad 1.5 mm 10 well green comb

- Fisher Scientific Methanol Code No. A412-4, Lot No.053816

- Fisher Chemalert® Fisher Scientific® Acrylamide Code No. BP-170-500

- BioRad Ammonium Persulphate (APS)- Cat No. 161-0700

- BioRad TEMED-N,N,N'-N'-tetra-methylethylenediamine Cat No. 161-0801

- Sigma 2-Mercaptoethanol M6250 Batch No. 12328HH

- Fisher scientific Isobutanol Code No. A399-4, Lot No. 031612

- Bio-Rad Trans-Blot® SD. Semi-Dry transfer cell Serial No.153BR 0146032

- Bio-Rad extra thick blot paper mini blot size 7x 8.4cm, Cat No.1703966

- Bio-Rad nitrocellulose or PVDF membrane

- Cell signalling technology™ Cyclin D2 Antibody Code No. 2924 (1:1000)

- Santa Cruz Biotechnology p27^{KIP} Antibody Cat No. SC-528 (1:1000)

- Research and Development Systems phosphos-p27 (1:1000)

- Cytoskeleton Anti-Actin (Beta-Actin) Cat No. AANO1 Lot No. 095 (1:2000)

- Cell signalling technology™ MST-1 Antibody Code No. 3682 (1:1000)

- Cell signalling technology™ Phospho-SAPK/JNK Antibody Code No. 92515 (1:500)

- Cell signalling technology™ PI3 kinase p110 gamma antibody Code No.4252 (1:300)

- Cell signalling technology™ Phospho-PDK1 Code No. 30615 (1:100)

- Cell signalling technology™ Akt antibody Code No. 9272 (1:2000)

- Cell signalling technology™ phospho-Akt rabbit monoclonal antibody (p-AKT) Code No.4058L (1:500)

- Cell signalling technology™ FOXO1 Antibody Code No. 9462 (1:2000)

- Cell signalling technology™ Phospho-FKHR / Phospho-FOXO1 Antibody Code No. 94615 (1:1000)

- Cell signalling technology™ Phospho-p70S6 kinase Antibody Code No. 92055 (1:1000)

- Santa Cruz Biotechnology C/EBP beta (C19) Antibody Cat No. SC-150 (1:300)

- Cell signalling technology™ phospho-STAT5 Antibody Code No. 9359 (1:500)

- Cell signalling technology™ Phospho-p44/42 MAP kinase (Thr202/Tyr 204) Antibody / Phospho-ERK antibody Code No. 91015 (1:1000)

- Biolabs New England p44/42 MAP kinase Antibody /Total ERK Code No. 9102 (1:1000)

- ECL^{TM} Anti-Rabbit IgG HorseradishPeroxidase linked whole Antibody from donkey Cat No.NA934V Lot No.362613

- Western Super Signal® West Pico chemi-iluminescent substrate/ enhanced chemi-iluminescent substrate for detection of HRP (Solution A-Western Super Signal® West Pico Stable peroxide solution, Solution B-Western Super Signal® West Pico luminal/enhancer solution)

- GE Healthcare, UK Limited Enhanced Chemical Luminescence (ECL) Lumigen^{TM} PS-3 detection reagent Solution A (RPN2132V1 lot 74) and 25uls of Lumigen^{TM} PS-3 detection reagent Solution B (RPN2132V2, lot 74)

- Optical density quantifier: Molecular Dynamics Machine with Scanner control software: Image Quant 5.2

Solutions

- 1 X SDS Electrophoresis Tank Buffer: Tris Base 3.02g, Glycine 14.40g, SDS 1.00g, dissolve this in approx 0.4mls of dH$_2$O and then make this up to 1 litre (pH 7.5).

- Semi-Dry Transfer Buffer: 5.82g Tris Base, 2.93g Glycine, 20%methanol, 3.75g 10% SDS, dissolve this in approx 0.4mls of dH$_2$O and then make this up to 1 litre.

- Milk Blocking Buffer: 10g milk powder, 200mls TBST.

- Stripping Buffer: 15mls dH$_2$O, 5mls 4 X Tris/SDS ph 6.8, 0.14mls Betamercaptoethanol.

- Tris Buffer Saline with Tween (TBST): 100ml of 10X TBS- (200ml 1M Tris pH 7.5, 300ml 5M NaCl, 500ml H$_2$O) + 900ml H$_2$O + 2mls 50% Tween 20.

Western Blot Gels

8% lower resolving gel

	Mls
30% Acrylamide	1.040
4 X Tris/SDS ph 6.8	1.000
dH$_2$0	1.960
10% APS	0.040
TEMED	0.004

5% upper stacking gel

	Mls
30% Acrylamide	4.24
4 X Tris/SDS ph 8.8	4.00
dH$_2$0	7.76
10% APS	0.16
TEMED	0.01

3.3 H&E Materials

- PFA fixed, processed, paraffin embedded 7-um ovary sections

- MENZEL-GLASER® Super frost® Plus Microscope Slides. Art no. J1800AMNZ.

- Histolab® Xylene. Art Nr. 02080 Fisher Scientific® Xylene- Code No. X5-4 UN1307, Lot No. 040649

- Histolab® 99.5% ethanol

- Histolab® 95% ethanol

- Histolab® 80% ethanol

- dH$_2$0

- Histolab® Mayers Haematoxylin. Art Nr. 01820

- Eosin, DPX mounting media

3.4 Immunofluorescence Staining

Materials

- Frozen sections embedded in OCT compound (Sakura Finetek USA, INC) fixed in 4% PFA and sectioned at 7 μm

- DAKO pen

- Primary Antibodies: Santa Cruz Biotechnology p27^{kip} (1:100) Cell Signalling MST1 (1:100), pSAPK/JNK (1:100) pFOXO1 (1:100)

- Invitrogen Molecular Probes, Eugene, Oregon, USA: Alexa Fluor® 594 Goat anti-rabbit IgG (H+L) (red) A11080 Lot No:93E1-1

- Invitrogen Molecular Probes, Eugene, Oregon, USA: Alexa Fluor® 488 Goat anti-rabbit IgG (H+L) (green) A11008 Lot No:45601A

- Dapi counter stain (10μg/ml)

- MENZEL-GLASER® Super frost® Plus Microscope Slides. Art no. J1800AMNZ

- Light-proof humidity chamber

- 80% Glycerol

- Nail polish

Solutions

- 10 X Phosphate Buffered Saline (PBS): 80g NaCl, 2g KCL, 14.4g Na$_2$HPO$_4$, make up to 1 litre with milli-Q water.

- Paraformaldehyde (PFA) 4%: To make up 30mls, 1.2g of paraformaldehyde EMD™ UN2213, Code No.PX0055, Lot No. 45105621 dissolved in 30mls of 1 X PBS solution or in 90°C water bath for 1 hour. Solution kept at 4°C and used within two weeks.

- PBS/0.3% Triton X-100 (PBS/Triton)1.5ml of 20% Triton X-100 added to 100mls of 1XPBS

- Amersham Life Science BSA- Bovine serum albumin albumin fraction V RIA grade Cat No. 70244

3.5 Genotyping Materials

- Extracted DNA

- DNA digestion buffer with proteinase K

- Phenol: chloroform: Isoamyl alcohol (25:24:1)

- 100% ,70% Ethanol

- Taq polymerase, dNTPs,

- TE Buffer(10mM Tris & 1mM EDTA)

- Amhr-2-Cre primers (Invitrogen)

- Cyp-19-Cre primers (Invitrogen)

- dH_2O, $MgCl_2$, 10X buffer

- Heating block and eppendorf tubes

- 1.5% agarose gel

- PCR machine

- ISC Bio Express Gene Pure LE Quick Dissolve Agarose 500g Lot C405030

3.6 RNA Extraction Materials

- Qiagen RNeasy Mini® Kit Lot 42141618

- Qiagen RNeasy Mini® Kit RLT buffer: Add 100µls of beta-mercaptoethanol to 10mls of RLT buffer (1:100).

- 20.5 gauge syringes

METHODS

3.7 Animal and Hormone Treatment Methods

All mouse genotypes under study were housed under a 16 hour light : 8 hour dark schedule in the transgenic mouse facility in the centre of comparative medicine at Baylor College of Medicine, Houston, Texas. Immature female C57BL/6 mice were obtained from Harlan, Inc. (Indianapolis, IN), weighing between 20-30gms. The rat strain used was Holtzman, Sprague-Dawley, 26-28 days old females which weighed between 50-90gms. The rats were housed in the Taub Vivarium facility in Baylor College of Medicine. All animals were provided with food and water which they could take freely. Animals were treated under the National Institutes of Health Guide for the Care and Use of Laboratory Animals as approved by the Animal Care and Use Committee at Baylor College of Medicine. The knock-out/knock-in mice used are explained in Section 1.14.

3.7(i) Method for mouse treatment

Immature (day 21 of age) mice were sacrificed prior to and at 48 hours following injection of PMSG. 0.1ml of PMSG (4IU dissolved in phosphate buffered saline) was administrated through the intraperitoneal route in order to stimulate development of preovulatory follicles. The mice killed prior to PMSG injection served as the not-treated samples (NT). PMSG-treated mice were killed at 2,4,8,12 and 16 hours following hCG (human chorionic gonadotrophin) administration. 0.1ml of LH analogue, hCG (5IU dissolved in saline) was injected into mice via the intraperitoneal route. hCG functioned to stimulate ovulation and luteinisation. For each time point ovaries were isolated and pooled from 5 mice. Mice were euthanised by manual restraint followed by cervical dislocation to prevent rapid stress related release of pituitary hormones (FSH, LH and ACTH) and brain peptides that occurs during the relatively slow process of chemically induced anaesthesia. The bolus release of

hormones in response to anaesthesia can impact and acutely alter the function of the tissues being studied, namely the ovaries. As well as this, the chemicals used for anaesthesia can themselves exert effects so this is why more rapid physical methods that did not involve chemicals were used. If the ovary was to be paraffin wax embedded, it was immediately frozen at -80°C once removed from the mouse. If the ovary was to be used for western blot analysis it was placed in an eppendorf containing 4mls of blue RIPA protein lysis buffer and 40μl of Roche mini protease inhibitor (1:100 dilution). It was important to keep everything on ice at this stage. A homogenous solution was made by sonicating the ovary emmersed in the RIPA lysis buffer, protease inhibitor cocktail. The ovary lysate was then centrifuged in a 4°C centrifuge for 5 minutes at top speed. 200μl of the supernatant was removed and put in a fresh eppendorf. Protein concentration was determined using Bradford Assay. 5 ovaries were pooled for each time point in the western blots. 30μg protein was added to each well of an 8% acrylamide gel: volume of 20μl diluted in RIPA protein lysis buffer 1.5μg/μl. This 8% acrylamide gel was run at 150volts until molecular weight marker had separated sufficiently so that protein of interest could be seen (approx 45 minutes).

3.7(ii) Method for rat treatment

Immature rats were injected with 0.2ml estradiol (1.5mg of estradiol was dissolved in 0.2ml propylene glycol) subcutaneously for three consecutive days and sacrificed on day 3. Rats were euthanised by manual restraint followed by decapitation to prevent rapid stress related release of pituitary hormones (FSH, LH and ACTH) and brain peptides that occurs during the slower process of chemical induced anaesthesia. The chemicals themselves and also the release of such pituitary hormones may alter the natural state of the ovary being studied. At the time of sacrifice the ovaries were removed and granulosa cells isolated to use for cell culture. Four rats (8 ovaries) generated 12 mls of granulosa cell suspension.

3.7(iii) Method for collecting ovarian granulosa cells

Rats were selected and sacrificed by decapitation. Cells were collected from rat ovary by puncturing the follicles with a 26.5 gauge needle while it was suspended in Gibco culture medium with penicillin streptomycin antibiotic added (1:100). Cells were centrifuged for 3.5 minutes at 2800rpms to get rid of apoptotic cells and cell fragments which were present in supernatant. Supernatant was poured off and cells were re-suspended in fresh medium. 1ml of trypsin was added to the cells and monitored for approx 1 minute until precipitation occurred and cell fragments were evident in the supernatant. Immediately 1ml of trypsin inhibitor was added along with 1.5ml of DNase. Cells were washed twice with fresh culture medium at 2800rpms for 3.5 minutes and re-suspended in an appropriate amount of culture medium depending on how many cell culture plates were being used. Cells were cultured at a density of 1×10^6 cells in 1 ml of culture medium in 12-well culture dishes that were serum coated to allow the attachment of the granulosa cells.

3.7(iv) Method for culturing rodent granulosa cells

Granulosa cells were cultured and used for western blotting analysis. This was carried out using 12 well culture dish coated with a 1% fresh bovine serum solution for rats or a 5% fresh bovine serum solution for mice. This solution was made up using Gibco culture medium with penicillin streptomycin antibiotic added (1:100) warmed to 37°C in the water bath. Approx 1ml of serum solution was added to each well in the 12-well culture dish. The dish was then incubated at 37°C for at least 30 minutes. The serum contained fibronectin which coated the plastic culture dish and allowed the granulosa cells to attach easily. When the granulosa cells were isolated and ready to be used, the serum solution was pipetted off and wells were washed three times with serum free Gibco culture medium with penicillin streptomycin antibiotic added (1:100). Cell were cultured in this medium overnight to allow their attachment to the dish bottom and the next day were treated with a

variety of different hormones such as Follicle Stimulating Hormone, Interleukin-6, Amphiregulin and Forskolin (100ng/ml). After these various treatments, 100µl of boiling RIPA lysis buffer was added to each well. This was pipetted up and down so as to ensure maximum protein extraction from all the granulosa cells. Samples from each well were then placed in labelled eppendorf tubes and boiled for a further 5 minutes. These samples were kept in the -80°C freezer until they were ready to be used for western blot analysis or if more appropriate 20µl of sample were loaded to each well of an 8% acrylamide gel and ran at 150 volts for 45 minutes or until protein of interest has separated out.

3.8 Performing Western Blot

Ensuring glass plates are aligned straight, the gel apparatus for two 1.5mm thick gels was assembled. Resolving gel for two 1.5mm thick acrylamide gels were made up as outlined in the materials section, adding TEMED last. Before adding the 10µl of TEMED, two 200µl aliquots of the gel mixture was taken out and placed in two separate eppendorf tubes. 2.5µl of TEMED was added to each tube. This 202.5µl was pipetted into each gel apparatus and allowed sit for 5 minutes to seal the bottom of the gel. 10µl of TEMED was added to the rest of the mixture which was then distributed between each gel apparatus. 200µl of iso-butanol was added to the top of each gel mixture to remove air bubbles and ensure a straight line top on each gel. Gel was left to set for approx 15 minutes. After this time gels were checked macroscopically to ensure interface had formed and iso-butanol was poured off. 2 mls of stacking gel was then added on top of the resolving gel. Immediately well comb was put in place and gels were allowed to sit for a further 15 minutes. The tank was filled with 1 X SDS Electrophoresis Buffer and gels placed inside. Comb was removed and SDS extracts containing 30µg protein were resolved by SDS-PAGE (8%) and transferred to nitrocellulose/PVDF membrane. Gels were ran at 150 volts for approx 45 minutes and then removed and placed in semi-dry transfer buffer. The

hydrophobic PVDF membranes were washed in a few drops of methanol in order to make them permeable. Membranes were then washed in semi dry transfer buffer and immersed in fresh semi-dry transfer buffer with two lots of blotting towels. A few drops of transfer buffer were placed on the surface of the transfer chamber surface. One lot of blotting towels followed by the membrane and then the gel and a further lot of blotting towels were subsequently placed on the transfer chamber surface. During this procedure excess air was removed between each layer using a rolling pin. More semi-dry transfer buffer was placed on top of this sandwich and excess buffer from around the sides sucked up using a Pasteur pipette. The gel was transferred to the membrane on the transfer chamber at 15 V for 45mins. Once fully transferred the membrane was blocked in 5% milk blocking solution for 30 minutes. During this time the primary antibody was made up. The membrane was incubated in the appropriate primary antibody diluted in blocking solution as outlined in the materials section to a final volume of 10mls. The membrane was agitated in a plastic pouch overnight at 4°C and subsequently washed three times in TBST for 10 minutes each. The membrane was then incubated in HRP-conjugated secondary antibody diluted (1:5000) in 10ml of 5% milk with gentle agitation for 30 minutes at room temperature. The membrane was washed in TBST three times for 10 minutes each and then patted dry on a paper towel. The ECL substrate (1ml Solution A and 25μls of Solution B) was applied to membrane for two minutes. Membrane was blotted on tissue paper and wrapped in a plastic sleeve. This was taped inside a labelled metal cassette that had been exposed to light for 10 minutes. In the dark room Kodak film was placed on top of the membrane and an initial 10 second exposure indicated the proper exposure time. Subsequently the membrane was exposed with new film for the appropriate time and developed. Membrane was rinsed briefly in TBST if it was to be probed with a different primary antibody. If the second protein overlapped with the first one then the membrane was stripped. Stripping Buffer was placed in a 50ml tube and allowed to heat up in 55°C incubator. Once buffer

had heated up the membrane was placed inside the tube. The membrane was left rotating inside the incubator for 30 minutes. After this time it was washed in TBST for 10 minutes, rinsed in milk and the new primary antibody was applied. Each western band was then measured on an optical density quantifier (Molecular Dynamics Machine) and analysed using scanner control software (Image Quant 5.2).

3.9 Haematoxylin and Eosin Staining

The required mouse ovary paraffin tissue sections were deparaffinised in three changes of xylene for ten minutes each. The sections were hydrated through a graded alcohol series, which included two changes in 100% ethanol for two minutes each and further rehydration through 95%, 70%, 50% ethanol and water for two minutes each. The sections were stained in new and unfiltered haematoxylin for 20-30 seconds and then transferred to running water to blue up. The eosin was applied to the sections for 20 seconds and rinsed. The sections were dehydrated through 95% ethanol for two minutes and through two changes of 100% ethanol for a minute each. The dehydrated sections were cleared in two changes of xylene for a minute and mounted in DPX mounting media.

3.10 Immunofluorescence Method

Frozen ovarian sections were selected and the outside of the tissue section was circled using a DAKO pen. Slides were fixed in 4% PFA in PBS for 30 minutes at room temperature. The PFA was aspirated off using a pipette and the sections were rinsed three times in PBS in a coplin jar for three minutes each. Sections were blocked and made permeable with 5% Bovine Serum Albumin (BSA) in PBS/Triton for 30 minutes at room temperature. The BSA was aspirated off and the primary antibody applied. The primary antibody was diluted 1:100 in PBS/Triton and approx 150 µls was applied per tissue section. The slides were placed in a light proof humidity chamber and left at 4°C overnight.

The primary antibody was aspirated off and slides were washed three times with 1 X PBS in a coplin jar. Slides were incubated for 1 hour in the dark at room temperature with fluorochrome conjugated secondary antibody diluted in PBS/Triton. Secondary antibody Invitrogen Molecular Probes Alexa Fluor® 594 Goat anti-rabbit IgG (H+L) was typically diluted 1:200 (red colour)/ Invitrogen Molecular Probes Alexa Fluor® 488 Goat anti-rabbit IgG (H+L) (1:200) (green colour). Slides were counter stained with DAPI (10µgs/ml) diluted 1:200 in PBS for 5 minutes (blue colour). Slides were rinsed once with 1 X PBS for 5 minutes in a coplin jar. Slides were mounted in 10µl of 80% glycerol and the edges of the coverslips were sealed with nail varnish to stop the sections drying out. The slides were left in a light-proof box at 4°C and examined within a week using appropriate excitation wavelength.

3.11 Genotyping AMHR2-CRE and CYP19-CRE mice

Mouse-tail snips were digested in 600 µl of DNA digestion buffer/ proteinase K solution at 55°C overnight. Tubes were centrifuged at full speed for 5 minutes so as to force the mouse fur to the bottom of the tube. 400µl of supernatant was pipetted off and added to 1ml of 100% Ethanol. This was mixed well and centrifuged at 1400rpm for 10 minutes. The supernatant was discarded and the pellet washed with 200µls of 70% Ethanol. The ethanol supernatant was then discarded and the pellet was left to dry in the 55°C incubator for 10 minutes. 500µl of TE Buffer (10mM Tris & 1mM EDTA) was added to the pellet to help the DNA dissolve. Tubes were vortexed vigorously and placed in the 55°C incubator for 10 minutes again. This genomic DNA was used to genotype the mice by PCR using standardised reagents and optimised PCR programmes. 1µl of genomic DNA was added to each tube along with 19µls of premix to give a final volume of 20µls per tube as outlined in the table below:

Reagent	Volume per tube (µl)
Genomic DNA (1µg/µl)	1
Primer mix: Amhr2-Cre (100ηg/µl) Cyp-19-Cre (80ηg/µl)	3
Invitrogen 5 X First Standard Buffer Lot 1172260	4
Promega MgCl₂ (25mM)	2
dNTPs (2mM)	1
DNase free H₂0	8.8
Promega Go Taq® Flexi DNA Polymerase 5U/µl	0.2

NOTE: *Amhr2-Cre primers:* CGCATTGTCTGAGTAGGTGT (forward)
GAAACGCAGCTCGGCCAGC (reverse)
Cyp19-Cre primers: TCTGATGAAGTCAGGAAGAACC (forward)
GAGATGTCCTTCACTCTGATTC (reverse)

The two PCR programmes used were as follows:

Cyp-19-Cre Programme	Amhr2-Cre Programme
1. 94°C- 2 minutes	1. 94°C-3 minutes
2. 94°C-30 seconds	2. 94°C-30 seconds
3. 94°C-30 seconds	3. 60°C-30seconds
4. 72°C-30 seconds	4. 72°C-1 minute
5. Go to step 2-30X (cycles)	5. Go to step 2 -35X (cycles)
6. 72°C- 5 minutes	6. 72°C-5minutes
7. 15°C forever	7. 15 °C forever

Note: The genotype of *Pten* and *R26R* mice were previously confirmed.

After PCR was complete, 10µl of each sample was loaded on a 1.5% agarose gel with a positive and negative control. Gel was run at 200 volts for 20 minutes. Samples were allowed to run half way down the gel and were then photographed under a UV light.

Making 1.5% agarose gel

2.5g of agarose powder was dissolved in 150mls of 1 X TBE in a 500ml conical flask and microwaved for 2 minutes. The mixture was removed and swirled for 30 seconds before placing it back in the microwave for a further 2 minutes. When agarose mixture started to boil, flask was removed from microwave and allowed to cool at room temperature (22°C) on magnetic stirrer. 12µl of Ethidium Bromide was added to agarose mixture and gel was poured in 4°C cold room and allowed to set for approx 30 minutes. Samples for genotyping were loaded along with molecular weight marker.

3.12 RNA Extraction

m-RNA Extraction from cultured cells using Qiagen RNeasy Mini® Kit

After incubation and cell treatment the culture medium was aspirated off and 350µls of RLT clear cell lysis buffer was added to each well. Samples were put into separate eppendorf tubes and placed in -80°C freezer. m-RNA was then extracted from these samples using RNeasy kit. Firstly samples were passed at least five times through a 20.6 gauge needle (0.9mm diameter) fitted to an RNase-free syringe. 350µl of 70% ethanol was added to this homogenised lysate and mixed well with a pipette. This was then transferred to an RNeasy mini column placed in a 2ml collection tube. The tube was closed gently and centrifuged for 15 seconds at 8000 x g. The flow through was discarded and 700µl of buffer RW1 was added to RNeasy column. The tube was closed gently and centrifuged for

15 seconds at 8000 x g in order to wash column. The flow through and collection tube were then discarded. The RNeasy column was transferred to a new 2ml collection tube. 500µl of buffer RPE was pipetted onto column and the tube was closed gently and centrifuged for 15 seconds at 8000 x g to wash column. The flow through was discarded and another 500µl of buffer RPE was added to the column. The tube was closed gently and centrifuged for 2 minutes at 8000 x g to dry RNeasy gel membrane. 30µl of RNase free water was pipetted directly onto membrane and tube closed gently and centrifuged for 1 minute at 8000 x g. The m-RNA was stored at -80°C until RT-PCR was performed.

3.13 RT-PCR: Obtaining c-DNA from m-RNA using rodent granulosa cells

Final volume of 40µls per tube

Reagent	Volume per tube (µl)
Mouse mRNA (Rat mRNA use 5 µl)	20
Invitrogen 5 X First Standard Buffer Lot 1172260	8
Invitrogen DTT Lot 1382315 (100mM)	4
Oligo dT Lot 316256 (500ng/µl)	2
dNTP (2mM)	2
Invitrogen RT enzyme Lot 1393450 (200U/µl)	1
DNase RNase free H₂0 (Rat use 18 µl)	3

20µl of each mRNA sample (or 5µl in the case of rat granulosa cells) was placed into separate eppendorf tubes and a premix of the other components was made up depending on how many samples were being reverse transcribed. Each tube contained a final volume of

40µl. PCR was then carried out: 37°C for 45 minutes, 65°C for 15 minutes and 15°C forever. If the signal proved to be too weak, ^{32}P isotope was used to amplify the response as outlined in Section 3.14 below.

3.14 Method to determine linear range of genes of interest
(kit ligand, p15, p16. p19)

Linearisation tests were carried out using template c-DNA. A series of dilutions of the c-DNA template was made to determine the optimum number of cycles to use in radioactive PCR as outlined in table below.

Sample No.	Sample	H$_2$0 (µl)	Corresponding Cycle Number
1	12 µl Template cDNA	0	34
2	3 µl Template cDNA	9	32
3	3 µl Sample 2	9	30
4	3 µl Sample 3	9	28
5	3 µl Sample 4	9	26

2µl of c-DNA Samples No.1-5 were added to 18µl of premix. 1µl of ^{32}P (MP dCTP –Alpha 32P Lot J8YdCX4 3000Ci/mmol) was added to 9µl of H$_2$0 and then 1µl of this diluted ^{32}P mixture was added to the premix. 18 µl of premix and 2µl of c-DNA were then added to each tube as below:

Reagent	Volume per tube (μl)
cDNA	2
Invitrogen C/EBPβ /Cyclophilin /kit ligand / p15 / p16/ p19 primers (100ηg/μl)	2
dNTPs (2mM)	2
Promega 5 X Green Go Taq® Flexi Buffer	4
Promega MgCl₂ (25mM)	2
Promega Go Taq® Flexi DNA Polymerase (5U/μl)	0.2
DNase RNase free H₂0	7.8

PCR was carried out as follows:

Step	Temperature (°C)	Time (Minutes)
1	94	3
2	94	0.3
3	60	0.3
4	72	0.3
5	Step 2,3,4	Repeated 33 times
6	72	5
7	15	Forever

Each sample was then ready to be loaded onto a polyacrylamide gel.

Making 17.5cm X 16cm Polyacyrlamide gel

Solution	Mls
30% acrylamide	6.64
dH20	29
5 X TBE	4
10% APS	0.4
TEMED	0.04

The gel was poured between two clean glass plates and allowed set for 30 minutes. Comb was then removed and gel was placed in tank buffer. 10µl of sample was loaded into each well. Loading dye was loaded into the last well in order to know which direction samples were going in. The gel was run at 150 volts for 30 minutes. It was then removed from the glass plates and placed on top of two sheets of filter paper. The gel and two sheets of filter paper were placed on the gel dryer (filter paper on the bottom) and covered with cling film. The valve on the gel dryer was closed and temperature set to 80°C. The gel was allowed dry for 55 minutes. At this point the gel attached to one sheet of filter paper. The other sheet of filter paper was disposed of in the radioactive waste. The sheet of filter paper with the gel attached was then placed inside a cassette and exposed to an x-ray film in the darkroom. An initial 10 second exposure helped to determine how long the x-ray film should have been left on for. If no bands were seen the cassette with x-ray film was placed in -80°C freezer for 2 hours. Each band was then examined macroscopically and the optimum number of cycles for a particular set of primers was determined. A PCR using the optimum cycle number and the appropriate primers was then carried out as outlined above (start of Section 3.14).

4.0 RESULTS

The pituitary gonadotrophins follicle stimulating hormone (FSH) and luteinising hormone (LH) are the primary stimuli for the growth and differentiation of preovulatory follicles. FSH stimulates granulosa cell proliferation, follicle growth, and the production of estrogen. Once follicles reach the preovulatory stage the LH surge initiates the meiotic resumption of oocytes, the expansion of cumulus cells, and ovulation. After ovulation the remained follicle cells differentiate into luteal cells and produce progesterone to prepare for pregnancy. The pituitary gonadotrophins have the capability of activating various molecular pathways such the PI3K/AKT/FOXO pathway and the Ras/Raf/MEK/ERK pathway. Such pathways are involved with cell proliferation, cell survival and cell cycle arrest. G_1 is the start of the cell cycle and this is entered when the cell senses growth signals or mitogens such as FSH or LH.

Fig 4.1: The pituitary gonadotrophin function in the ovarian cycle
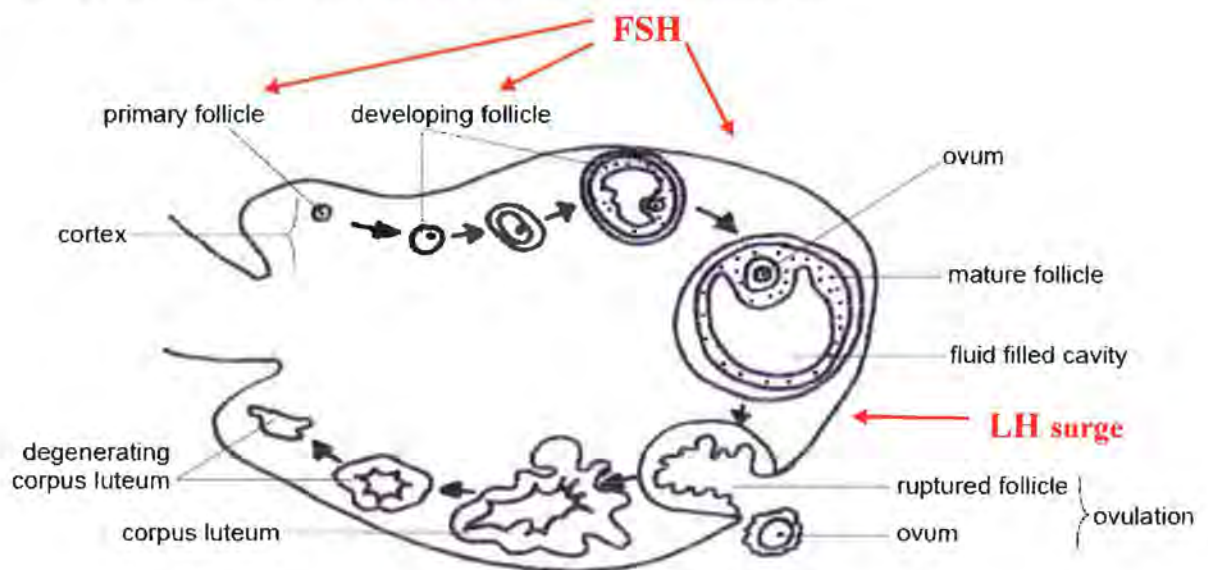

Fig 4.1: Follicle Stimulating Hormone (FSH) functions in the maturation of the ovum over time from primary follicle to antral follicle, followed by a Luteinising Hormone (LH) surge which results in ovulation and the development of the corpus luteum in the empty follicle.

Cellular proliferation and differentiation are fundamental biological processes controlled by extracellular signals that impinge upon cell cycle regulatory machinery and modulate gene expression. Two key classes of regulatory molecules, Cyclins and Cyclin-Dependent Kinases (CDKs), determine a cells' progress through the cell cycle. CDKs are constitutively expressed in cells whereas cyclins are synthesised at specific stages of the cell cycle, in response to various molecular signals from different molecular pathways. In the ovary, hormones control the development of individual follicles by triggering sequential, dynamic changes in granulosa cell proliferation and gene expression (Hadley *et al* 2007). In the case of the PI3K/AKT/FOXO and the Ras/Raf/MEK/ERK pathway, FSH/LH bind to its G-protein coupled receptor which in turn activates Cyclin D2 to start the cell cycle. Cyclin D2 has been shown to be specifically localised to the granulosa cells of growing follicles (Robker and Richards 1998b). The Cyclin D2/CDK4 complex can then activate E2F responsive genes by phosphorylation. Different Cyclin-CDK combinations determine which downstream proteins will be targeted. A protein called p27^{Kip1} can inhibit Cyclin D2 and thus cause cell cycle arrest. Protein kinase B (AKT) can repress p27^{Kip1}. In the ovaries, Cyclin D2 expression has been shown to be crucial for the proper growth of granulosa cells with the follicle. Female mice carrying a null mutation on the Cyclin D2 gene are infertile because of impairment in granulosa cell proliferation in response to FSH, resulting in small follicles with trapped oocytes (Robker and Richards 1998b). In non-growing, primordial follicles, a single layer of granulosa cells arrested in G_0 surrounds the oocyte. In response to unknown signals, primordial follicles leave the resting pool and granulosa cells initiate a phase of growth in which proliferation is exceedingly slow.

To investigate the importance of the PI3K/AKT/FOXO and Ras/Raf/MEK/ERK cascades in this process of proliferation and differentiation, downstream targets from each pathway were selected and studied. This was accomplished

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by injecting 3 week old mice with PMSG and 48 hours later with hCG at various time points to mimic the effects of FSH and LH as explained in Section 3.7(i). Protein was then extracted from whole ovary lysates and western blot analysis carried out. Firstly the expression pattern of critical cell cycle regulators Cyclin D2 and p27^{Kip1} was examined.

Fig 4.2a: Western blot analysis of cell cycle regulators
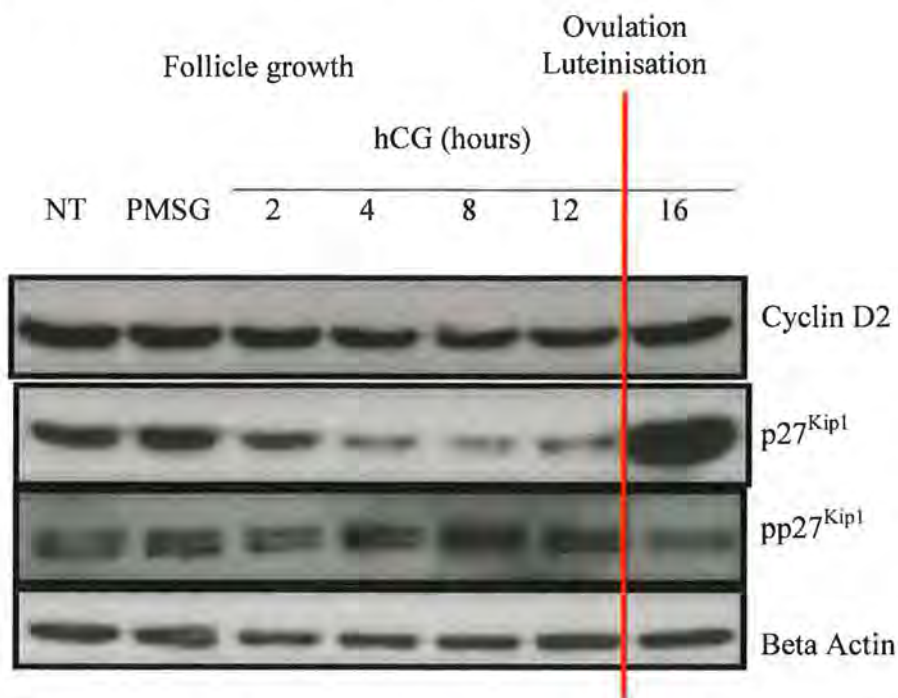
Fig 4.2a: Shows a western blot of wild type mouse whole ovary lysate for Cyclin D2, p27^{Kip1} and phosphorylated p27^{Kip1} (pp27^{kip1}) expression. Expression is shown for immature non-treated (NT), pregnant mare serum gonadotrophin (PMSG) treated and hCG (human chorionic gonadotrophin) treated wild type mice at different time points (2,4,8,12 and 16 hours) post treatment. Beta Actin is used as a loading control.

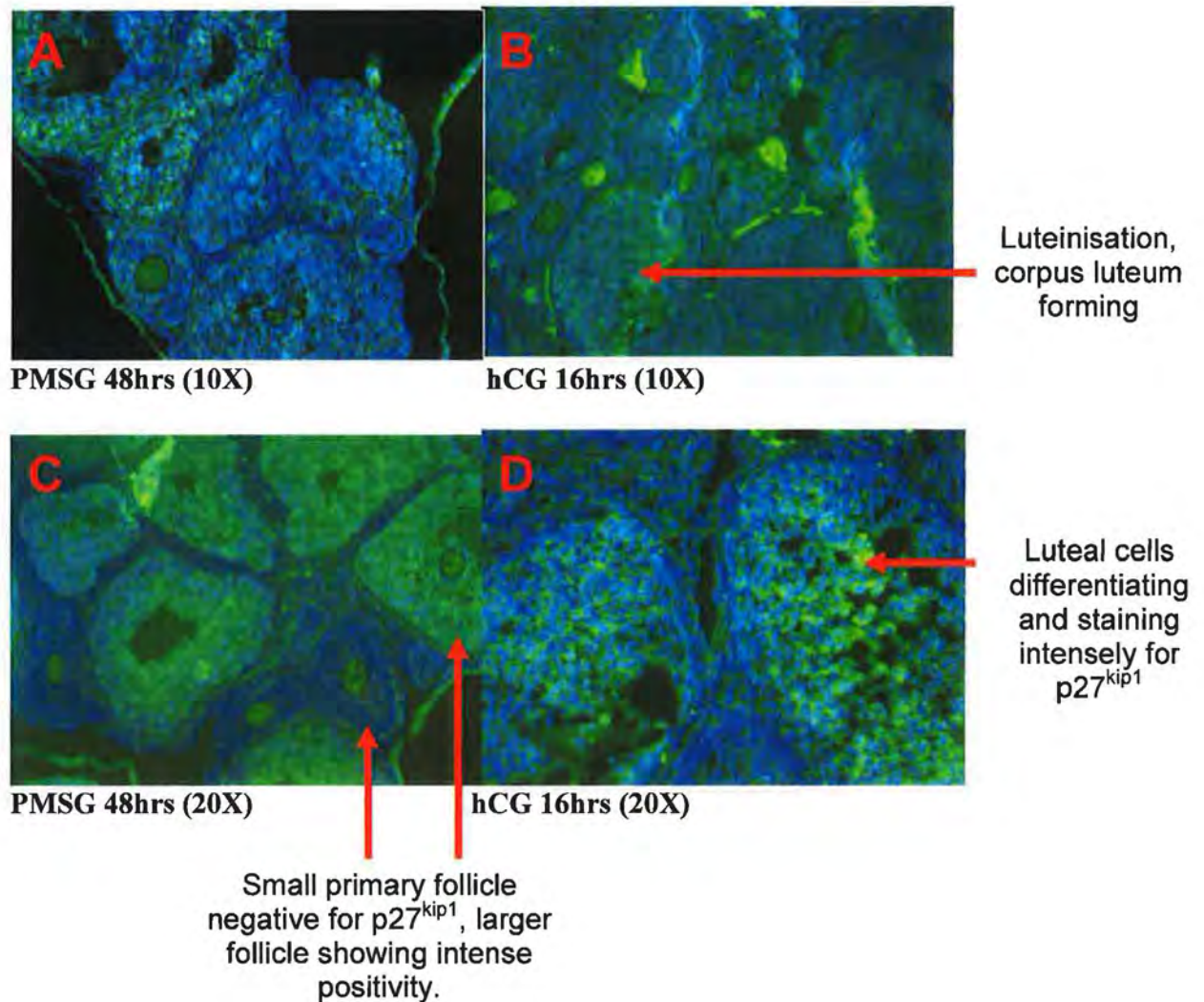**Fig 4.2b: Optical Density Quantification of Western Blots shown above**
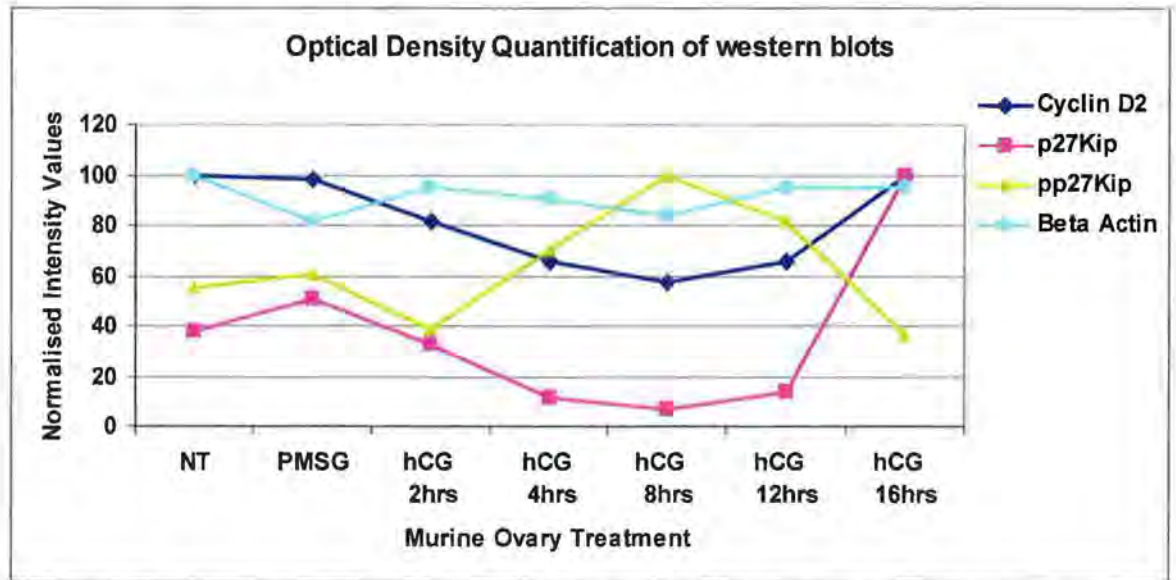
Fig 4.2b: Each western blot band was measured on an optical density quantifier (Molecular Dynamic Machine) and analysed using Scanner Control Software Image Quant 5.2. A volume and an area measurement were obtained for each band. An intensity index was then calculated using these results and a normalised value which was obtained for each band was plotted on this graph.

Levels of Cyclin D2 remain relatively stable throughout the hCG series. It is known that Cyclin D2 is a critical factor needed for cell cycle initiation and progression. p27^{Kip1} is down regulated first at 2, 4, 8, 12 hours post hCG but is then up regulated at 16 hours when cells have started to luteinise (Fig 4.1). It is at this time point that ovulation occurs and the oocyte is released. The ovulated follicle now develops into a corpus luteum. The cell cycle stops after hCG treatment so essentially granulosa cells stop proliferating and start to differentiate. Phospho-p27^{Kip1} (pp27^{Kip1}) protein levels decrease slightly at 16 hours post hCG which is mirrored by an increase in total p27^{Kip1}. From the hCG 2 hour time point onwards there is an inverse relationship between total p27^{Kip1} levels and phospho-p27^{Kip1} levels (Fig 4.1b). To determine the localisation of p27^{kip1} within the ovary some immunofluorescence staining was carried out on PMSG treated and 16 hours post hCG treated frozen ovary sections. Results showed that p27^{Kip1} was present in some of the developing primary follicles, mainly the larger more developed ones (Fig 4.3) and also in

the oocyte after PMSG treatment for 48 hours. 16 hours post hCG treatment, luteal cells

stained positively for p27^{Kip1} (Fig 4.2).

Fig 4.3: p27^{Kip1} immunofluorescence staining of wildtype mice frozen ovary sections

PMSG 48hrs (10X)   hCG 16hrs (10X)

Luteinisation, corpus luteum forming

PMSG 48hrs (20X)   hCG 16hrs (20X)

Luteal cells differentiating and staining intensely for p27^{kip1}

Small primary follicle negative for p27^{kip1}, larger follicle showing intense positivity.

Fig 4.3: Showing p27^{kip1} staining (green colour) and DAPI (blue colour) counter staining in PMSG treated (A, C) and 16 hours post hCG (B, D) treated ovary sections. p27^{kip1} staining more intensely 16 hours post hCG after luteinisation has occurred and luteal cell differentiation commenced. A negative control which had not been treated with any primary antibody was included showing no green p27^{kip1} positive staining.

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Apoptotic markers

The PI3K/AKT/FOXO and Ras/Raf/MEK/ERK pathways provide signals for many types of somatic cells. One function of these pathways in the ovary may be the survival of granulosa cells. To understand this better, two factors connected with apoptosis were investigated: Mammalian Ste20-like kinase (MST1) and stress-activated protein kinase/Jun-amino-terminal kinase (Phospho-SAPK/JNK). Oxidative stress activates the MST1 JNK pathways that phosphorylate FOXO1 at a conserved site within the forkhead domain that promotes FOXO nuclear translocation and thereby induces cell death (Lehtinen *et al* 2006) *(Fig 1.10(i))*. To examine the effects of FSH/LH treatment on MST1 and p-SAPK/JNK, 3 week old mice were injected with PMSG first and then 48 hours later with hCG at different time points.

Fig 4.4: Western blot analysis of some apoptotic markers
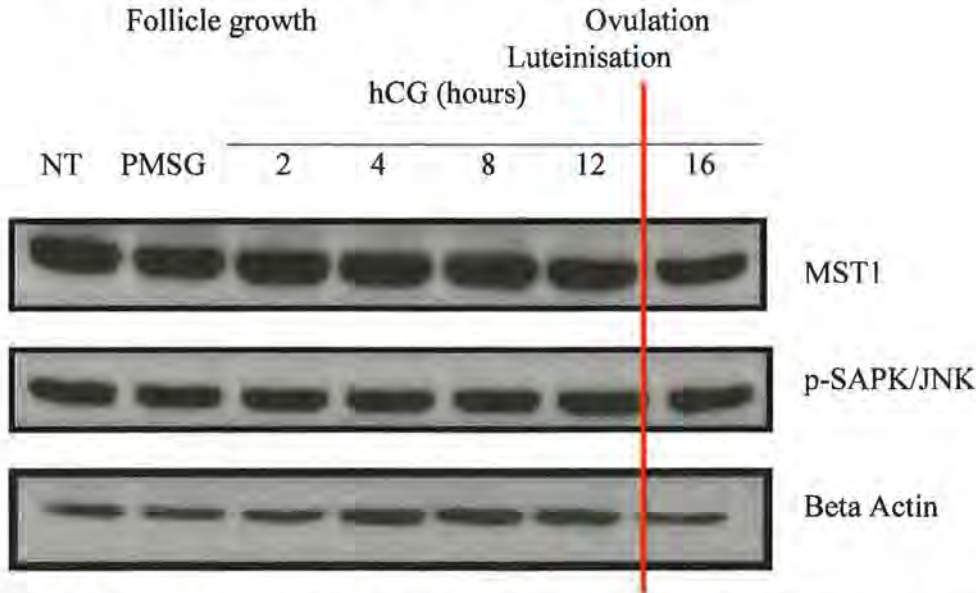
Fig 4.4: Shows a western blot of wild type (WT) whole mouse ovary lysate using mammalian Ste20-like kinase (MST1) and phosphorylated stress-activated protein kinase/Jun-amino-terminal kinase (p-SAPK/JNK) antibodies. Protein expression is shown for immature non treated (NT), pregnant mare serum gonadotrophin (PMSG) treated and hCG (human chorionic gonadotrophin) treated wild type mice at different time points (2,4,8,12 and 16 hours) post treatment. The red line symbolises where ovulation has occurred. Beta actin was used as a loading control.

The protein levels of both apoptotic markers remained unchanged at each time point in the western blot hCG series. It would appear that ovarian cells rely on apoptotic signals other than MST1 and phospho-SAPK/JNK to control their apoptosis. To investigate the localisation of MST1 and pSAPK/JNK within the ovary, immunofluorescent staining was carried on out some frozen ovary sections. MST1 and pSAPK/JNK gave a very similar staining pattern. Theca cells stained more intensely than other cells within the ovary. PMSG treated ovaries show a slightly more positive staining pattern than non-treated immature follicles. In the PMSG treated ovary section, the oocyte stained intensely with the pSAPK/JNK antibody.

Fig 4.5: Immunofluorescence staining of apoptotic markers MST1 and pSAPK/JNK
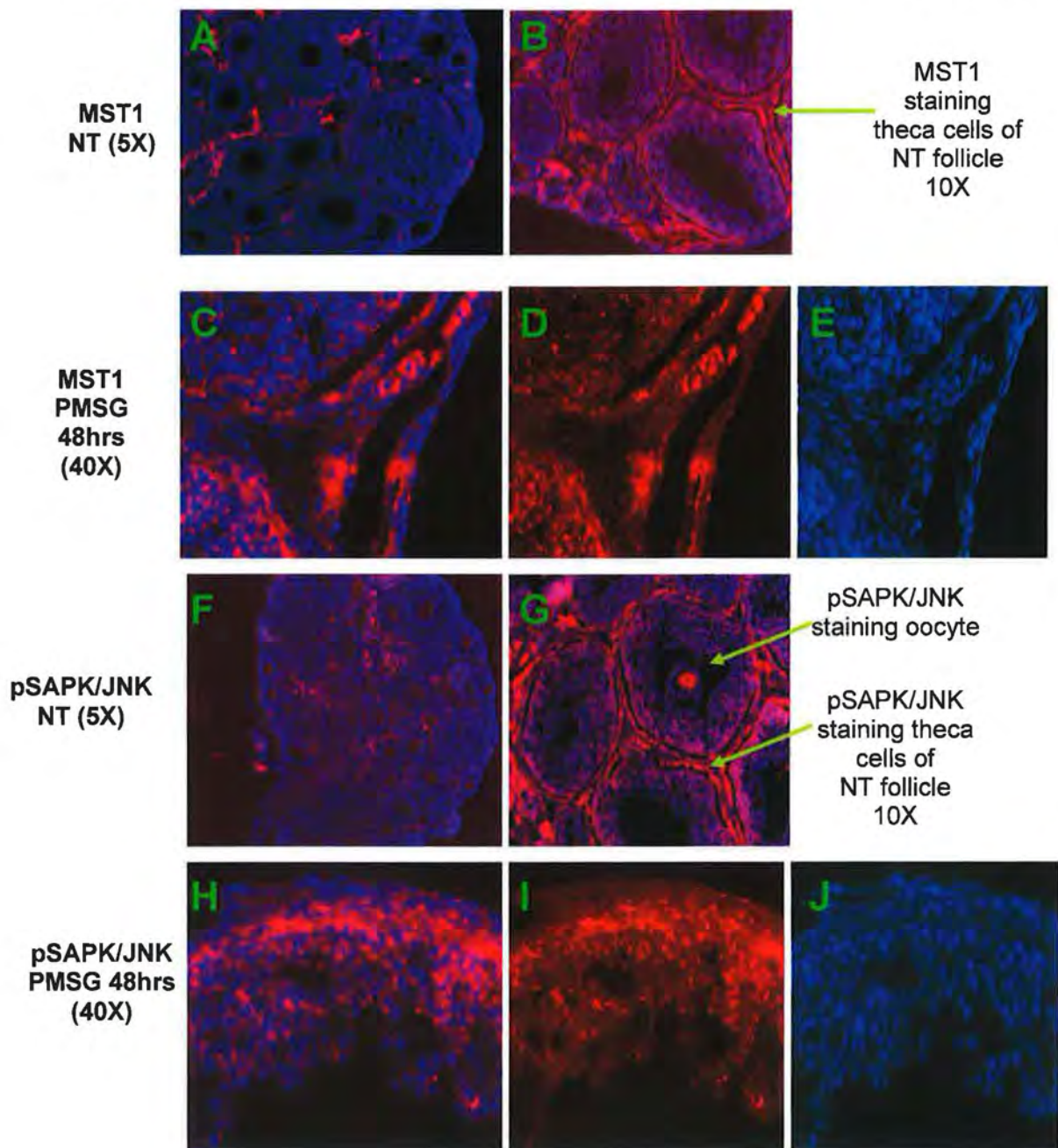
Fig 4.5: Mammalian Ste20-like kinase (MST1) red staining of frozen non-treated (NT) ovary under 5X magnification (A) and 10X magnification (B). MST1 staining non-treated (NT) ovary sections viewed under 40X magnification (C), with (D) and (E) separately showing the red MST1 staining and blue DAPI staining respectively. (F) depicts phosphorylated stress-activated protein kinase/Jun-amino-terminal kinase (pSAPK/JNK) red staining of frozen non-treated (NT) sections viewed under 5X magnification and (G) 10X magnification. (H) PMSG treated section viewed under 40X magnification, clearly showing theca cell positivity with (I) and (J) separately showing red pSAPK/JNK staining and blue DAPI staining respectively.

Investigation of the phosphorylation pattern of downstream targets of PI3K/AKT/FOXO pathway

Having looked at a number of cell cycle regulators and some markers of apoptosis it was now necessary to investigate the phosphorylation of specific PI3K components downstream of the FSH/LH receptor. The mechanism by which FSH/LH receptors activate PI3K/AKT pathway has as yet not been determined. In other systems however, Ras has been reported to interact with PI3K directly and activates the whole pathway as explained in Section 1.10(ii). PI3K is a lipid kinase that catalyses the transformation of PIP_2 into PIP_3. As a membrane-associated second messenger, PIP_3 induces the activation of PDK1/2 and AKT sequentially. AKT is a multi-functional kinase which promotes cell proliferation and cell survival by phosphorylating many target molecules. One important target of AKT in granulosa cells is the transcription factor FOXO1. FOXO factors repress cell proliferation and promote apoptosis. AKT activation induces the nuclear export and degradation of FOXO1 by phosphorylation. When AKT is phosphorylated it can also phosphorylate p70S6kinase which is concerned with regulating protein translation and synthesis *(Fig 4.6)*.

Fig 4.6: Overview of downstream phosphorylation pattern in the PI3K/AKT/FOXO pathway
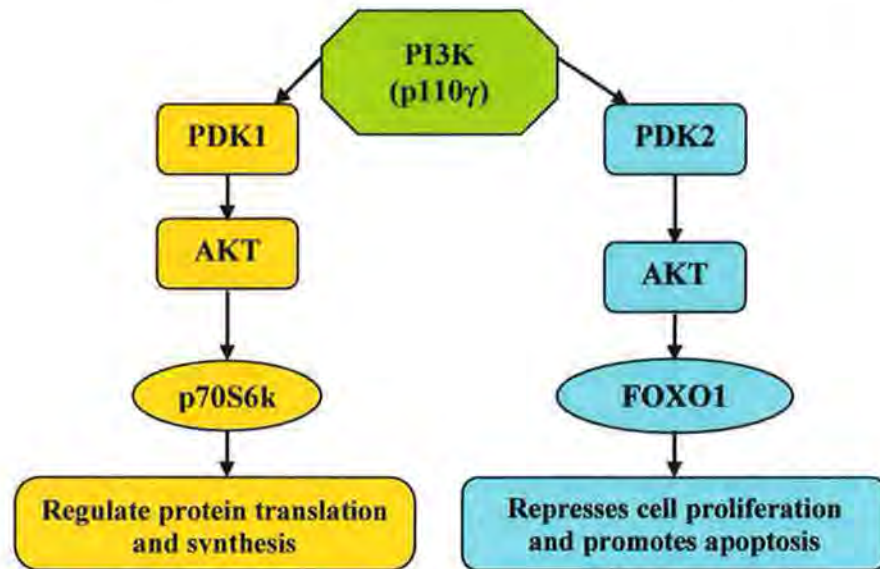
Fig 4.6: p110gamma is the catalytic subunit of PI3K. PDK1/2 are the activating kinases which phosphorylated protein kinase B (AKT). pAKT then activates p70S6K or it can phosphorylate the transcription factor forkhead box O1 (FOXO1) rendering it inactive.

A wildtype hCG series was used to look more closely at some key intermediates in the PI3K/AKT/FOXO pathway and to monitor the changes in protein expression. Firstly to consider the step of the pathway where p110γ the catalytic subunit of PI3K results in PDK1 phosphorylation, p110γ levels and the phosphorylated form of PDK1 (pPDK1) were investigated using western blot analysis.

Fig 4.7: Western blot analysis to investigate the phosphorylation pattern of downstream targets of the PI3K/AKT/FOXO pathway
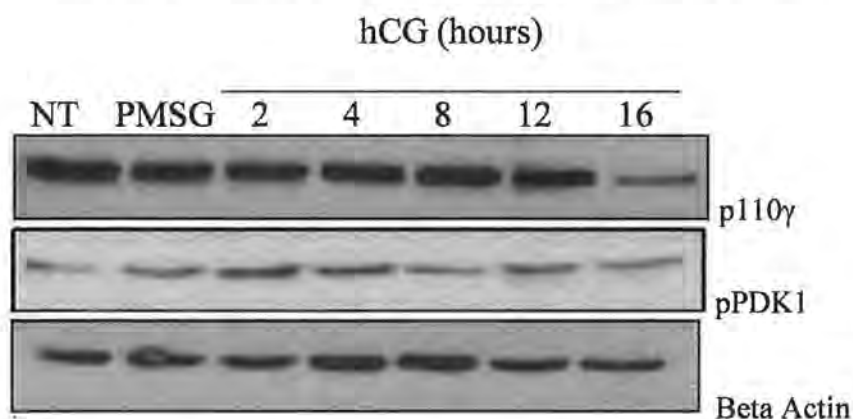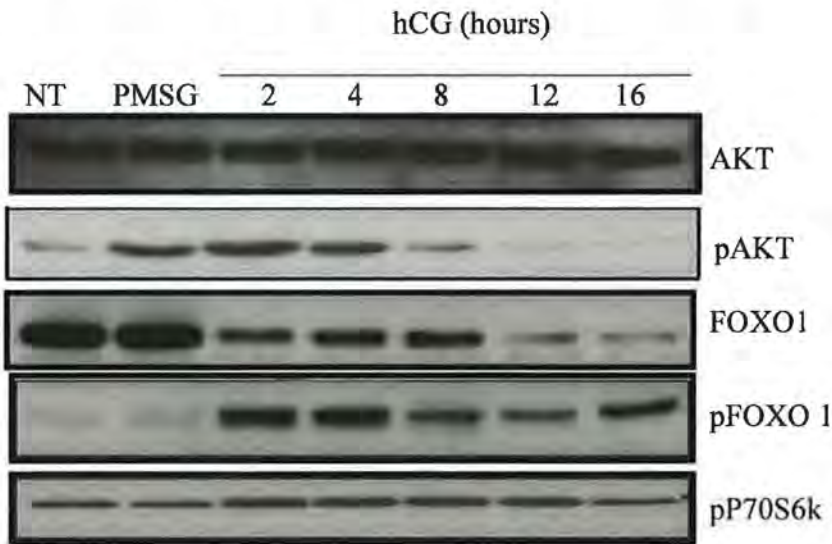

Fig 4.7: Shows a western blot of wild type (WT) whole mouse ovary lysate for p110γ (phosphorylated 110 gamma subunit) and pPDK1 (phosphoinositide dependant protein kinase 1) expression. Expression is shown for immature non-treated (NT), pregnant mare serum gonadotrophin (PMSG) treated and hCG (human chorionic gonadotrophin) treated wild type mice at different time points (2, 4, 8, 12 and 16 hours) post treatment. Beta Actin is used as a loading control.

p110γ levels remain constant until the 16 hour post hCG time point indicating once ovulation occurs there is a decrease in p110γ protein level as a result of granulosa cells beginning to develop into luteal cells. Phosphorylated PDK1 levels are at their highest at 2 and 4 hours post hCG as this is the point when cells start to proliferate and follicles start to develop from the pre-antral stage. Protein expression then reverts back to NT, PMSG levels at the 8, 12 and 16 hour post hCG time points. This would indicate that the PI3K/PDK1/AKT pathway is at its most active when follicular cells are proliferating and growing before luteinisation occurs. Further downstream targets of PDK1 were also investigated using a hCG timecourse *(Fig 4.8)*.

Fig 4.8a: Western blot analysis to investigate the phosphorylation pattern of downstream targets in the PI3K/AKT/FOXO pathway

Fig 4.8a: Shows a western blot of wild type (WT) whole mouse ovary lysate for total unphosphorylated Protein Kinase B (AKT), which is also used in this case as a loading control, phosphorylated protein kinase B (pAKT), total unphosphorylated forkhead box O1 (FOXO1), phoshorylated forkhead box O1 (pFOXO1) and phosphorylated p70S6 kinase (pP70S6k) expression. Expression is shown for immature non-treated (NT), pregnant mare serum gonadotrophin treated (PMSG) and hCG (human chorionic gonadotrophin) treated wild type mice at different time points (2, 4, 8, 12 and 16 hours) post treatment.

Fig 4.8b: Optical Density Quantification of Western Blots shown above
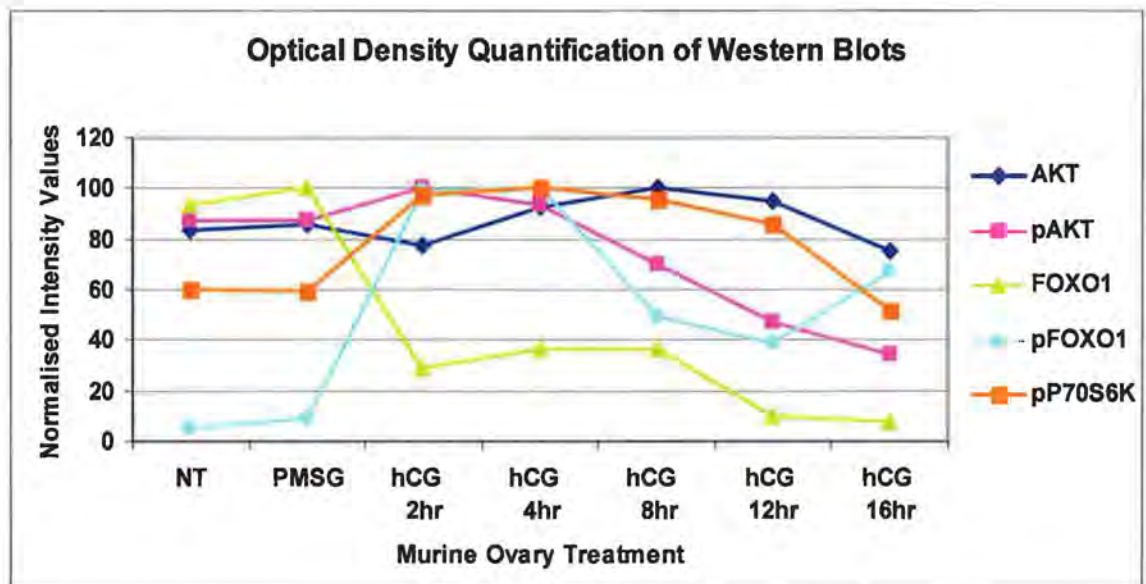
Fig 4.8b: Each western blot band was measured on an optical density quantifier (Molecular Dynamic Machine) and analysed using Scanner Control Software Image Quant 5.2. A volume and an area measurement were obtained for each band. An intensity index was calculated from these results and a normalised value obtained for each band and plotted on this graph.
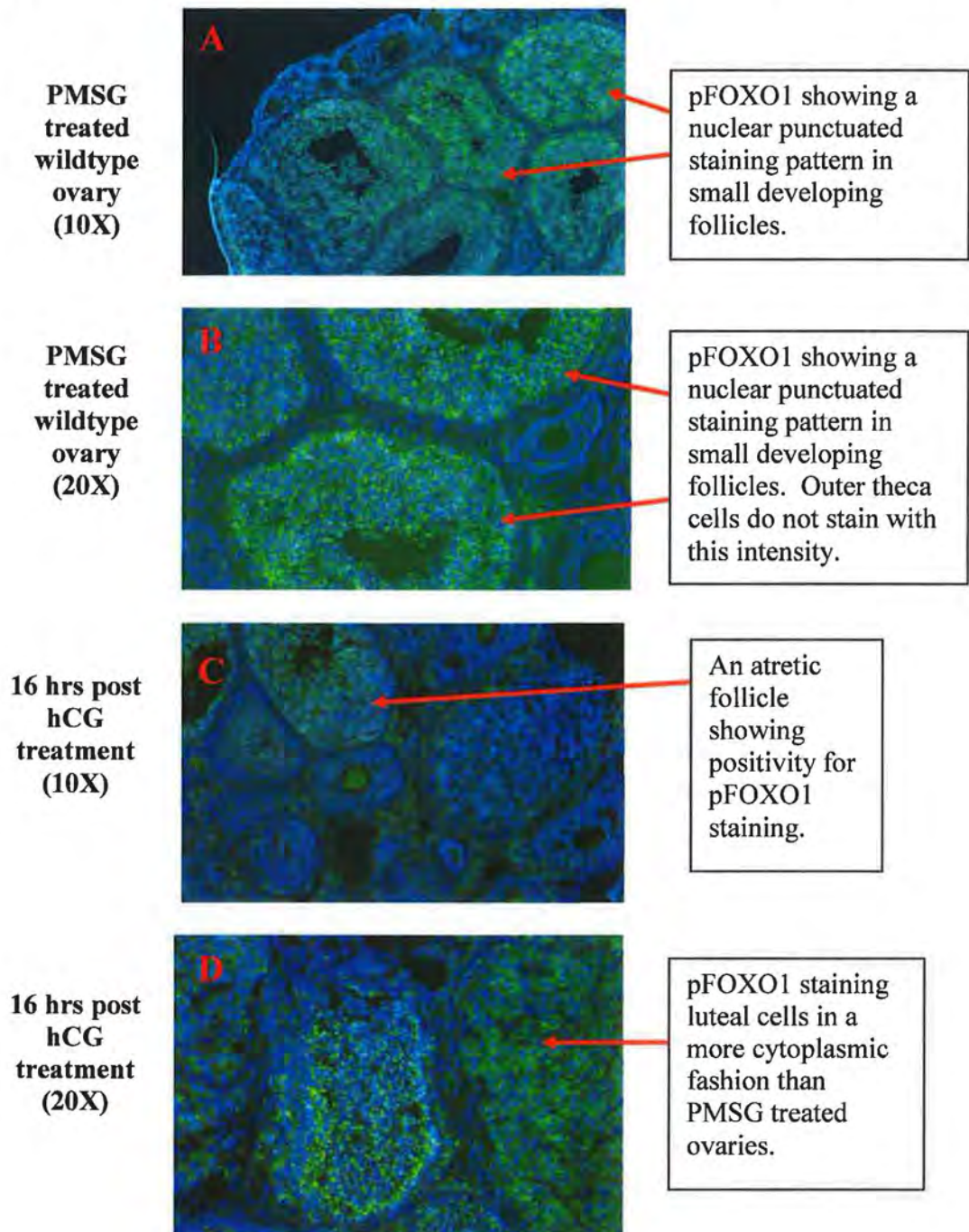
In the immature untreated wild type ovaries, phosphorylated AKT expression was low to absent. pAKT, the activated form of the protein kinase B intermediate, was induced by PMSG, 2 and 4 hours post hCG treatments in the wild type mouse. p-AKT expression was shown to be moderately induced by PMSG treatment. A greater induction was evident two hours post hCG treatment and at 4 hours post hCG treatment induction was down regulated. Expression was therefore highest 2 hours post hCG treatment in the wild type ovary lysate. At 12 hours and 16 hours post hCG treatment pAKT expression decreased to almost absent levels in the wild type mouse *(Fig 4.8a, 4.8b)*. There is perhaps a transient phosphatase acting to downregulate pAKT.

Total FOXO1 levels are highest in the immature non-treated and PMSG treated ovaries. After hCG treatment the levels of FOXO1 start to fall and at the 12 and 16 hour post hCG time points, the protein expression is nearly undetectable indicating that once differentiation and luteinisation occurs, total FOXO1 is not longer needed in such abundance as when follicles are developing. The removal of total FOXO1 correlates with the increase in the phosphorylated form of FOXO1 (pFOXO1) at 12 and 16 hours post hCG. In fact, once hCG has been administered, even at the 2 hour time point there is an increase in pFOXO1 protein expression. The smaller immature follicles of the non-treated and PMSG treated ovaries do not experience this rapid phosphorylation of FOXO1. In these earlier developing and proliferating follicles, total FOXO protein levels rather than pFOXO1 levels are high. The transient increase of pAKT suggests that AKT phosphorylation is important in getting the ball rolling as regards FOXO1 phosphorylation but then perhaps another kinase takes over. pP70S6 kinase protein levels remain relatively constant after hCG treatment, decreasing slightly at the 16 hour time point where luteinisation occurs.

Frozen ovary sections were stained with pFOXO1 antibody to assess if pFOXO1 was changing localisation in the PMSG treated ovaries as opposed to ovaries that

had been treated with hCG for 16 hours. Both PMSG treated and hCG treated sections however showed a punctuated cytoplasmic type staining pattern *(Fig 4.9)*.

Fig 4.9: pFOXO1 immunofluorescence staining of wildtype frozen ovary sections

PMSG treated wildtype ovary (10X) — pFOXO1 showing a nuclear punctuated staining pattern in small developing follicles.

PMSG treated wildtype ovary (20X) — pFOXO1 showing a nuclear punctuated staining pattern in small developing follicles. Outer theca cells do not stain with this intensity.

16 hrs post hCG treatment (10X) — An atretic follicle showing positivity for pFOXO1 staining.

16 hrs post hCG treatment (20X) — pFOXO1 staining luteal cells in a more cytoplasmic fashion than PMSG treated ovaries.

Fig 4.9: Showing pFOXO1 staining (green colour) and DAPI (blue colour) counter staining in PMSG treated (A) and (B) and 16 hours post hCG treated (C) and (D) frozen ovary sections.

To investigate how extracellular ligands affect the PI3K/AKT/FOXO pathway

To understand AKT phosphorylation better, some cell culture work using rat granulosa cells was carried out. Amphiregulin (Areg), Follicle Stimulating Hormone (FSH) and Interleukin 6 (IL6) are thought to be strong activators of the PI3K/AKT/FOXO pathway. Areg is an epidermal growth factor (EGF) which is induced in the rat ovary by gonadotrophin treatment. The role of rat amphiregulin in the ovary is unknown. Previous studies have shown that ovarian expression of rat amphiregulin coincides with that of cyclo-oxygenase-2 (COX2) and progesterone receptor (PR), both of which are known to play integral roles in ovulation. These observations suggest that rat amphiregulin is induced by the preovulatory LH surge, and may be associated with ovulatory events since amphiregulin and epiregulin act as mediators of LH action in the mammalian ovulatory follicles (Sekiguchi *et al* 2004). Regulation of the expression of these factors may open new possibilities in treatment of ovarian malfunction implicated with ovarian hyper-stimulation. FSH is the principal regulator of follicular growth and maturation. As mentioned in Section 1.13 (i), Interleukin 6 (IL6) has previously been demonstrated to directly influence FSH differentiated functions of ovarian granulosa cells (Van der Hoek *et al* 1998). To investigate AKT phosphorylation from an extracellular point of view, granulosa cells were treated with these inducers (Areg, FSH and IL6) for 5, 10 and 30 minutes.

Fig 4.10: Western blot results of rat granulosa cells cultured overnight and then treated with extracellular ligands
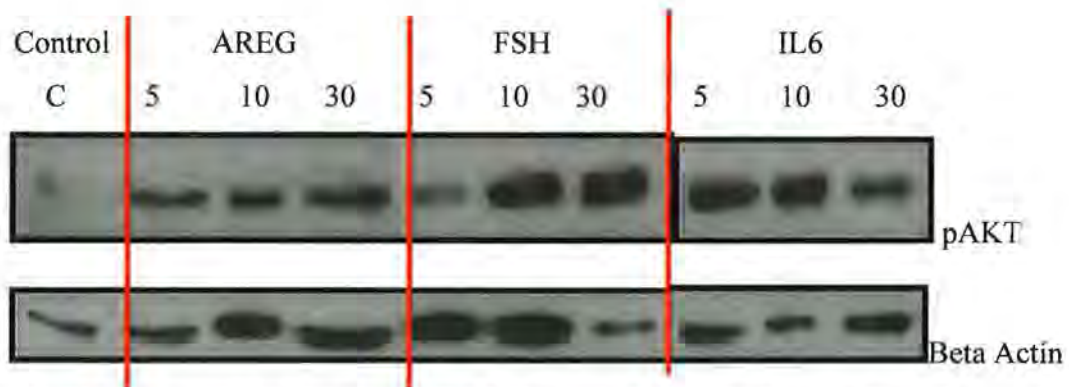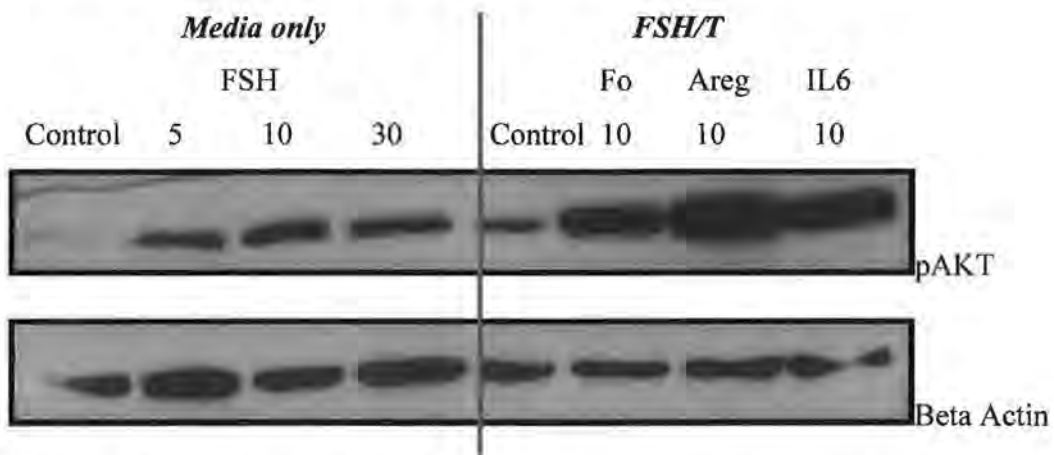

Fig 4.10: Rats were injected with estradiol for 3 consecutive days before granulosa cells were isolated and cultured overnight in culture media at 37°C. Ligands Amphiregulin (Areg), Follicle Stimulating Hormone (FSH) and Interleukin 6 (IL6) were added for 5, 10 or 30 minutes just before cells were harvested. The control lane on the left was not treated with any ligands. Beta-Actin was used as a loading control.

All the ligands display the ability to induce AKT phosphorylation when compared to the control lane. Areg shows a uniform induction of AKT phosphorylation at 5, 10 and 30 minute time points. FSH has the strongest ability out of the three ligands used to induce AKT phosphorylation, showing most phosphorylation between the 5 minute and 10 minute treatment time points. IL6 also displays the ability to induce AKT phosphorylation, showing the largest amount of pAKT expression at 5 and 10 minute time points and decreasing slightly after 30 minutes of treatment (Fig 4.10).

Rat granulosa cells were also cultured with culture media alone overnight and with culture media containing FSH and Testosterone (FSH/T) overnight. Treatment with FSH/T results in androgen being converted to estrogen by aromatase. This treatment means better induction of the LH receptor and better cytokine cleavage. Estradiol is important for facilitating induction of many genes which are present in the pre-ovulatory follicle. Rat granulosa cells were again cultured overnight at 37°C and then treated with FSH, IL6, Areg, and Forskolin (Fo) for ten minutes. Forskolin is commonly used to raise levels of cyclic AMP (cAMP) in the study and research of cell physiology.

Forskolin resensitises cell receptors by activating the enzyme adenylyl cyclase and increasing the intracellular levels of cyclic Adenosine Monophosphate (cyclic AMP or cAMP). Cyclic AMP is an important signal carrier that is necessary for the proper biological response of cells to hormones and other extracellular signals. It is required for cell communication in the hypothalamus/pituitary gland axis and for the feed-back control of hormones. It acts by activating protein kinase A.

Fig 4.11: Western blot results of rat granulosa cells cultured overnight in media only and in media containing FSH and Testosterone

Fig 4.11: Rats were injected with estradiol for 3 consecutive days before granulosa cells were isolated and cultured overnight in culture media only at 37°C and in culture media containing FSH and testosterone (FSH/T). FSH was added to media only samples for 5, 10 and 30 minutes just before cells were harvested. Forskolin (Fo), Amphiregulin (Areg) and Interleukin 6 (IL6) were added to granulosa cells in FSH/T media for 10 minutes just before cells were harvested. Both control lanes were not treated with any ligands. Beta-Actin was used as a loading control.

FSH induces AKT phosphorylation in the cells that have been cultured overnight in media only at 5, 10 and 30 minute time points. Fo, Areg and IL6 all show the capability to induce AKT phosphorylation compared to the control sample which was not treated with any ligand. If we compare the 10 minute Areg time point in Fig 4.10, where culture media alone has been used, with the Areg 10 minute time point in Fig 4.11 where granulosa cells have been incubated with FSH/T overnight, Areg shows a stronger ability to induce AKT

phosphorylation when cells are incubated with FSH/T overnight. The same could be said for IL6 results in Fig 4.10 and Fig 4.11, i.e. granulosa cells incubated in FSH/T containing media results in an increase in pAKT levels.

C/EBPβ

The transcription factor C/EBPβ is induced promptly in the ovary, as a response to an ovulatory dose of gonadotrophins. A specific reduction in ovarian C/EBPβ expression inhibits ovulation. In such ovaries, oocytes appear to be entrapped within the follicle. It has been shown that there is a correlation between the expression level of the activating isoform of C/EBPβ and the number of oocytes ovulated in response to gonadotrophins (Pall *et al* 1997). Since a reduction in C/EBPβ expression does not affect the level of the ovulatory mediator cyclo-oxygenase-2 (COX2), these findings support the view of C/EBPβ being an important factor in the ovulatory process and highlight a C/EBPβ-dependent and COX-2-independent pathway that takes part in the regulation of ovulation (Sternick *et al* 1997). C/EBPβ is induced very promptly in response to an ovulatory dose of LH. Within 30 minutes after administration of an ovulatory dose of hCG, C/EBPβ mRNA can be detected in the ovary. C/EBP-β acts as a regulator of COX2 expression (Sirios *et al* 1993). To investigate how C/EBPβ compares to other downstream targets in the PI3K/AKT/FOXO pathway and potentially elucidate the role it plays in ovarian cell proliferation and differentiation, western blot analysis was carried on a hCG series.

**Figure 4.12: Western blot analysis to investigate C/EBPβ expression in the ovary**
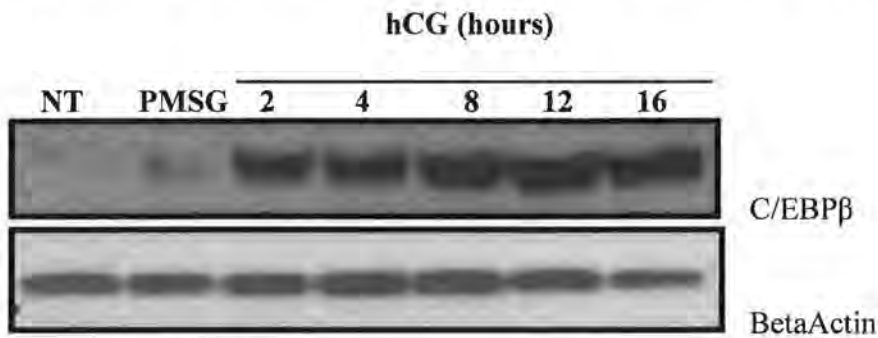
Fig 4.12: A western blot of wild type mouse whole ovary lysate for C/EBPβ expression. Expression is shown for non- treated immature (NT), pregnant mare serum gonadotrophin (PMSG) treated and hCG (human chorionic gonadotrophin) treated wild type mice at different time points (2, 4, 8,12 and 16 hours) post treatment. Beta Actin is used as a loading control.

C/EBPβ is present at 2, 4, 8, 12 and 16 hour time points. Previous studies show ERK is present in NT and PMSG treated samples (Fan *et al* 2008b). C/EBPβ comes later than this so we hypothesise that it is downstream of ERK. It was now necessary to look at a longer hCG time course to see if C/EBP beta is still present after 16 hours hCG treatment. m-RNA was extracted from rat whole ovary lysates. Reverse Transcriptase PCR was carried out using this m-RNA in order to make c-DNA. A PCR was then carried out on this c-DNA, using C/EBP beta primers and a radioactively labelled ^{32}P dCTP isotope. The samples were run in a polyacrylamide gel, dried on a gel dryer and transferred to a sheet of filter paper. This was then placed in a cassette and developed in the dark room using x-ray films.

Fig 4.13: C/EBPβ expression using a longer hCG time course.

hCG (days)

NT PMSG 16h 2 3 4 5 6

LAP 21 kDa

LIP 14 kDa   **C/EBPβ**
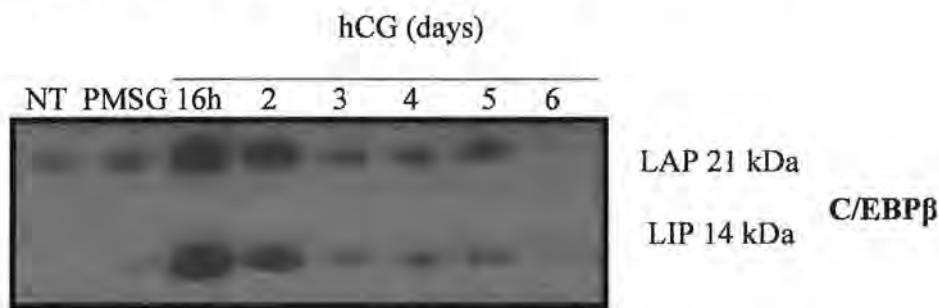
*Fig 4.13:* mRNA was extracted from mouse granulosa cells and a RT-PCR carried out to make c-DNA. A PCR using radioactively labelled ^{32}P dCTP was then carried out to investigate C/EBPβ expression using a longer time course of hCG treatments. Upper band is LAP 21-kDa and lower band is LIP 14-kDa.

C/EBPβ is induced at 16 hrs and decreases progressively as the days go on. There are two forms of C/EBPβ, LAP (liver-enriched transcriptional activator protein), and LIP (liver-enriched transcriptional inhibitory protein). In Fig 4.13 the upper band is LAP 21-kDa and the lower band is LIP 14k-Da. LIP is the truncated form of LAP which acts as an internal negative regulator. LIP lacks an N-terminal domain so cannot bind to DNA. C/EBPβ functions mainly up to 2 days post hCG and is then quickly down-regulated after this time point onwards.

As discussed earlier, FSH and Areg are extracellular ligands with the ability to induce AKT phosphorylation. We now want to investigate, using cell culture techniques, if these ligands have the ability to induce the expression of C/EBPβ protein.

Fig 4.14: Western Blot to investigate if FSH and Areg can induce C/EBPβ expression
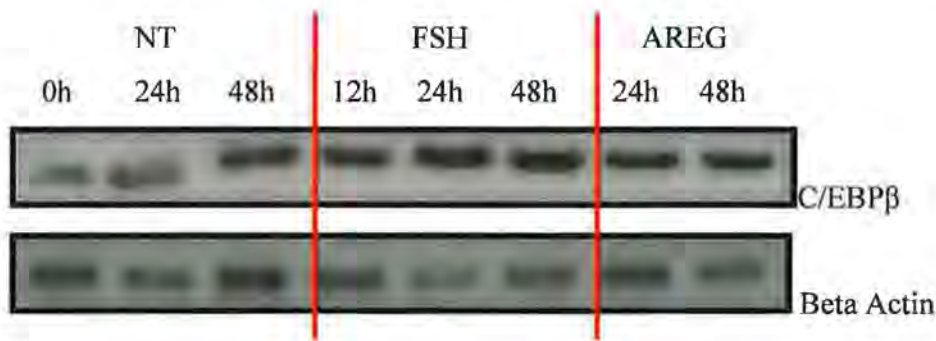

Fig 4.14: Western Blot analysis of rat granulosa cells which were cultured overnight and then treated with extracellular ligands Follicle Stimulating Hormone (FSH) and Amphiregulin (Areg) to investigate if these ligands can induce C/EBPβ expression. The non-treated samples (NT) on the left were cultured overnight and not treated with any ligand at all. The middle column of samples were cultured at 37°C overnight in media and then treated with FSH for 12, 24 and 48 hours. The samples on the right were cultured at 37°C overnight in media and then treated with Areg for 24 and 48 hours. Beta Actin was used as a loading control.

FSH and Areg display the ability to induce C/EBPβ when compared to non-treated cells (NT). This would suggest that C/EBPβ may be actively involved in ovarian cell proliferation and differentiation. Published data suggests that C/EBPβ is in fact involved in the cell cycle. Cyclin D1 and p21 can be regulated by C/EBPβ (Luedde *et al* 2004).

Pten: The negative regulator of the PI3K pathway

In view of the importance of the PI3K pathway to the differentiation of ovarian granulosa cells and ovulation, we sought to identify the physiological role of *Pten* in developing ovarian follicles and its regulation of the PI3K pathway in granulosa cells. A conditional *Pten* knockout mouse strain was generated as described in Section 1.14. To generate a granulosa cell specific conditional knockout, Pten^flox/flox^ mice were mated to Cre recombinase expressing mice, either the Amhr-2-Cre or the Cyp-19-Cre mouse. Amhr-2-Cre and Cyp-19-Cre genotyping was carried out.

Fig 4.15 Genotyping for the Amhr2-Cre and Cyp19-Cre genotypes
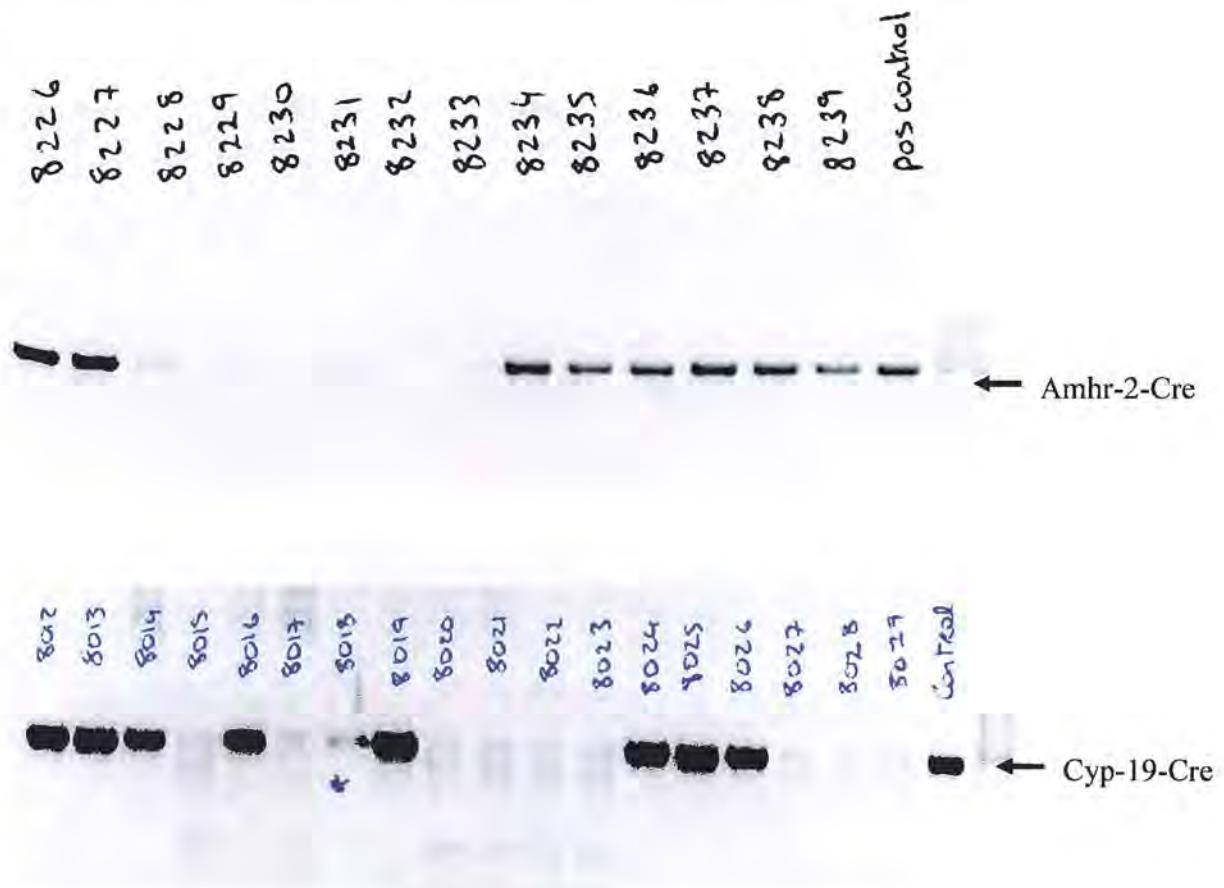
Fig 4.15: showing the genotyping results of a representative number of mice tested for the Amhr 2-Cre and Cyp-19-Cre genotypes. The bands represent PCR amplified mouse tail DNA run out and visualised on a 1.5% agarose gel. The Amhr 2-Cre and Cyp-19-Cre genotypes are both represented by bands of approximately 300 base pairs. Lane 8018 in the Cyp-19-Cre gel is a very immature mouse in which Cre is still present. This will eventually disappear. On the far right of the gel a molecular weight marker is shown.

Pten mutations often lead to tumours in different tissues as the PI3K pathway is uncontrolled and hyperactive, however in the Pten conditional knockout (CKO) model, tumour formation does not occur, even at 10 months of age. Moreover, the Pten$^{flox/flox}$Cyp19-Cre mice are actually fertile. Studies in our lab have shown that the Pten mutant mice give birth to ~20% more pups than wildtype in a 6-months breeding period. In super-ovulation experiments, the Pten$^{flox/flox}$Cyp19-Cre mice ovulate more oocytes than wildtype do, as examined at 16 hours after hCG treatment. When the mice were sacrificed

at 10 hours post-hCG treatment, on average 10-15 oocytes were recovered from the oviducts of Pten mutant mice, while no oocytes had been ovulated in wildtype. The blood estradiol and progesterone levels were shown to be no different between wildtype and Pten mutant mice, suggesting that the ovarian endocrine functions are not affected by Pten knockout (Fan *et al* 2008a). The Pten CKO ovaries are exhausted quicker as a result of the PI3 kinase cascade being hyperactive and shown abundant corpora lutea at 3 months of age as opposed to only a few corpora lutea being present in wildtype mice at this age (Fig 4.16). To investigate this ovarian histology, some haematoxylin and eosin staining was carried out on wildtype and Pten CKO mouse ovaries.

Fig 4.16: Haematoxylin and Eosin staining of wildtype versus Pten CKO paraffin wax embedded ovaries

3 month old wildtype ovary showing few corpora lutea

3 month old Pten CKO ovary showing abundant corpora lutea
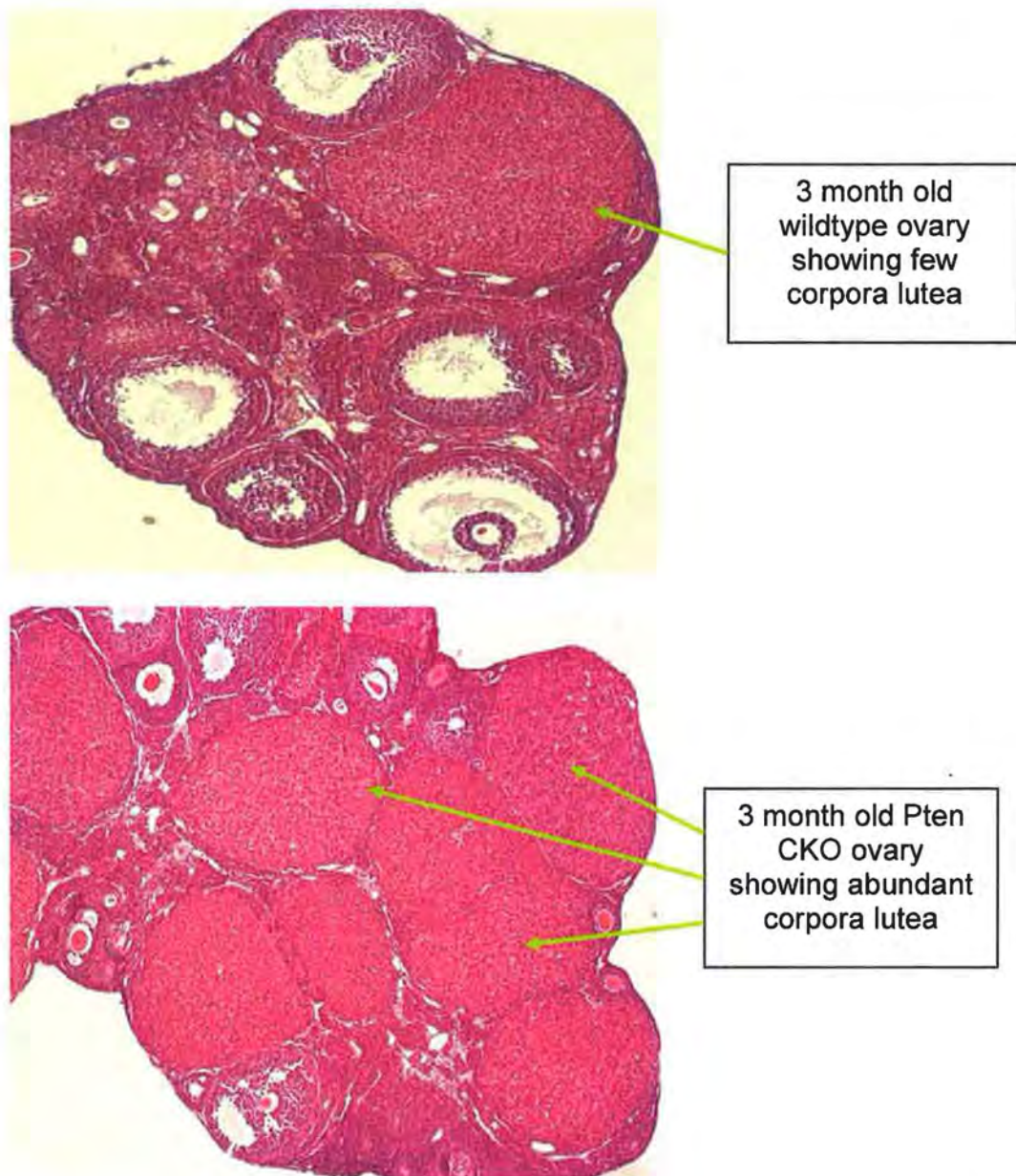
Fig 4.16: H and E staining of 3 month old wildtype ovary (top) versus 3 month old Pten CKO ovary, both viewed under 10X magnification.

After 3 months of age the ovaries of *Pten* CKO mice are larger and heavier and contain more corpora lutea than wild-type mice. Usually corpora lutea begin to regress by day 4 post hCG. Recent studies in our lab have shown that the regression of corpora lutea in the *Pten* mutant ovaries is impaired and corpora lutea can still be present at day 15 post-hCG.

These observations provided the novel evidence that *Pten* appears to control the life-span of luteal cells and that its loss results in the persistence of luteal structures in the mouse ovary (Fan *et al* 2008a). In light of this knowledge we wanted to study how key intermediates, AKT and FOXO1 in the PI3K pathway differed in wildtype mice versus the *Pten* CKO mouse strain.

Fig 4.17: Western Blot showing PI3K pathway intermediates using wildtype versus Pten CKO mouse granulosa cells.
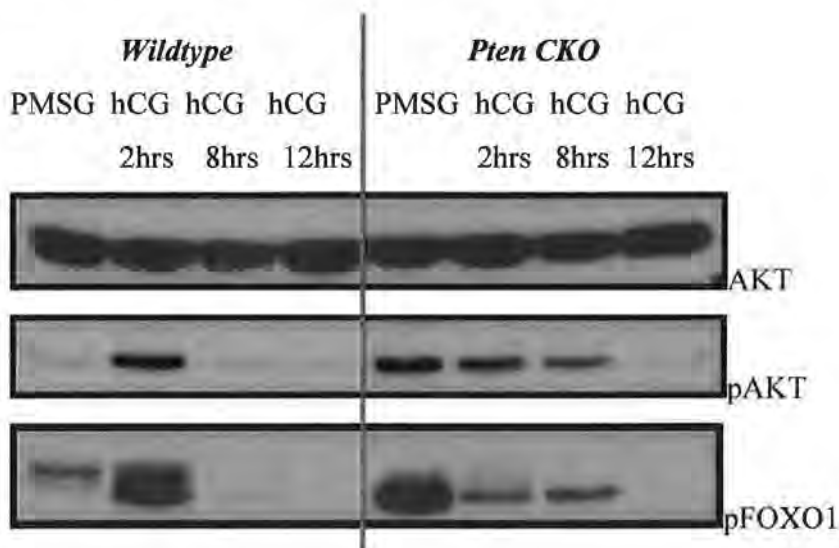
Fig 4.17: hCG series showing that phosphorylated AKT and phosphorylated FOXO1 are still present 8 hours post hCG injection in the Pten CKO whereas both are absent in wildtype mice at this time point. Total AKT was used as a loading control.

pAKT and pFOXO1 proteins are still present at 8 hours post hCG treatment in the Pten CKO where as both are gone in wildtype granulosa cells at this time point. This implies that the PI3K/AKT/FOXO cascade is still active at this stage in the Pten mutant as a result of the negative regulator of this pathway being knocked out in the granulosa cells. This uncontrolled cascade may result in the proliferation of granulosa cells and exhaustion of follicles in the Pten CKO ovaries. In addition to the PI3K/AKT/FOXO cascade, the Janus kinase/Signal transducer and activator of transcription (JAK/STAT) cascades pathway is also concerned with ovarian proliferation and differentiation.

The JAK/STAT pathways role in ovary development

As explained in Section 1.10(iii), the JAK/STAT pathway functions in cell survival responses and is possibly linked to granulosa cell proliferation, differentiation and apoptosis. Stat5 is one of the downstream components of the JAK/STAT pathway. The mechanisms controlling the progression of granulosa cell fate (growth vs. atresia) and terminal differentiation (luteinisation) are not well understood. During the ovarian cycle numerous cytokines modulate gene expression and function, while luteinisation involves the acquisition of prolactin induced Stat5 responsiveness, a process requisite for maintenance of luteal cell function (Russel *et al* 1999). It has also been reported that luteinisation of PMSG primed ovaries by the administration of hCG is accompanied by an induction of Stat5 protein (Ruff *et al* 1996). Some cell culture work was carried out using rat ovaries that had been treated with estradiol for 3 days to investigate whether extracellular ligands Amphiregulin, FSH, IL6 and Forskolin had the ability to increase pStat5 levels or not. Some of the samples were treated with FSH/T (Follicle Stimulating Hormone and Testosterone) resulting in further differentiated, more mature granulosa cells and were then treated with various ligands to determine if pStat5 levels showed any variation between immature and more developed granulosa cells.

Fig 4.18a: Western blot results of rat granulosa cells cultured overnight in media only and in media containing FSH and Testosterone
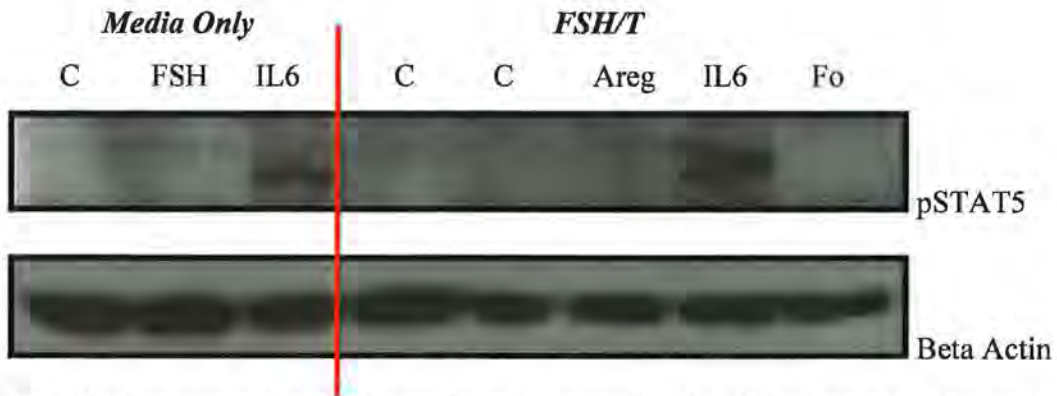
Fig 4.18a: Rats were injected with estradiol for 3 consecutive days before granulosa cells were isolated and cultured overnight at 37°C in culture media only and in culture media containing Follicle Stimulating Hormone and Testosterone (FSH/T). FSH and IL6 were added to media only samples for 10 minutes. Amphiregulin (Areg), Interleukin 6 (IL6) and Forskolin (Fo) were added to FSH/T samples for 10 minutes. Control lanes (C) were not treated with any ligands. Beta-Actin was used as a loading control.

Fig 4.18b: Western blot results of rat granulosa cells cultured overnight and then treated with extracellular ligands
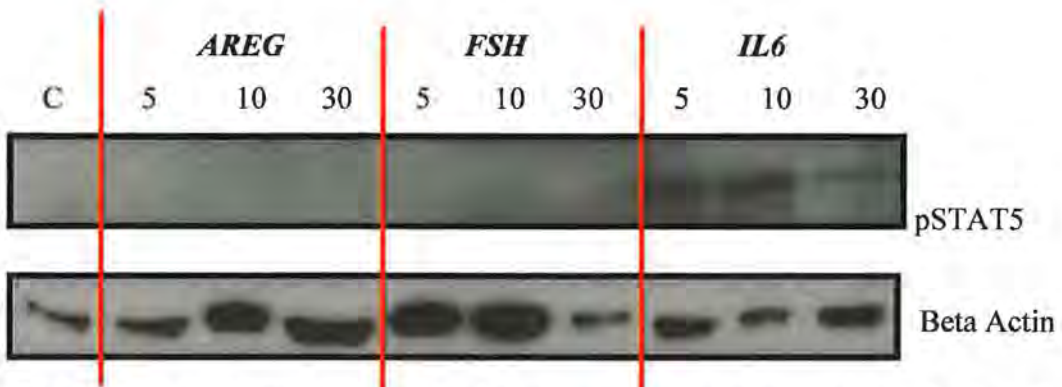
Fig 4.18b: Rats were injected with estradiol for 3 consecutive days before granulosa cells were isolated and cultured overnight in culture media at 37°C. Ligands Amphiregulin (Areg), Follicle Stimulating Hormone (FSH) and Interleukin 6 (IL6) were added for 5, 10 or 30 minutes just before cells were harvested. The control lane (C) on the left was not treated with any ligands. Beta-Actin was used as a loading control.

The western blot results from Figure 4.18 demonstrate that IL6 has the ability to induce STAT5 phosphorylation when compared to granulosa cell samples that have not been treated with any ligand and those treated with AREG and FSH. Most phosphorylation occurs after 10 minutes of treatment with IL6. Amphiregulin, Forskolin or FSH cannot induce STAT5 phosphorylation. FSH/T treatment does not result in a higher level of

STAT5 phosphorylation indicating pSTAT5 levels are independent of the stage of development of the granulosa cells.

Ras/Raf/MEK/ERK pathways role in ovary development

In conjunction with the PI3K/AKT/FOXO pathway, FSH also has the ability to stimulate the Ras/Raf1/MEK/ERK1/2 cascade. As mentioned in the introduction, Section 1.10(ii), this pathway is also concerned with ovarian cell proliferation and differentiation through FSH signal transduction. FSH can activate the (Extracellular Signal Related Kinase) ERK pathway in a unique time dependant and synergistic manner. We have already looked at Areg, FSH and IL6 ability to induce AKT phosphorylation and C/EBP-β expression, it was now necessary to assess how these ligands functioned in activating ERK pathway intermediates. Cell culture work was carried out using granulosa cells which were isolated from estradiol treated rat ovaries.

Fig 4.19a: Western blot results of rat granulosa cells cultured overnight and then treated with extracellular ligands
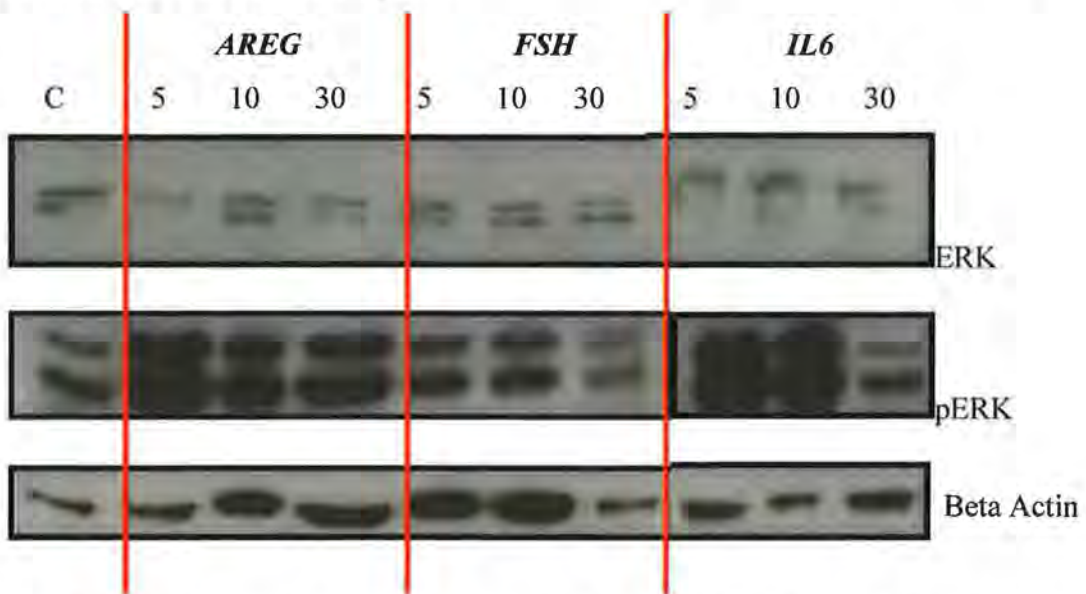
Fig 4.19a: The left control lane (C) was not treated with any ligand. Each of the other lanes were treated with Amphiregulin (Areg), Follicle Stimulating Hormone (FSH) and Interleukin 6 (IL6) ligands respectively for 5, 10 and 30 minutes. Beta actin was used as a loading control.

Fig 4.19b: Western blot results of rat granulosa cells cultured overnight and then treated with extracellular ligands
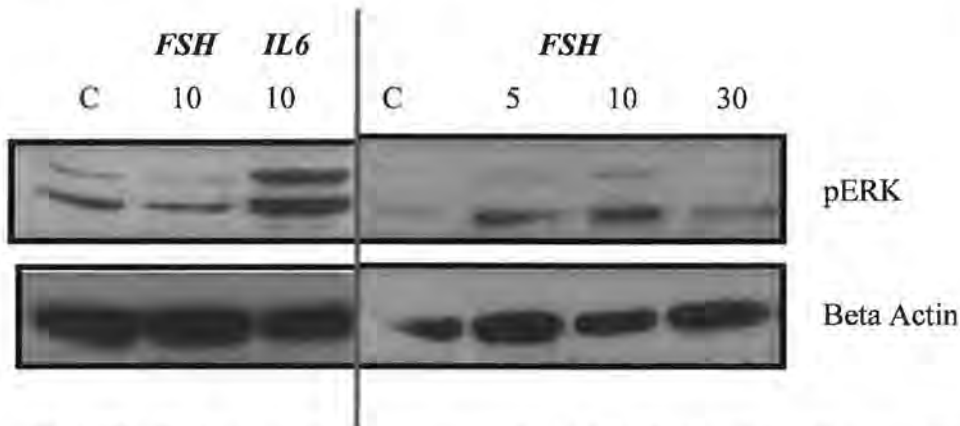
Fig 4.19b: The lanes on the left were treated with Follicle Stimulating Hormone (FSH) and Interleukin 6 (IL6) for 10 minutes. The other lanes were treated with Follicle Stimulating Hormone (FSH) for 5, 10 and 30 minutes. The control lanes (C) were not treated with any ligands. Beta actin was used as a loading control.

Areg, IL6 and FSH all display the ability to induce the phosphorylation of ERK when compared to the control lane which has not been treated by any ligand at all. IL6 is the strongest inducer, followed closely by Areg and then FSH. IL6 and FSH show similar induction patterns, showing highest ERK phosphorylation at 5 and 10 minutes post ligand treatment and then decreasing at the 30 minute time point. Areg shows a similar level of ERK phosphorylation at 5, 10 and 30 minute time points.

FSH/T causes better induction of the LH receptor and improves cytokine cleavage. Cells differentiate more after FSH and testosterone treatment so are more mature. To investigate if further differentiated, more mature cells respond differently to such ligands (Areg, IL6, Fo), rat granulosa cells were incubated overnight at 37°C in media containing FSH/T and phospho-ERK expression was measured.

Fig 4.20: Western blot results of rat granulosa cells cultured overnight in FSH/T containing media and then treated with extracellular ligands
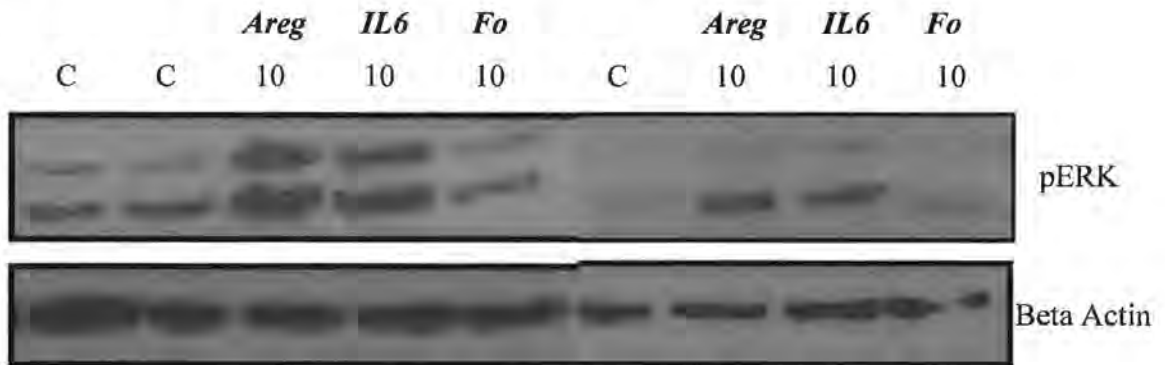
Fig 4.20: Rat granulosa cells cultured overnight in Follicle Stimulating Hormone/Testosterone (FSH/T) containing media. The control lanes (C) were not treated with any ligands. The other lanes were treated with Amhiregulin (Areg), Interleukin 6 (IL6) and Forskolin (Fo) for 10 minutes. Beta actin was used as a loading control.

The addition of FSH/T to the media did not necessarily yield better phosphorylation of ERK. Again IL6 and Areg display the ability to induce pERK protein levels. However, forskolin does not result in an induction of pERK expression when compared to the control lanes. Perhaps more mature granulosa cells are not responsive to forskolin. As mentioned in the introduction Section 1.10(i)(ii), Ras functions to activate both the PI3K/AKT/FOXO and Ras/Raf/MEK/ERK pathways. Gene targeting studies have determined the K-Ras isoform to be essential for normal development in mice as opposed to other H-Ras and N-Ras isoforms (Koera K *et al* 1997). Some reports show that binding between Ras and down stream Raf is strengthened in the fibroblast cells of the *K-ras* [G12D] knock-in mouse strain (Tuveson *et al* 2004) where as other reports suggest that *K-ras* [G12D] models induce tumourigenesis by activating PI3K/AKT and p38 MAPK cascades (Cespedes *et al* 2006). Haematoxylin and Eosin staining of ovary sections from wildtype and K-ras [G12D] knock in mice was carried out to investigate the histological difference between the two *(Fig 4.21).*

Fig 4.21: Haematoxylin and Eosin staining of wildtype ovary versus K-ras^{G12D}-Amhr2-Cre knock-in ovary

Wildtype hCG 48 hrs   *K-ras^{G12D}-Amhr2-Cre hCG 48 hrs*

| Corpus luteum which has ovulated | Corpus luteum with trapped oocyte inside |
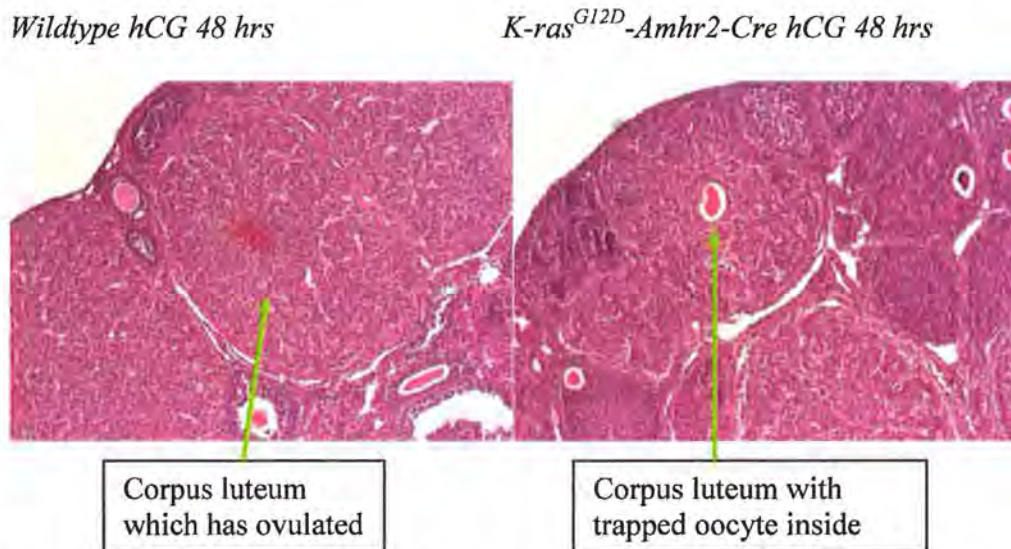
Fig 4.21: H and E staining of wildtype ovary on the left which has been treated with hCG for 48 hours and K-ras^{G12D}-Amhr2-Cre mutant ovary on the right which has also been treated with hCG for 48 hours (Fan *et al* 2008b).

Ovaries of immature *KrasG12D* mutant mice did not exhibit tumourigenic cells but instead the ovaries contained numerous, abnormal, follicle-like structures, devoid of mitotic and apoptotic cells as well as cells expressing granulosa cell marker genes. Most follicles that proceeded to antral stage failed to ovulate and exhibited impaired responses to PMSG and hCG. Fan et al have shown that these strange corpora lutea-like structures with trapped oocytes inside are negative for proliferating cell nuclear antigen (PCNA), a mitotic marker. Bromodeoxyuridine (BrdU) is also gone in this knock in model (Fan et al 2008b). BrdU is commonly used in the detection of proliferating cells in living tissues. BrdU can be incorporated into the newly synthesised DNA of replicating cells during the S phase of the cell cycle, substituting for thymidine during DNA replication.

Previous work done in the lab included carrying out a microarray on these *K-ras* knock-in mice. Results showed Cyclin A, Cyclin E and E2F1 genes were all

decreased by 10 fold. Each of these cyclin genes are involved with the G_1 phase of the cell cycle. E2F1 can mediate both cell proliferation and p53/dependent/independent apoptosis (NCBI database). Since each of these genes are associated with cell progression through the cell cycle and each one was decreased on the microarray, it suggested that the *K-ras* knock-in models consisted of abnormal, non-mitotic and non-apoptotic cells. Inappropriate, premature expression of *Kras* in granulosa cells blocks the granulosa cell differentiation pathway and leads to the persistence of these corpora lutea-like structures (Fan *et al* 2008b). These structures appear to be senescent.

To investigate if these knock-in mice models were associated with tumourigenesis, a variety of tumour suppressor genes were investigated: *p15, p16, p19*. The *p15(INK4B)* gene encodes a CDK inhibitor. *p15* is critically involved in cell cycle regulation and can be hyper-methylated in Acute Myloid Leukaemia. Functional inactivation of the retinoblastoma and p53 pathways appears to be a rite of passage for all cancerous cells and results in disruption of cell-cycle regulation and deactivation of the apoptotic response that normally ensues. The *INKa/ARF* locus sits at the nexus of these two growth-control pathways, by virtue of its ability to generate two distinct products: the $p16^{INKa}$ protein, a cyclin-dependent kinase inhibitor that functions upstream of RB; and the $p19^{ARF}$ protein, which blocks MDM2 inhibition of p53 activity. This 'one gene-two products-two pathways' arrangement provides a basis for the prominence of *INKa/ARF* in tumourigenesis (Chin *et al* 1998). *p16* inhibits CDK4 and commonly functions in cell cycle G_1 control. This gene increases in cells with age and accumulates and modulates specific age-associated human stem cell functions (Jansen *et al* 2006).

The human *KIT ligand* gene, known also as human stem cell factor, is the ligand of the c-kit transmembrane tyrosine kinase receptor (KIT). This ligand-receptor interaction is known to play important roles in mouse germ cell migration and proliferation.

In preparation for ovulation, paracrine communication between the preovulatory follicle and overlying theca/stromal cells and ovarian surface epithelium must take place to facilitate the degradative and apoptotic events associated with ovulation. Kit tyrosine kinase receptors and their ligand kit ligand are expressed within ovarian follicles, and ligand-induced receptor activation appears to account for some of the cell - cell interactions important for oocyte development (Driancourt *et al* 2000). As a consequence of this, the kit ligand gene was also investigated in the knock-in mouse model to see if it was down-regulated.

Firstly linearisation assays were carried out to determine the best number of cycles to use for these particular genes of interest within the ovary. m-RNA was extracted from mouse cell culture samples and an RT-PCR carried out to produce c-DNA. The optimum number of cycles was then determined using this c-DNA by performing a PCR using radioactively ^{32}P dCTP. Once the PCR was carried out these samples were then loaded on a 1.5% acrylamide gel and dried on a gel dryer. This dried gel was then placed in a cassette and exposed to an x-ray film in the darkroom similar to how western blots are developed.

Fig 4.22: Linearisation assay to determine optimum number of PCR cycles using a two step PCR method
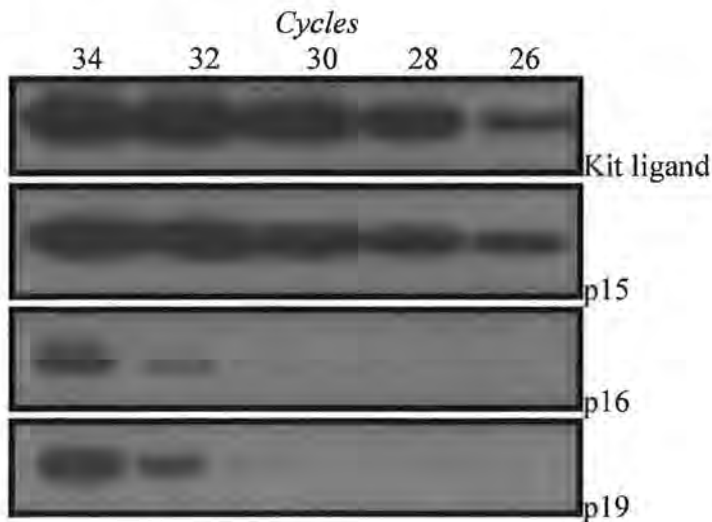
Fig 4.22: Wildtype mouse ovaries were used to determine the linear range of particular genes of interest associated with senescence.

Judging from the results in Fig 4.22, it appears the optimum number of cycles for kit ligand and p15 is 28. The optimum number of cycles for p16 is 34 and for p19 is 32. It was now possible to go ahead and compare how wildtype ovary lysates compared to K-ras^{G12D}-Amhr2-Cre mutant ovary lysates and to see if tumour suppressor genes are in fact altered in the mutant ovaries as a result of these strange corpora lutea like structures with trapped oocytes inside.

Fig 4.23: Two step PCR method to compare markers of senescence between wildtype and the K-ras^{G12D}-Amhr2-Cre knock-in mice
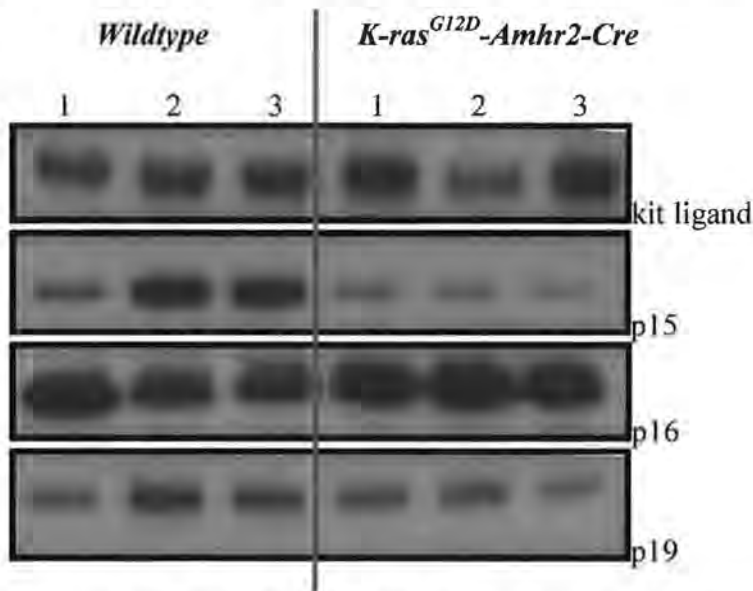
Fig 4.23: Radioactive PCR using ^{32}P dCTP. The three samples to the left of the red line are wildtype ovary lysates from three different mice that have been treated with hCG for 48 hours. To the right of the red line are three ovary lysate samples for three different K-ras^{G12D}-Amhr2-Cre mutant mice that have been treated with hCG for 48 hours.

Kit ligand expression in the *Kras* mutant mouse does not seem to be affected. During early folliculogenesis, kit together with kit ligand controls oocyte growth and theca cell differentiation, and protects preantral follicles from apoptosis. Formation of an antral cavity requires a functional kit-kit ligand system. In large antral follicles, the kit-kit ligand interaction modulates the ability of the oocyte to undergo cytoplasmic maturation and helps to maximise theca androgen output. Hence, many steps of oogenesis and folliculogenesis appear to be, at least in part, controlled by paracrine interactions between these two proteins (Draincourt *et al* 2000). An increase in tumour suppressor genes was perhaps expected in the *Kras* mutant mice samples as these mice are non-mitotic and display senescent qualities. The *Kras* mutant however showed a decrease in *p15* and *p19* indicating there must be some other mechanism taking place that induces senescence. *p16* expression remained relatively similar to the wildtype. It has been shown that the initiation of oocyte growth, which seems to lead follicle growth, is associated with diminished p16

expression in the mouse ovary. Increased expression of the p16 gene occurs early in the development of ovarian carcinomas and then levels begin to decrease (Chiesa-Vottero *et al* 2007).

5.0 DISCUSSION

Cell proliferation and differentiation is necessary for the progressive growth of ovarian cells. Although granulosa cells are the main cell type within the ovary, granulosa cell tumours are extremely rare, accounting for ~5% of all ovarian cancer. This suggests that there is a very strict, controlled regulatory system influencing the growth and differentiation of these cells. The cell cycle itself is regulated by the balance between signals which promote the cell cycles progression and apoptotic or inhibitory signals which stop the cell cycle. These signals are as a result of interplay between many signalling pathways. This project looked mainly at two such pathways, the PI3K/AKT/FOXO and the Ras/Raf/MEK/ERK pathway. This study provided the novel insight into the expression pattern of key components from these cell signalling pathways, allowing us to investigate which component was expressed at which particular stage of follicular growth during normal ovarian development.

Cell cycle regulators play a big role in ovarian follicular development and determine if ovarian cells are to proliferate, perhaps resulting in ovulation, or differentiate into luteal cells and eventually become atretic. Cyclin D2 levels do not seem to increase or decrease throughout the hCG series *(Fig 4.2a)*. This would imply that Cyclin D2 plays a critical role in all aspects of ovarian cell development. Whether the cells are growing to a pre-antral stage, as is the case with the PMSG treated cells or whether the cells are more developed and differentiating into luteal cells after hCG treatment, there is no major change in Cyclin D2 protein level. It has previously been shown in the absence of Cyclin D2, the mitotic activity of granulosa cells is markedly impaired and growing follicles remain small with few (usually only one or two) layers of granulosa cells (Robker *et al* 1998a). Despite the reduced number of cells, these cells respond to LH in a normal pattern of differentiation. For instance, progesterone receptor and prostaglandin synthase-2, two

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regulators of ovulation (Richards JS 1994) (Sirois J *et al* 1992a) are induced by the LH surge in a pattern similar to that of normal ovulating follicles (Robker *et al* 1998a). Yet the "preovulatory" follicles of cyclin D2 null mice do not ovulate. This raises the intriguing question whether or not the number of granulosa cells is critical for stimulating some event associated with ovulation. The temporal expression pattern for p27[Kip1] at 16 hours post hCG treatment *(Fig 4.2a)* suggests that a second mechanism by which LH terminates granulosa cell proliferation is by increasing the level of this CDK inhibitor. In addition, the increase in p27[Kip1] may control some aspect of granulosa cell differentiation or maintenance of luteal cell differentiation. In the absence of p27[Kip1], differentiation characteristic of luteinisation appears impaired (Robker *et al* 1998a). However, despite the key role of p27[Kip1] in checking cell cycle progression and its presence in granulosa cells and luteal cells, the absence of p27[Kip1] does not lead to rampant uncontrolled proliferation of these cells. Thus, other inhibitors of cell division appear to be more critical during follicular growth. Considering the immunofluorescence pictures *(Fig 4.3)*, p27[Kip1] is not present in every growing follicle 48 hours after PMSG treatment. Some of the larger follicles stain intensely for p27[Kip1] but the smaller, less developed primary follicles do not show this intense staining pattern. Once cells begin to luteinise after 16 hours hCG treatment, the p27[Kip1] staining pattern is more uniform. Luteal cells and apoptotic follicles stain positively for this cell cycle inhibitor. The cell cycle has somewhat stopped once granulosa cells reach this stage of development and differentiation has commenced.

Oxidative stress can activate the MST1 SAPK/JNK apoptotic pathway. This can result in the phosphorylation of FOXO1 promoting nuclear translocation and inducing cell death (Lehtinen *et al* 2006). Non treated, developing and luteinising follicles all display a similar protein level of these apoptotic markers *(Fig 4.4)*. Both these markers show positivity in the theca cells of follicles showing quite a similar staining pattern *(Fig

4.5). In each estrous cycle, only one or a few follilces are successfully ovulated so more than 90% of the recruited follicles stop growth at various stages of development and are eliminated by apoptosis. This phenomenon is called follicle atresia and is still not very well understood. The MST1 and pSAPK/JNK results imply that follicles rely on apoptotic signals other than these two apoptotic markers during follicle atresia.

The PI3K/AKT/FOXO pathway is at the heart of one of the major pathways of intracellular signal transduction. The PI3K signalling pathway has been shown to be required for an extremely diverse array of cellular activities, most notably cellular proliferation and survival. As a consequence of this, components of the pathway are high on the list of most pharmaceutical companies as good targets for cancer therapy. It has also been demonstrated that up-regulation of the PI3K pathway by chronic treatment with hCG can induce changes in ovaries that stimulate polycystic ovary syndrome (POS) (Lima *et al* 2006). The PI3K subunit p110γ is mutated in many cancers, causing it to be more active (Ward *et al* 2003). Downstream of PI3K, mTOR and AKT are being explored as potential targets for cancer therapy and perhaps POS treatment. Better targets in the PI3K/AKT/FOXO pathway are constantly been looked for as toxicity is often an issue in drug design. Before proposing to establish potential drug targets within the pathway, it is important to understand the expression of pathway components during normal ovarian development. Once FSH/LH binds its receptor, ras can activate the PI3K pathway by directly binding to the IGF receptor and resulting in the activation of p110y, the catalytic subunit of PI3K *(Fig 1.11).* p110y protein levels show a slight decrease at the 16 hour time point, after ovulation has occurred *(Fig 4.7).* The PI3K pathway is perhaps not as active at this time point as granulosa cells are differentiating into luteal cells as opposed to proliferating. The next step in the pathway is the sequential phosphorylation of PDK and AKT. pPDK1 levels remain relatively constant, showing a slight increase once hCG is

administered at the 2 and 4 hour time points *(Fig 4.7)*. The initiation of the PI3K pathway appears to be most important once hCG is administered. The phosphorylation of AKT occurs once PMSG is administered, FOXO1 being a downstream target of AKT shows a later phosphorylation induction at 2 hours post hCG treatment *(Fig 4.8a)*. As mentioned, the functional FOXO1 remains in the nucleus promoting apoptosis but when phosphorylated by AKT is translocated to the cytoplasm and rendered inactive. This phosphorylation is important once hCG is administered and lasts right up to the 16 hour time point after where ovulation has occurred. pFOXO1 therefore plays a role in early follicular development right up to the preovulatory, ovulatory and luteinisation stage *(Fig 4.8a)*. Total FOXO1 is present at high levels in the non treated and PMSG treated samples, promoting apoptosis at this stage *(Fig 4.8a)*. This constant translocation and phosphorylation of FOXO in a time dependent manner appears to be essential for the growth and development of follicles as well as ovulation and luteinisation. If we consider the immunofluorescence staining in *Figure 4.9* we can see the pFOXO1 displays a more nuclear punctuated cytoplasmic staining pattern in the PMSG treated sections and shows a more cytoplasmic staining pattern in more mature luteal cells. This higher abundance of pFOXO1 at 16 hours post hCG treatment than in the PMSG treated sample again solidifies the idea that pFOXO1 is playing a role in at the luteinisation stage. The low levels of pFOXO1 at the PMSG time point in both the western and the immunofluorescence pictures *(Fig 4.8a, Fig 4.9)*, implies that the intense phosphorylation of this key component is not essential at early stages of follicular development. P70S6k is another possible target of AKT. Phospho-P70S6k (pP70S6k) levels show a slight increase once hCG is administered which drops back to NT/PMSG treated levels at 16 hours post hCG *(Fig 4.8a)*. The phosphorylation of P70S6k therefore is a key event in the growth of follicles up to the ovulatory stage but is not needed in such abundance once luteinisation begins to occur at the 16 hours post hCG time point.

Rat granulosa cell culture work was carried out to investigate how isolated granulosa cells reacted to extracellular ligands such as AREG, FSH, IL6 and Fo. AREG is a growth factor involved in cancer development, but its potential role in signalling in the gonads is still obscure. It has been reported that basal expression of this growth factor is evident in human granulosa cells obtained from women treated for in vitro fertilisation. Expression of these factors was elevated concomitantly with elevation of progesterone production in these cells upon stimulation with LH, and to a lesser extent with FSH. AREG gene expression was dose- and time-dependent when measured subsequent to LH stimulation (Freimann *et al* 2004). Moreover, Fo which activates adenylate cyclase, was as efficient as LH in stimulating expression of these growth factors. It is suggested that upregulation of AREG expression is part of the signal transduction pathway which leads to ovulation and luteinisation in the human ovary. IL6 can also directly influence FSH differentiating functions of ovarian granulosa cells. All three ligands show the capacity to induce AKT phosphorylation to more or less the same degree that FSH does *(Fig 4.10)*. The more mature granulosa cells which have been treated with FSH and Testosterone display a greater level of pAKT protein after treatment with Fo, AREG and IL6 *(Fig 4.11)* than the granulosa cells which have been cultured in culture media only *(Fig 4.10)*. These results suggest that these three ligands may be players in signalling pathways that influence granulosa cell proliferation and differentiation such as the PI3K pathway and the ERK pathway, perhaps having a bigger role influencing the more mature granulosa cells than the immature developing cells.

C/EBPβ mRNA expression is induced rapidly in granulosa cells 30 minutes after stimulation with LH/hCG in vivo (Sirios *et al* 1993). This indicates the C/EBPβ gene is an immediate target of LH-receptor signalling. It has recently been shown that C/EBPβ is expressed upon induction of differentiation of preadipocyte cells, initiating

a transcription cascade. C/EBPβ immediately undergoes a priming phosphorylation on Thr188 by MAPK/ERK. The acquisition of DNA binding and transactivation capacity of C/EBP is delayed until further phoshorylation on Ser184 and Thr170 by glycogen synthase kinase-3β (GSK3β) occurs. Phosphorylation by GSK3β induces S phase entry and thereby mitotic clonal expansion, a requirement for terminal differentiation (Li *et al* 2007). In this project C/EBPβ was induced at 2 hours post hCG treatment and remained until 5 days after hCG had been administered *(Fig 4.13)*. This indicates that C/EBPβ is important in events surrounding the cell cycle ie. growing follicles where granulosa cells move to form the antrum and also at the granulosa cell differentiation into luteal cell stage. FSH and AREG both showed the ability to induced C/EBPβ gene expression *(Fig 4.14)*. This would suggest the C/EBPβ is a downstream target of the FSH/LH receptor.

PTEN is the negative regulator of the PI3K pathway. *Pten* CKO mice display abundant corpora lutea at 3 months of age *(Fig 4.16)*. The PI3K pathway is known to govern cell division, survival, size and apoptosis inhibition (Sulis M.L *et al* 2003). Without mural granulosa cell expression of the tumour suppressor PTEN, uncontrolled proliferation, evasion of apoptosis and possible tumourigenesis could prevail in the ovary as a result of the over activation of the PI3K cascade. PTEN under normal circumstances is expressed intensely in the mural granulosa cells and theca cells (Fan *et al* 2008a). This identifies PTEN to have an important role in cell signalling and morphology in these cells. In wildtype mice PTEN expression is constant over all stages of development. The presence of corpora lutea at 3 months of age would suggest that the conditional knock-out of Pten in granulosa cells did not alter follicular development, ovulation or luteinisation. The PI3K pathway components were not altered or dysfunctional in the Pten CKO but just uncontrolled and overactive as a result of this. As seen in our study *(Fig 4.8)* and also reported previously in this laboratory (Richards et al

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2002), normally activation of the PI3K pathway by FSH/PMSG has shown to increase the expression of the pro-apoptotic factor FOXO1. Activated PI3K can phosphorylate AKT. Subsequent phosphorylation of downstream FOXO by pAKT inactivates the active FOXO by restricting its nuclear localisation (Richards *et al* 2002). In the Pten CKO it could be hypothesised that the transcription factor FOXO could not transcriptionally activate the cell cycle inhibitor p27^{kip1} resulting in excess growth and proliferation of the ovarian cells and hence abundant corpora lutea and ovary exhaustion. pAKT and pFOXO is still present 8 hours post hCG injection in granulosa cells of the *Pten* CKO model but not in the wildtype mouse granulosa cells providing further evidence that the PI3K pathway is over-active in the CKO *(Fig 4.17)*. The phosphorylation of AKT is highest after 2 hours of hCG treatment in both whole ovary lysates *(Fig 4.8a)* and isolated granulosa cells *(Fig 4.17)* of wildtype mice. PMSG treatment results in the strong phosphorylation of AKT in wildtype ovary lysate samples *(Fig 8a)* but induces this phosphorylation to a less extent in wildtype isolated granulosa cells *(Fig 4.17)*. Granulosa cells from the Pten CKO mouse show significant pAKT protein levels after PMSG treatment which lasts up to 8 hours post hCG. FOXO1 phosphorylation also is sustained for longer in the Pten CKO granulosa cells than isolated granulosa cells from wildtype mouse. The decline of pFOXO1 is greater in wildtype granulosa cells *(Fig 4.17)* than in whole ovary lysates *(Fig 4.8a)*. pFOXO1 is present at a significantly lower level in granulosa cells at 8 and 12 hours post hCG treatment when compared to pFOXO1 levels in whole ovary lysates. The phosphorylation of FOXO1 at 8 and 12 hours post hCG must therefore play a more important role in other cells of the ovary ie. theca cells and cumulus cells, than in isolated granulosa cells. However, both figures 4.8a and 4.17 both show the most intense phosphorylation of FOXO1 at the 2 hours post hCG timepoint. The Pten CKO granulosa cells in fig 4.17 exhibit enhanced responses to PMSG, hence the higher level in PMSG lane. We could hypothesis that the levels of total FOXO are already down in these mice by the time of

hCG, hence the poor response to hCG compared to wildtype granulosa cells. In the CKO the cells remained functional and progressed to the next stage of ovulation, differentiating into luteal cells. LH was released at the luteal stage of development causing a decrease in expression of the pro-apoptotic factor FOXO1 favouring cell survival. Eventually when the granulosa cells where induced to undergo luteinisation they possibly became resistant to apoptosis, pro apoptotic factors were lost and luteinisation factors like p21 and Sgk acquired, thus possibly giving rise to a luteal cell pre-tumour phenotype yet with relatively normal function. It is quite probable that this phenotype will eventually reach a tumourigenic stage that will impinge greatly on ovarian function.

The JAK/STAT pathway functions in cell survival responses and is possibly linked to granulosa cell proliferation, differentiation and apoptosis. Activation of downstream components of this pathway, STATs, is complex and involves many factors and in some situations gene regulation may be dependent on signal duration, indicating that the deactivation of signals is as important as the ligand-dependent activation in establishing and maintaining tissue responsiveness. Stat 5 null mice fail to produce corpora lutea at all (Russell *et al* 1999). Ligands such as FSH, IL6, AREG and Fo were used to treat cultured granulosa cells to investigate which ligand, if any, displayed the ability to induce the phosphorylation of Stat5. IL6 was the only ligand showing the ability to induce pSTAT5 and there was not difference in pSTAT5 protein levels in granulosa cells cultured in media alone versus further differentiated granulosa cells which had been treated with FSH/T overnight *(Fig 4.18a)*. Treatment with IL6 resulted in an increase in pSTAT5 levels at 5 and 10 minutes post treatment, decreasing slightly at 30 minutes *(Fig 4.18b)*. IL6 therefore appears to works in a time dependent manner.

As mentioned previously, the Ras/Raf/MEK/ERK pathway plays a major role in cell proliferation and differentiation. The ability of ligands such as FSH, IL6 and

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AREG were investigated to determine their ability at inducing ERK phosphorylation. All ligands showed the ability to induce levels of pERK, IL6 showing the strongest transient induction at the 5 and 10 minute post treatment time points before decreasing slightly after 30 minutes of treatment *(Fig 4.19a)*. FSH displays a similar induction pattern while AREG retained the same levels of pERK induction at 5, 10 and 30 minutes post treatment *(Fig 4.19a)*. Forskolin did not induce the phosphorylation of ERK *(Fig 4.20)*. Cells treated with FSH/Testosterone in order to make them more mature did not show any greater induction of pERK than the less mature cells *(Fig 4.20)*. This indicates that Fo is not capable of inducing the Ras/Raf/MEK/ERK pathway and the induction of pERK as a result of FSH, IL6 or AREG treatment is not effected by granulosa cells stage of development.

Another side to this study aimed to examine the location and some of the effects of constitutively active *K-ras* on ovarian morphology and function. Recent work done in the Richards lab indicates that ovaries expressing constitutively active *K-ras* are considerably larger in size when compared to wildtype ovaries. Microscopic examination of ovarian morphology via H&E staining showed that ovaries expressing constitutively active *K-ras* demonstrated aberrant tumour like follicles. 48 hours post hCG treatment the *K-ras* mutant showed corpora lutea with trapped oocytes inside *(Fig 4.21)*. Immunohistochemistry and in-situ hybridization experiments have also demonstrated the lack of vital ovarian FOXO1 protein expression and *Lrh-1* mRNA expression in these aberrant follicles (Fan *et al* 2008b). As FOXO protein transcription factors are known to play a vital role in the transcription and activation of pro and anti apoptotic factors (Richards *et al* 2002), the absence of FOXO may in part create an imbalance in cell growth favouring tumourigenesis and abnormal ovarian function. The observation that these *K-ras* mutant ovaries showed some morphologically normal follicles as well as some tumour-like follicles and that the only apparent fault of these morphologically normal follicles was an

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inability to progress to the next stage of ovulation and luteinisation when induced, indicated a possible hormone receptor complication. The *K-ras* knock-in models consist of abnormal, non-mitotic and non-apoptotic cells. Their phenotype is one of senescence. Some tumour suppressor genes as well as *kit ligand,* a gene associated with oocyte development, was investigated in the hope of elucidating the origin of these strange corpora lutea like structures. *Kit ligand* and *p16* showed similar results to wildtype models suggesting the problem is not associated with their expression *(Fig 4.23). p15* and *p19* are both decreased in the mutant *K-ras* ovaries. Perhaps this decrease in tumour suppressor genes is the reason some *K-ras* mutants have gone on to develop tumours (Fan *et al* 2008b). As these *K-ras* knock-in ovaries demonstrated tumour like follicles with highly condensed granulosa cells, absent follicle cavities and de-shaped oocytes pushed to the periphery, it was suggested that the abnormal structure identified translated into abnormal ovarian function and this then possibly related to a compromised fertility status (Fan *et al* 2008b).

In conclusion, the PI3K/AKT/FOXO pathway is undoubtedly a core pathway involved with follicular growth. Granulosa cell proliferation and luteal cell differentiation rely on signals from this pathway. Extracellular ligands can induce the activation of downstream targets in the PI3K pathway. Generating a *Pten* CKO mouse strain resulted in a luteal cell pre tumour phenotype in which the PI3K pathway was over-active and ovaries were exhausted earlier than would happen in a wildtype mouse. Engineering *ras* to be constitutively active in the mouse ovary results in ovulation malfunction and fertility problems. Considering these results, it is important for us to understand the time specific and downstream target specific nature of components in both the PI3K/AKT/FOXO pathway and the Ras/Raf/MEK/ERK pathway. From a drug design point of view, although no downstream inhibitors have reached trials, transcription factors have recently gained interest as the most direct and mechanistically relevant targets of the ERK pathway. Already a number of small kinase inhibitors against Raf and MEK have

entered clinical development. This has occurred despite the importance of these kinases in so many processes unrelated to cellular proliferation. Ultimately, as the pathway is further revealed, novel components will provide the researcher with new therapeutic strategies. This novel insight into the expression pattern of components from molecular pathways such as the PI3K and the ERK pathway in normal ovarian development may help us on the journey of drug design to potentially target and treat polycystic ovarian syndrome, ovarian cancer or perhaps even infertility.

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