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Regulation of Proliferation-Associated Leucine-Rich Protein 31 in the Rat Ovary

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Regulation of Proliferation-Associated Leucine-Rich Protein 31 in the Rat Ovary

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ABSTRACT

In neuronal and placental cells, proliferation-associated leucine-rich protein 31 is an anti-apoptotic, cell cycle-related protein in its intact form, and upon cleavage by caspase-3, is pro-apoptotic. However, whether PAL31 is expressed in the follicle and is modulated during follicular development and atresia is not known. The expression and regulation of ovarian PAL31 were investigated using immature rat models and a granulosa cell culture system. PAL31 was ubiquitously expressed in all follicular cell types. eCG caused a significant decrease in granulosa cell PAL31 protein content during granulosa cell differentiation and late stage follicle growth. Gonadotropin withdrawal prevented an increase in ovarian weight after eCG treatment, but failed to induce granulosa cell apoptosis and PAL31 cleavage. Finally, estradiol stimulated PAL31 expression in the granulosa cells of preantral and early antral follicles. These results demonstrate for the first time that PAL31 is regulated by key reproductive hormones during follicular development *in vivo*.

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LIST OF ABBREVIATIONS

A	Alanine
aa	Amino acid
ANOVA	Analysis of variance
APRIL	Acidic protein rich in leucines
APS	Ammonium persulfate
Arg	Arginine
Asp	Aspartic acid
BMP-15	Bone morphogenetic protein-15
C	Cysteine
cAMP	Cyclic adenosine monophosphate
cdk	Cyclin-dependent kinase
cDNA	Complimentary DNA
CBP	CREB-binding protein
cGMP	Cyclic guanylyl monophosphate
CREB	cAMP-response element-binding protein
Cdk	Cyclin-dependent kinase
D	Aspartic acid / aspartate
dNTP	Deoxynucleotide triphosphate
dUTP	Deoxyuridine triphosphate
DAB	3', 3'-diaminobenzidine
DES	Diethylstilbestrol
DNA	Deoxyribonucleic acid
dNTP	Deoxynucleotide triphosphate
DTT	Dithiothreitol
E	Glutamic acid / glutamate
eCG	Equine chorionic gonadotropin
EDTA	Ethylenediaminetetraacetic acid
EGF	Epidermal growth factor
ER	Estrogen receptor

FBS	Fetal bovine serum
FORKO	Follicle-stimulating hormone receptor knockout
FSH	Follicle-stimulating hormone
FSHR	Follicle-stimulating hormone receptor
G	Glycine
GDF-9	Growth differentiation factor-9
G_0 phase	Resting (or quiescent) phase of the cell cycle
G_1 phase	First gap of the cell cycle
G_2 phase	Second gap of the cell cycle
GAPDH	Glyceraldehyde-3-phosphate dehydrogenase
GC	Granulosa cell
GDF-9	Growth differentiation factor-9
GnRH	Gonadotropin releasing hormone
GST	Glutathione *S*-transferase
H&E	Hematoxylin and eosin
hCG	Human chorionic gonadotropin
HRP	Horseradish peroxidase
IAP	Inhibitor of apoptosis protein
IgG	Immunoglobulin G
IGF-I	Insulin-like growth factor I
IHC	Immunohistochemistry
i.p.	Intraperitoneal
Kb	Kilobase
kDa	KiloDalton
KL	Kit ligand
L	Leucine
M phase	Mitosis phase of the cell cycle
LANP	Leucine-rich acidic nuclear protein
LDH	Lactate dehydrogenase
LH	Luteinizing hormone
LHR	Luteinizing hormone receptor

LRR	Leucine-rich repeat
Lys	Lysine
mRNA	Messenger ribonucleic acid
NCBI	National Center for Biotechnology Information
NLS	Nuclear localization signal
nt	nucleotide
OC	Oocyte
P	Proline
PAGE	Polyacrylamide gel electrophoresis
PAL31	Proliferation-associated leucine-rich protein 31
PARP	Poly (ADP-ribose) polymerase
PBS	Phosphate buffered saline
PCNA	Proliferating cell nuclear antigen
PCR	Polymerase chain reaction
PHAP I	Putative HLA-associated protein I
p*I*	Isometric point
PKA	Protein kinase A
PL-I	Placental lactogen-I
PP32	Phosphoprotein 32
PRL	Prolactin
Rb	Retinoblastoma protein
RNA	Ribonucleic acid
RT	Room temperature
RT-PCR	Reverse transcriptase-polymerase chain reaction
RPMI-1640	Roswell Park Memorial Institute-1640 medium
S	Serine
s.c.	Subcutaneous
S phase	DNA synthesis phase of the cell cycle
SD	Standard deviation
SDS	Sodium dodecylsulfate
SEM	Standard error of the mean

T	Threonine
TBS-T	Tris buffered saline Tween-20
TEMED	Tetramethylethylenediamine
Tet	Tetracyclin
TGC	Trophoblast giant cell
TGFβ	Transforming growth factor β
TC	Theca cell
TNFα	Tumour necrosis factor α
TNFRI	Tumor necrosis factor receptor type I
TSH	Thyroid stimulating hormone
TUNEL	Terminal deoxynucleotidyl transferase-mediated dUTP nick end labeling
UV	Ultraviolet
V	Valine
WB	Western blotting
X	Any amino acid
XIAP	X-linked inhibitor of apoptosis protein

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CHAPTER 1: INTRODUCTION

1.1. Preamble

The female reproductive cycle is regulated on multiple levels by neuroendocrine, endocrine and local factors and involves the cyclical recruitment of resting ovarian follicles, subsequent growth in response to sex hormones, selection of a dominant follicle and ovulation. Each cycle produces a mature oocyte capable of being fertilized and is tightly coordinated so that the follicles in the resting pool at birth are conserved, and not exhausted prior to propagation of the species. To date, we and others have examined the cellular regulators involved in the control of follicular development and atresia. It is well established that gonadotropins from the anterior pituitary exert their effects within the ovary. The earlier stages of follicular growth proceed at a slower rate than the antral stage. At the penultimate stage of follicular development, the growing follicle is thought to achieve dominance through heightened follicle-stimulating hormone (FSH) sensitivity in the granulosa cells, thereby allowing the selected follicle to reach ovulation (Hirshfield, 1991). Meanwhile, ~99 % of the remaining cohort undergoes a degenerative process called atresia (Hsueh et al., 1994).

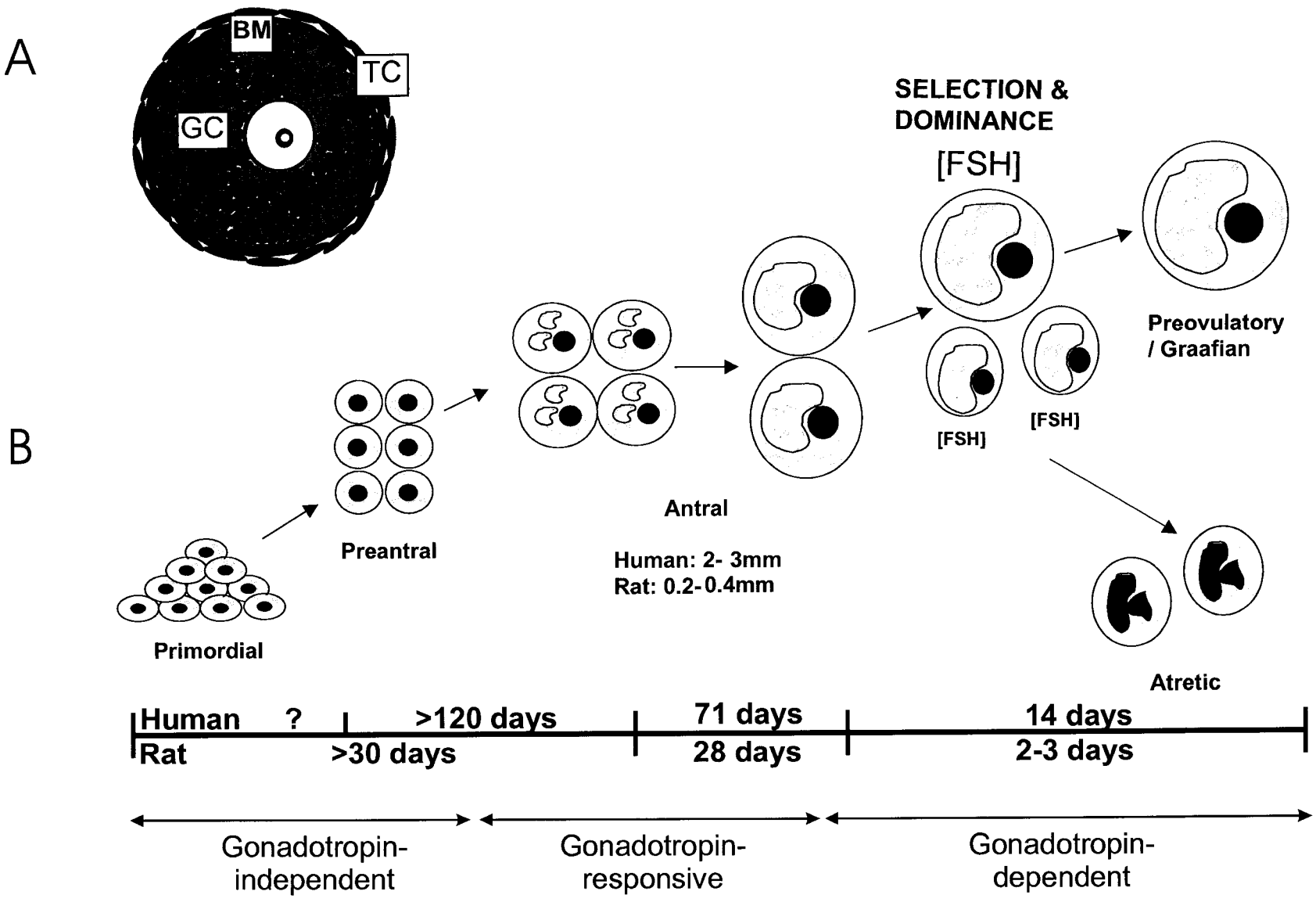
1.2. The Ovarian Follicle

1.2.1. Anatomy of the follicle

The ovarian follicle is the functional unit of the ovary and contains 3 major cell types: the granulosa cells (GC), the theca cells (TC) and the oocyte (OC; Figure 1). The follicular unit is surrounded by the stroma, and consists of connective tissue cells and interstitial cells, all of which are encapsulated by a single layer of epithelial cells.

Figure 1: Ovarian follicular development and atresia

A. The ovarian follicle is comprised of 3 principal cell types including the granulosa cells (GC), theca-interstitial cells (TC) and the oocyte. The oocyte is surrounded by GCs, of which the number of layers varies depending on the stage of follicular growth. At the late preantral stage, pools of follicular fluid join together and coalesce to form an antrum. The TCs are located outside of the basement membrane and play a key role in steroidogenesis. **B.** Duration of follicle recruitment and growth in human and rat ovaries. Quiescent primordial follicles are present at birth and, in response to growth signals, increase in size to become preantral follicles. Intraovarian regulators play an important role during early folliculogenesis and are required for progression to the early antral stage (gonadotropin-independent stage). At the late preantral / early antral stage, the follicles are gonadotropin-responsive and acquire FSH receptors. At the penultimate stage of follicular development, ~99 % of the growing cohort undergoes atresia, while the few remaining follicles continue to grow and acquire dominance (gonadotropin-dependent stage).



Adapted from McGee and Hsueh, 2000

The GC layer is devoid of vascularization, while the TCs are in close contact with vascular supply (Peters et al., 1980). The theca cells also play an important role in providing structural support to the follicle, and secrete androgen precursors for estradiol (E_2) synthesis in the GCs. The initiation and formation of the theca layer is not completely understood. However, it is well documented that the TCs are derived from immature fibroblast-like cells (Mossman H.W et al., 1973) and become a morphologically distinct cellular layer after the initiation of follicular growth (Hirshfield, 1991). Eventually, the TCs form 2 functionally distinct layers, the theca interna and the theca externa (Peters et al., 1980). The theca interna possesses steroidogenic potential and is a major source of androgens during late stage follicular growth, while the theca externa is comprised of contractile connective tissue and is thought to facilitate ovulation (Freeman, 1988; Peters et al., 1980). In the antral follicle, the GC layer becomes 2 functionally distinct cell types: the cumulus cells and the mural GCs. The cumulus cells are highly proliferative, mediate OC-GC communication through intra-zonal cytoplasmic processes and gap junctions, and undergo cumulus expansion prior to ovulation (Gilchrist et al., 2004). The mural GCs are located closest to the basement membrane and merge with the stratified epithelium to form a basal lamina along the wall of the follicle (Gilchrist et al., 2004); these cells have exited cell cycle, exhibit decreased proliferation and are more differentiated than the cumulus cells (Hirshfield, 1991). Thus, the different ovarian cell types within the follicle milieu are anatomically and functionally inter-dependent and promote follicular development in a coordinated fashion.

1.2.2. Classification of ovarian follicles

Pedersen and Peters elegantly characterized the stages of ovarian follicular development in the mouse ovary (Pedersen et al., 1968) and since that time, this mode of classification has been widely implemented in other species. In the rat, primordial follicles (25 μm, diameter) contain small oocytes, a single layer of simple squamous GCs and a basement membrane (Freeman, 1988). The nucleus of the oocyte is in the diplotene stage of meiotic prophase and remains arrested until shortly before ovulation (Peters et al., 1980). Preantral (primary) follicles (140-200 μm) have one or more layers of stratified cuboidal GCs and a growing oocyte (Freeman, 1988; McGee et al., 2000; Peters et al., 1980). Early antral (secondary) follicles (200-400 μm) contain multiple fluid-filled intracellular spaces, which coalesce to form an antrum (Freeman, 1988; McGee et al., 2000). During follicular development, the cells within the follicles undergo continuous differentiation as evident by marked biochemical, morphological and functional changes, including increased expression of gonadotropin receptors and steroidogenic enzymes (Richards, 1994). In late antral and preovulatory follicles (500-800 μm), the GCs are highly differentiated and have acquired the ability to express luteinizing hormone receptor (LHR), inhibin and aromatase, and to produce large amounts of E_2 (Hirshfield, 1991; McGee et al., 2000). By the late preovulatory stage and following ovulation, these cells are luteinized and are considered "terminally differentiated", and progestins become the predominant steroids produced. A full-sized pre-ovulatory follicle contains approximately 2000-2500 GCs in its largest cross-section (Hirshfield et al., 1987).

1.3. Gonadotropins and ovarian follicular development

1.3.1. Structure of the gonadotropins

FSH and LH are heterodimeric glycoprotein hormones that are secreted from the gonadotropes of the anterior pituitary gland, and are regulated by gonadotropin-releasing hormone (GnRH) (Hirshfield, 1991). The gonadotropins contain 2 non-covalently bound subunits: 1) a common α-subunit that is shared between FSH, LH, thyroid-stimulating hormone (TSH) and human chorionic gonadotropin (hCG), and 2) a β-subunit, which confers hormone specificity and biological activity (Catt et al., 1986; Lambert et al., 1998). The three-dimensional structures of the α and β subunits are maintained by cross-linked disulphide bonds (Catt et al., 1986) and form a "seat-belt" configuration where the β subunit is wrapped around the α subunit (Lambert et al., 1998). Upon association of the 2 subunits, a conformational change occurs and the subunits form an "active" heterodimer configuration (Combarnous, 1992). After FSH binding and receptor activation, the protein kinase A (PKA) pathway is activated through coupling of the FSH receptor (FSHR) to a G stimulatory (G_s) protein, stimulation of adenylyl cyclase and increased cyclic adenosine monophosphate (cAMP) production (Zhang et al., 1991). Activated PKA then phosphorylates proteins and transcriptional activators containing cAMP responsive element (CRE) binding proteins (Simoni et al., 1997). Terminal-end sialic acid residues enhance activity by lengthening the half-life of gonadotropins, thereby decreasing metabolic clearance in plasma (Morell et al., 1971; Wide, 1986). Although carbohydrate chains are not required for receptor binding (Valove et al., 1994), the sialic acid residue in FSH is important for hormone-receptor interaction (Flack et al., 1994; Lambert et al., 1998; Stanton et al., 1995).

1.3.2. Two-cell, two-gonadotropin theory in the control of follicular development

In 1959, Falck proposed the "two-cell, two-gonadotropin" model of estrogen synthesis (Figure 2). According to this model, TCs contain the 17α-hydroxylase and C17, 20-lyase required for androgen synthesis, and the GCs contain the aromatase enzyme, which converts androstendione to estradiol. Androgen precursors secreted from the TCs cross the basement membrane and are converted to estradiol in the granulosa cells. Thus, functional synergy between the TCs and GCs is essential for estradiol synthesis by the granulosa cells. During follicular E_2 production, LH stimulates TC synthesis of androgens, which are then aromatized to estrogens in the granulosa cells under the influence of FSH (Armstrong et al., 1979; Falck, 1959; Lipsett, 1986). The GCs are the only cell type in the ovary that possess FSH receptors (FSHR), while LH receptors (LHR) are abundant in TCs and in GCs of mature follicles (Armstrong et al., 1979; Falck, 1959; Hillier et al., 1994). Granulosa cells do not acquire FSHR until the secondary stage, although the cAMP pathway is functional in small follicles and stimulates aromatase activity and FSHR expression (Ahmed et al., 1986; McGee et al., 2000). Exposure of GCs to FSH has been shown to increase LHR expression and GC differentiation (Erickson et al., 1979; Zeleznik et al., 1974).

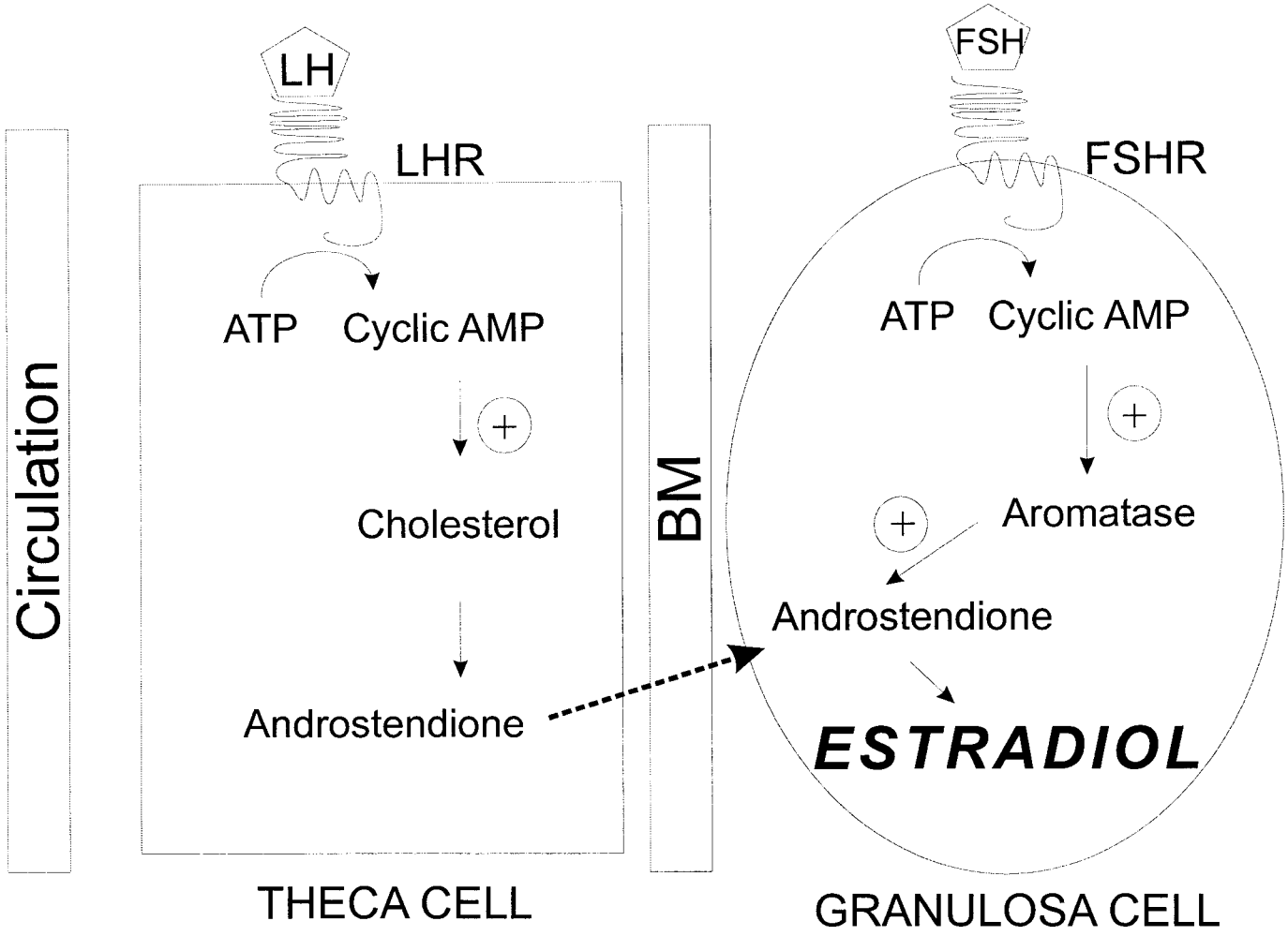
1.3.3. FSH levels in the immature rat

At birth, rat ovaries contain oocytes surrounded by somatic cell cords (McGee et al., 1997b). By postnatal day 3, quiescent primordial follicles begin the initial growth phase (Hirshfield, 1991). Although low levels of truncated FSHR mRNA have been

Figure 2: Two-cell, two-gonadotropin theory of estrogen synthesis

LH binds to theca cell LHR and increases cAMP levels, which in turn, leads to the conversion of cholesterol to androstendione. Androstendione then crosses the basement membrane and enters the granulosa cell. FSH binds to the GC FSHR and increases the expression of aromatase enzyme. Aromatase in the GC converts the theca-derived androstendione to estradiol. BM = basement membrane.

Late preantral follicle



Adapted from Lipsett, 1986.

detected in the ovaries of 1-day old rats (Rannikki et al., 1995), functional ovarian FSH binding occurs from days 4-7 and markedly increases from days 12-18 (Sokka et al., 1990). At day 11, many secondary follicles with 2-4 layers of GCs are present and by day 19, many well-formed antral follicles are present in the rat ovary (McGee et al., 1997b). From day 18 onward, there is increased apoptosis in medium-sized antral follicles, as indicated by low molecular weight DNA (McGee et al., 1997a). After day 25, there is a significant decrease in plasma FSH levels until the onset of puberty (Dohler et al., 1975; Hermans et al., 1980; Rivier et al., 1987; Sander et al., 1985; Taya et al., 1988).

1.3.4. Intra-ovarian factors and FSH regulate early stage follicular development

Preantral follicular growth is thought to be gonadotropin-independent since follicles can progress to the antral stage in hypophysectomized animals and in ovaries with defective FSHR (Aittomaki et al., 1995; Gulyas et al., 1977; Halpin et al., 1986; Hillier, 1994; Kumar et al., 1997). However, it is now accepted that preantral follicles are gonadotropin responsive. In preantral follicles, 8-bromo-cyclic guanidine monophosphate, but not FSH, is anti-apoptotic and promotes follicular growth *in vitro* (McGee et al., 1997a). A number of studies have shown that FSH alone has no effect on GC division or steroidogenesis (Boland et al., 1993; Li et al., 1995), whereas treatment of whole follicles stimulates GC mitosis and folliculogenesis (Cain et al., 1995; Roy et al., 1989; Wang et al., 2003), suggesting that interactions between the support cells and the oocyte are critical for folliculogenesis. It is well-documented that theca- and oocyte-derived growth factors regulate GC functions including cellular proliferation, survival

and steroidogenesis (Eppig et al., 1997; Hsueh et al., 1984; McGee et al., 1999; McGee et al., 2000; Vanderhyden et al., 1992; Wang et al., 2002).

1.3.4.1. Influence of oocyte-derived growth factors on GC function

Growth differentiation factor-9 (GDF-9) and bone morphogenetic protein-15 (BMP-15) are members of the transforming growth factor β (TGFβ) family and are key regulators of GC function. In mice and rats, GDF-9 and BMP-15 are highly expressed in OCs during preantral follicle growth and expression patterns are similar throughout development (Wu et al., 2002).

In rodents, GDF-9 is produced in healthy growing OCs beyond the preantral stage (Dong et al., 1996; Hayashi et al., 1999), whereas, in cows and horses, GDF-9 mRNA is present as early as the primordial stage (Bodensteiner et al., 1999). In GCs isolated from antral follicles, GDF-9 increased GC proliferation and decreased FSH-induced differentiation (Vitt et al., 2000). GDF-9 is also an important regulator of preantral follicle growth, and GDF-9-deficient animals exhibit a block in folliculogenesis at the primary stage (Dong et al., 1996). In rat preantral follicles (150-180 μm diameter), GDF-9 enhanced FSH-induced follicular growth *in vitro*, and decreased follicular cell apoptosis in the presence of FSH (Orisaka et al., 2005). Down-regulation of GDF-9 at the preantral / early antral transition induced GC apoptosis, and suppressed basal and FSH-induced preantral follicle growth. In addition, treatment of granulosa cell cultures with GDF-9 suppressed ceramide-induced apoptosis (Orisaka et al., 2005). These data

suggest that GDF-9 promotes follicular growth at the preantral/early antral transition by suppressing GC apoptosis.

BMP-15 is highly expressed in OCs of secondary and pre-ovulatory follicles (Otsuka et al., 2000; Yan et al., 2001). Like GDF-9, BMP-15 has been shown to increase GC proliferation in small antral follicles (Hayashi et al., 1999; Otsuka et al., 2000; Vitt et al., 2000). Additional functions of BMP-15 include: stimulation of GC kit ligand (KL), down-regulation of FSHR expression and inhibition of FSH-induced progesterone production (Otsuka et al., 2000; Otsuka et al., 2001). Treatment of oocyte-granulosa cell complexes (OGC) from murine preantral follicles with high concentrations of FSH (5 ng/mL) decreased BMP15 mRNA, and this is associated with an increase in OGC diameter indicative of GC proliferation (Thomas et al., 2005). These results suggest that the action of BMP-15 on GC proliferation is likely follicle stage-dependent. The Inverdale (*FecX*^{I}) sheep has a naturally occurring mutation in the mature coding region of BMP-15 and is infertile due to arrested folliculogenesis at the preantral stage (Galloway et al., 2000). In contrast, BMP-15$^{-/-}$ mice are subfertile with minor pathohistological ovarian defects (Yan et al., 2001), suggesting that BMP-15 plays a crucial role during early follicular development in the sheep ovary and has less importance during murine follicle development.

1.3.5. The penultimate stage of follicular development

Prior to the early antral stage, there is relatively low incidence of atresia in the ovary. At the penultimate stage (~300-400 μm diameter), the dominant follicle is rescued, while the subordinate follicles undergo degeneration (Hirshfield, 1991). The

absence of FSH cyclicity in immature rats and during lactation prevents follicles from growing beyond the early antral stage (> 400 μm diameter) (Read et al., 1979; Taya et al., 1982). To date, many theories have been proposed to explain the cause of follicular demise. In subordinate follicles, a combination of FSH insensitivity, hypoxia (due to increased follicular size) and/or presence of other hormone signals may destine them for atresia (Hirshfield, 1991). The dominant follicle is thought to grow faster than the rest of the cohort due to an increased number of FSHR and enhanced FSH responsiveness. Increased inhibin and estrogen production, in turn, coordinately deprives subordinate follicles of circulating FSH (McGee et al., 2000). The rat is a multiparous species and more than one dominant follicle emerges from the cohort during estrous, however, the mechanisms by which multiple ovulations occur remain unclear. It has been suggested that genetic determinants (McNatty et al., 1986; Spearow, 1986) and the size of preovulatory follicles (Baird et al., 1998; Cahill et al., 1981; Driancourt et al., 1996) may modulate the degree of negative feedback (e.g. inhibins, estrogens) on FSH secretion.

1.4. Apoptosis and ovarian function

1.4.1. Apoptotic versus necrotic cell death

Apoptosis, or programmed cell death, is an important mechanism for cell elimination, and occurs in a controlled temporal fashion (Kerr et al., 1972; Palumbo et al., 1995). It is characterized morphologically and biochemically by nuclear and cytoplasmic shrinkage, chromatin condensation, internucleosomal DNA fragmentation [180-190 base-pair (bp) multiples] and nuclear pyknosis (Palumbo et al., 1995). Nuclear debris and intracellular organelles are then packaged into apoptotic bodies and

phagocytosed by neighbouring macrophages, without induction of inflammatory response (Tilly, 1996). Biochemically, internucleosomal DNA degradation is mediated through Ca^{+2}/Mg^{+2}-dependent endonuclease (Boone et al., 1997b) and caspase-activated DNase activities (Enari et al., 1998; Sakahira et al., 1998).

Necrosis is a form of "uncontrolled" cell death that occurs in response to toxic or ischemic insult and is characterized by cell swelling, loss of ATP or membrane pumps, destruction of cellular organelles, membrane obliteration and induction of inflammatory response (Lockshin et al., 2004; Palumbo et al., 1995). Currently, the precise cellular mechanisms of necrotic cell death are not well understood. Upon inhibition of caspases, certain cell types undergo a Fas or tumour necrosis factor α (TNFα)-mediated form of cell death that resembles apoptosis; cells exhibit swelling of mitochondria and endoplasmic reticulum and disruption of mitochondrial membrane potential (Matsumura et al., 2000; Vercammen et al., 1998). Although Fas classically activates an apoptotic pathway (as discussed below in Section 1.4.2.), Fas activation for a prolonged period of time without phagocytosis of apoptotic cells can lead to necrosis (Matsumura et al., 2000).

1.4.2. Apoptotic cell death pathways in the ovary

Three cell death signaling pathways including the receptor-, mitochondria- and endoplasmic reticulum-mediated cell death pathways converge on caspase activation to stimulate apoptotic machinery and lead to irreversible cell death. Although each of these pathways is able to function independently, the apoptotic response of the cell is likely dictated by cross-talk between all three signaling cascades, and convergence on caspase-3

activation. The downstream importance of caspase-3 in the induction of apoptosis will be discussed in further detail in Section 1.4.4.

Fas antigen (APO-1/CD95) is a member of the tumour necrosis factor / nerve growth factor receptor family. Fas and the TNF receptor type I (TNFRI) share high homology in the cytoplasmic death domain region (Nagata et al., 1995), although the mechanism by which they induce apoptosis differs. Fas is activated by Fas ligand (FasL) binding (Nagata et al., 1995) and the complex associates with Fas-associated death domain (FADD) and pro-caspase-8 to form the death inducing signaling complex (DISC), which in turn, activates caspase-8 (Chinnaiyan et al., 1995; Nagata, 1997). FasL exists in the cell as a membrane-associated form and is proteolytically processed to a soluble form (Nagata et al., 1995). Upon ligation, TNFRI undergoes trimerization, recruits TNF receptor-associated death domain (TRADD) and activates caspase-8 (Chen et al., 2002). Unlike Fas signaling, TNFRI action is also mediated by the nuclear factor κB (NFκB) cell survival pathway (Chen et al., 2002).

Mitochondrial cell death is facilitated by: 1) disruption of the electron transport chain, 2) release of mitochondrial apoptogenic factors [cytochrome-c, Smac/DIABLO, apoptosis inducing factor (AIF)], and 3) release of reactive oxygen species (ROS) and disturbance of redox reactions in the cell (Green et al., 1998). A number of reports have demonstrated that decreased ATP is a feature of late-stage apoptosis (Bossy-Wetzel et al., 1998; Eguchi et al., 1997). In response to an apoptogenic stimulus such as ultraviolet (UV)-radiation, cytochrome-c is released from the mitochondria and associates with

Apaf-1 and pro-caspase-9 to form the apoptosome, thereby activating downstream caspases (Green et al., 1998). Murine Smac and its human ortholog DIABLO (Smac/DIABLO) are mitochondrial precursor proteins that are processed to a mature peptide prior to release during apoptosis (Du et al., 2000; Verhagen et al., 2000). The primary function of Smac/DIABLO is the inhibition of X-linked inhibitor of apoptosis protein (XIAP) through physical binding to the baculoviral IAP repeat 3 (BIR3) domain (Wu et al., 2000). Upon receipt of an apoptotic signal such as poly (ADP-ribose) polymerase (PARP) activation, mature AIF is released from the mitochondria, migrates to the nucleus and causes chromatin condensation leading to DNA damage (Susin et al., 2000; Yu et al., 2002). Similar to disruption in electron transport, generation of ROS in response to pro-apoptotic agents is a relatively late apoptotic event (Jacobson et al., 1995).

Endoplasmic reticulum-induced apoptosis is a recently identified death pathway that requires caspase-12 as an effector caspase (Nakagawa et al., 2000). In response to endoplasmic reticulum-specific stress-inducing stimuli such as brefeldin A, tunicamycin and thapsigargan, caspase-12 is cleaved and activated. However, the same effect is not observed after staurosporine, Fas and TNF challenge, suggesting that caspase-12-mediated cell death is mediated through the endoplasmic reticulum (Nakagawa et al., 2000). In addition, caspase-12 deficient murine cells are resistant to apoptosis induced by ER stressors, further establishing a role for caspase-12 in this process (Morishima et al., 2002).

1.4.3. Apoptosis is the physiological mechanism of follicular atresia

In 1885, Flemming was the first to report a "chromatolytic" cell death process in the GCs of degenerating antral follicles in the rabbit ovary (Flemming, 1885). Since that time and taken together with Kerr's observations of apoptotic morphology (Kerr et al., 1972), a clear relationship has been established between apoptotic cell death and follicular atresia. In untreated immature rats, there is high incidence of follicular atresia and these follicles contain apoptotic GCs, as indicated by DNA fragmentation analysis and *in situ* labeling (Hughes, Jr. et al., 1991; Palumbo et al., 1995; Tilly et al., 1992). In the prepubertal rat ovary, apoptosis occurs primarily in the GCs, although theca cell and oocyte apoptosis has also been reported (Morita et al., 1999; Palumbo et al., 1994; Palumbo et al., 1995). In preantral follicles, there is minimal GC apoptosis and follicular atresia (Hirshfield, 1988; Palumbo et al., 1994; Roy et al., 1989). However, when atresia does occur, it is usually initiated by the oocyte and spreads to the GCs and TCs (Morita et al., 1999). In contrast, apoptotic cell death at the penultimate stage begins in the GC layer (Hughes, Jr. et al., 1991), and is followed by death of the TCs and the oocyte (Logothetopoulos et al., 1995; Morita et al., 1999; Palumbo et al., 1994; Tilly et al., 1991). Although the cellular and molecular mechanisms of follicle atresia are now becoming clear, the precise relationship between cell survival and cell death molecules is incomplete.

1.4.4. Caspases and induction of apoptosis

1.4.4.1. Caspases

Caspases (cysteine aspartate-specific proteases) are mammalian *CED-3* homologues constituting the family C14, clad CD peptidases (Rawlings et al., 1993), and

were originally characterized in the nematode *C. Elegans* (Thornberry et al., 1992; Yuan et al., 1993). To date, at least 14 caspases have been identified and have been classified into 2 sub-families, the ICE family (caspases-1, -4, -5, -11) and the CED-2 family (caspases-3, -6, -7, -8, -9) (Thornberry et al., 1998). Caspase-3 is the most well characterized member of the effector caspases and will be the focus of this section. The relationship between caspases and apoptosis was established by mutational analysis in which *CED-3* mutations resulted in lack of apoptosis during nematode development (Ellis et al., 1991). Further insight has since been gained from caspase-3, caspase-9 and Apaf-1 knockout animals, which exhibit late embryonic or perinatal lethal deficiencies due to defects in apoptosis during development of the central nervous system (Ranger et al., 2001).

1.4.4.2. Regulation of caspase activation

Caspases are present in the cell as inactive zymogen precursors (pro-form) and contain 3 domains: 1) a NH_2-terminal prodomain, 2) a large subunit (~20 kDa) and 3) a small subunit (~10 kDa) (Nicholson et al., 1997; Thornberry et al., 1998). Activation of the enzyme involves proteolytic cleavage at Asp-x bonds between the domains, and subsequent heterodimerization of the large and small subunits (Thornberry et al., 1998). During apoptosis, caspases function as initiators and effectors of cell death (Alnemri, 1997). Activation of initiator caspases is thought to occur through the interaction of 2 or more caspase precursors, and is termed the "induced proximity / oligomerization" theory (Salvesen et al., 1999). According to this model, pro-caspases-8 and –9 exist as monomers in the latent state and are activated through dimerization. Positive cofactors

such as Apaf-1 and FADD may facilitate the interaction between the precursors depending on proximity of the factors, and this association is thought to cause autoproteolytic activation (Salvesen et al., 1999; Thornberry et al., 1998). In contrast, the presence of negative cofactors such as flice-like inhibitory protein (FLIP) or the inhibitor of apoptosis proteins (IAPs) may modulate the conformation of the precursors, thereby preventing caspase activation (Thornberry et al., 1998). Compartmentalization of caspases within the cell may also contribute to regulation of caspase activation (Thornberry et al., 1998). Effector caspase activation involves a cascade of proteolytic processes, which begins with activation of initiator caspases and ends in cellular disassembly (Thornberry et al., 1998). Upon receipt of a pro-apoptotic stimuli, interaction with cofactors that stimulate activation or decrease inhibition have been proposed to activate downstream caspases (Shi, 2004; Thornberry et al., 1998).

1.4.4.3. Caspase-3 (CPP32) substrate recognition and cleavage

In general, caspase-3 substrates have a very strict requirement for an aspartate residue (D) at the P1 position in order for cleavage to occur (Nicholson et al., 1997). In addition, recognition of at least 4 amino acids upstream of the cleavage site is a requirement for substrate recognition (Thornberry et al., 1998). However, the presence of a caspase concensus sequence does not ensure physiological cleavage at this site, suggesting that tertiary protein structure may influence the enzyme-substrate interaction (Thornberry et al., 1998). Many known substrates for caspase-3 have a DXXD motif within their protein sequences (Nicholson et al., 1997). Some of these targets include: 1) essential nuclear targets (e.g. PARP) (Kaufmann et al., 1993; Tewari et al., 1995), 2)

structural proteins (e.g. actin, lamins) (Rosen et al., 1997), 3) anti-apoptotic proteins such as cIAP-1 (Clem et al., 2001) and Bcl-2 (Cheng et al., 1997), and 4) the inhibitor of caspase-activated DNase (ICAD) (Enari et al., 1998; Sakahira et al., 1998).

1.4.4.4. Caspase-3 localization and function in the ovary

In the mouse, the active form of caspase-3 is immunolocalized to the GCs of late preantral and antral-sized follicles, and this pattern is consistent with follicular selection (Fenwick et al., 2002). Biochemical and morphological analyses have demonstrated that caspase-3 function is required for induction of GC apoptosis (Matikainen et al., 2001). In healthy rat follicles, caspase-3 levels in the GCs are low, however, after induction of follicular atresia, there is a marked increase in caspase-3 expression in the GCs and TCs, and apoptosis (Boone et al., 1998). These data suggest an important function for this death protease in follicular demise. Despite their low apoptotic capacity, the TCs of preovulatory-sized follicles express higher levels of procaspase-3 than the GCs (Boone et al., 1998; Yacobi et al., 2004). Although these findings seem contradictory, it is worth noting that TCs do not contain DNaseI, the endonuclease responsible for apoptotic DNA fragmentation in the ovary, and hence, may be unable to complete the apoptotic program (Boone et al., 1997b; Boone et al., 1998). These findings suggest that levels of pro-caspase-3 in the follicular cells may not correlate to induction of apoptosis. Recently, it has been proposed that limited TC apoptosis may facilitate stigma formation and follicle rupture during ovulation (Yacobi et al., 2004), however, further studies are required to confirm this possibility.

Understanding of the role of caspase-3 during follicular atresia has been further enhanced by the generation of caspase-3 knockout animals (Kuida et al., 1996; Matikainen et al., 2001). In caspase-3 deficient mice, GC apoptosis and follicular atresia are defective, as indicated by a lack of GC nuclear pyknosis and low TUNEL staining in the follicle (Matikainen et al., 2001). Moreover, GCs cultured in serum-free conditions lacked the capacity to undergo nuclear fragmentation despite initiation of DNA condensation, and failed to undergo programmed cell death (Matikainen et al., 2001). Interestingly, the apoptotic process is not entirely absent in caspase-3-null animals, suggesting the compensatory activity of other downstream caspases or caspase-independent cell death in the ovaries of these mutants (Matikainen et al., 2001). Although a number of caspase-3 substrates have been identified in the granulosa cell, the precise roles of these proteins during induction of apoptosis are not completely understood.

1.5. FSH is a survival factor and promotes follicular maturation in antral follicles

During late stage follicular growth, FSH modulates GC function in two ways: 1) FSH causes GC proliferation and stimulates expression of LHR and aromatase in the peri-ovulatory period, including those of LHR and aromatase enzyme (Hirshfield, 1991), and 2) FSH promotes follicular growth by inhibition of GC apoptosis and rescue of the dominant follicle from follicular atresia (McGee et al., 2000; Richards, 1994; Wang et al., 2003). The importance of gonadotropins during follicular growth is further confirmed by the lack of large pre-ovulatory follicles and mature corpora lutea in the FSHR-knockout (FORKO) mouse (Dierich et al., 1998). The precise mechanisms by which

FSHR aberration leads to infertility are not completely understood. However, one hypothesis is that the profound estrogen and progesterone deficiencies in the null mutants severely hinder follicular maturation (Balla et al., 2003). Another possible contributor to impaired follicular growth beyond the secondary stage, is the downstream FSH-responsive gene cyclin D2, the expression of which is decreased in FORKO ovaries (Dierich et al., 1998). Since cyclin D2 is an important regulator of GC proliferation (Robker et al., 1998a), disruption of FSH signaling may hinder the proliferative pathway in the FORKO mouse.

1.5.1. Action of equine chorionic gonadotropin (eCG) in the immature rat ovary

Equine chorionic gonadotropin is a glycoprotein hormone obtained from the endometrial cups of a pregnant mare, and has FSH- and some LH-like activities (Gospodarowicz et al., 1967; Papkoff et al., 1978b). In the rat, eCG has a half-life of approximately 6-7 h in circulation (Aggarwal et al., 1981). The prolonged availability of eCG in blood is attributed to its high glycoprotein content (Aggarwal et al., 1981; Licht et al., 1979). In immature rats, eCG significantly increases FSHR and LHR mRNA expression, promotes follicular maturation and rescues small antral follicles from atresia (Braw et al., 1980; Camp et al., 1991a; LaPolt et al., 1992). However, high concentrations of gonadotropins can desensitize GCs to further stimulation by FSH and down-regulate the number of FSHR (Jonassen et al., 1980; LaPolt et al., 1992; Rao et al., 1977; Richards et al., 1976), suggesting a biphasic effect of FSH on the regulation of FSHR. During FSH-induced follicular growth, eCG primarily acts on antral follicles with more than 200 GCs in the largest cross-section, and increases the number of preovulatory follicles at 48 h (Braw et al., 1980). Based on these findings, the eCG-

primed immature rat model is useful for the study of GC function during follicular development *in vivo*.

1.5.2. Gonadotropin withdrawal induces GC apoptosis and follicular atresia

Atresia in preovulatory-sized follicles can be achieved by metabolic clearance of a single injection of eCG in immature rats, with the onset of atresia occurring 4-5 days after injection (Hughes, Jr. et al., 1991; Kim et al., 1999). In addition, hypophysectomy on day of proestrus induces follicular atresia starting 8 h after the procedure (Nahum et al., 1996). More recently, the use of a gonadotropin withdrawal method using anti-eCG antibody has provided a more rapid means of inducing follicular atresia (Bill et al., 1981; Boone et al., 1997a). Gonadotropin withdrawal in eCG-primed immature rats causes apoptosis and follicular atresia in small- and medium-sized antral follicles *in vivo* (Boone et al., 1997a). Morphologically, the formation of pyknotic nuclei occurs 4 h after anti-eCG treatment in the hamster, and becomes quite prominent by 24 h after gonadotropin withdrawal (Bill et al., 1981). Low molecular weight DNA is detectable as soon as 1 h of antibody treatment and further increases 6 and 24 h thereafter (Boone et al., 1997a). Similarly, a dramatic decrease in E_2 production occurs within 1 h of treatment (Bill et al., 1981), suggesting that changes in DNA integrity and steroidogenesis in response to gonadotropin withdrawal precede morphological signs of atresia. Fas and FasL are co-localized in apoptotic cells and are highly expressed in the GCs of atretic follicles (Kim et al., 1998). Gonadotropin withdrawal caused a marked increase in GC Fas and FasL contents after 24 h of anti-eCG treatment (Kim et al., 1998), and increased caspase-3 expression in TUNEL-positive follicles (Boone et al., 1998). These events are associated

with decreased expression of cell survival factors (e.g. XIAP, phospho-Akt), which may further shift the balance toward apoptosis (Asselin et al., 2001).

1.6. Role of estrogens during follicular growth

1.6.1. Estrogen synthesis

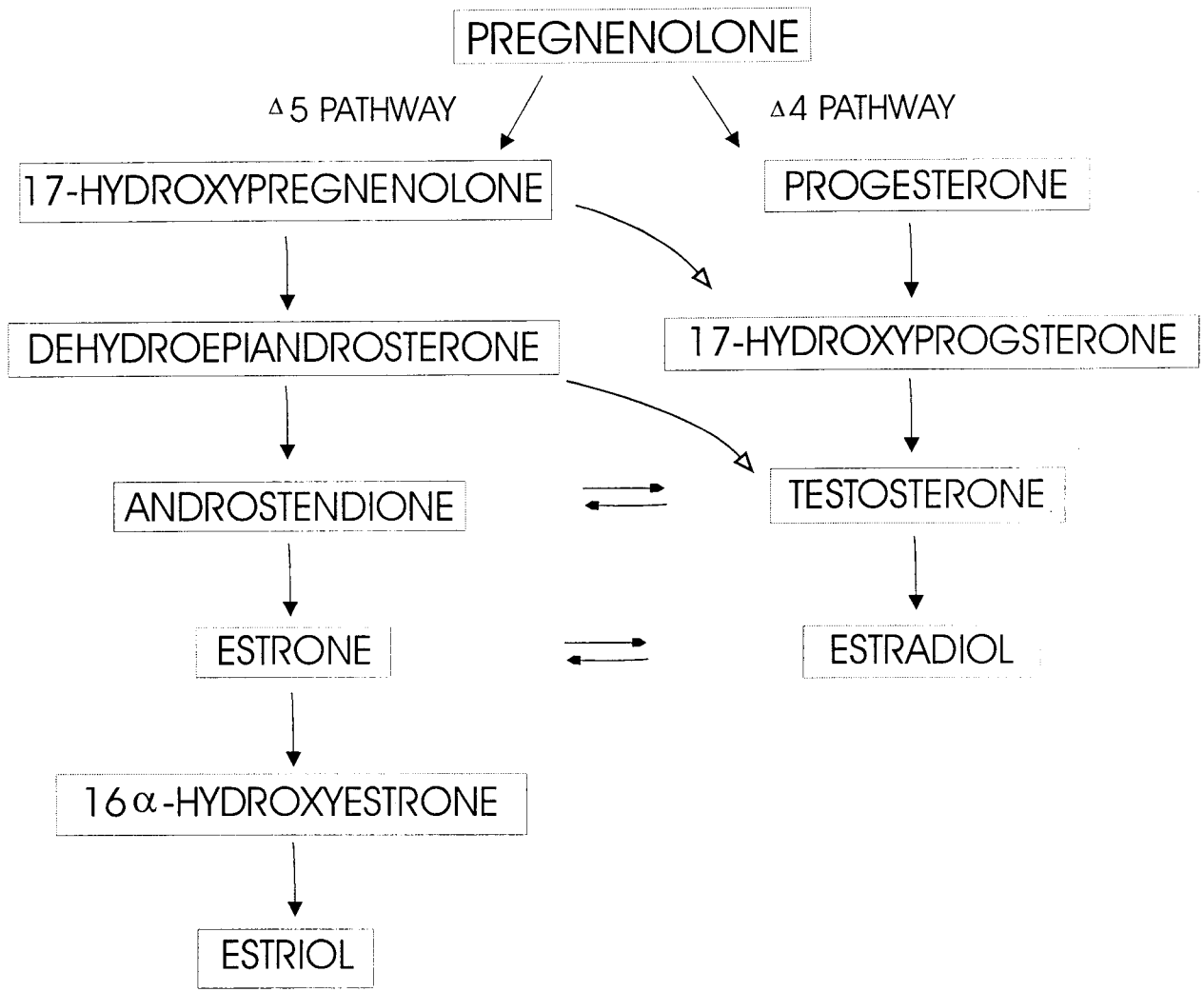
As shown in Figure 3, theca-derived androstendione is converted to estrogens in a step-wise manner (Lipsett, 1986). The action of P450 aromatase converts androstendione to estrone, which is then converted to E_2 through the action of 17β-HSD-1 enzyme. Theca-derived androgens are essential intermediates of estradiol synthesis, but excessive follicular androgen levels cause follicular arrest and anovulation. At the cellular level, high androgen inhibits estrogen-stimulated follicle growth (Louvet et al., 1975) and increases atresia of rat ovarian follicles (Bagnell et al., 1982). In rat GCs, FSH causes the induction of LHR (Zeleznik et al., 1974), while androgens inhibit induction of LHR (Farookhi, 1980; Jia et al., 1985). Dihydrotestosterone (DHT), a 5α-reduced metabolite of testosterone, inhibits GC proliferation by down-regulation of cyclin D2 and cell cycle arrest at G_1 (Pradeep et al., 2002).

1.6.2. Mechanisms of estrogen action

The estrogen receptor (ER) subtypes, α and β, are part of the nuclear receptor superfamily of transcription factors, and are closely related to the glucocorticoid, mineralocorticoid and progesterone receptors (Baker, 1997). Although the rat ovary expresses both ERα and ERβ, the GCs predominantly express the ERβ subtype (Byers et al., 1997). In contrast, ERα is highly expressed in the nuclei of TCs and germinal

Figure 3: Estrogen synthesis in the ovary

Estrogens are synthesized from pregnenolone through the Δ4 and the Δ5 steroidogenic pathways. Estrone and estriol are synthesized in a step-wise fashion from 17-hydroxypregnenolone, while E_2 is synthesized from progesterone. Bioconversion between androstendione and testosterone, as well as between estrone and E_2 also accounts for some estrogen production. Evidence suggests that the Δ5 pathway predominates in the human follicle for estrogen synthesis while the Δ4 pathway prevails in the corpus luteum for progesterone and estrogen synthesis.



Adapted from Lipsett, 1986.

epithelial cells (Sar et al., 1999). The classical estrogen responsive element (ERE) contains a palindromic inverted repeat 5'-GGTCAxxxTGACC-3' (Klein-Hitpass et al., 1988). Upon ligand binding, contact between the ER dimer and sugar phosphate backbone of the ERE are important for sequence recognition and high affinity binding (Koszewski et al., 1991). Classically, estrogen action occurs through the targeting of ERE on downstream genes. However, more recent evidence suggests that non-classical estrogen action occurs through: 1) transcription factor and co-activator binding to ERE (Feng et al., 1998), 2) growth factor activation (Smith, 1998), and 3) non-genomic membrane receptor signaling (Levin, 1999). Activated ERs have been shown to interact with Jun and Fos at AP-1 binding sites during activation of insulin-like growth factor-I (IGF-I) (Umayahara et al., 1994) and cyclin D1 (Liu et al., 2002; Sabbah et al., 1999). Similarly, genes with GC-rich promoter regions are regulated through ER-Sp1 interactions (Porter et al., 1997). In chicken and pig GCs, non-genomic estrogen activation occurs through the rapid increase of intracellular calcium concentrations (Morley et al., 1992).

1.6.3. Importance of estrogen during folliculogenesis

Estradiol and FSH play an obligatory role during follicular growth and differentiation. Short-term treatment of rats with diethylstilbestrol (DES; a potent synthetic estrogen) stimulates GC proliferation, enhances the sensitivity of GCs to FSH, and increases estrogen synthesis (Reilly et al., 1996). During emergence of the dominant follicle, increased E_2 production inhibits FSH secretion from the anterior pituitary. In addition, E_2 has been shown to modulate GC function through: 1) stimulation of gonadotropin receptors (Richards, 1975), 2) formation of gap junctions (Yu et al., 1994),

3) altered testosterone and progesterone production (Fortune et al., 1979; Leung et al., 1979), and 4) suppression of GC apoptosis (Billig et al., 1993). Estrogen-deficient animals including ER knockout (ERKO) and aromatase knockout (ArKO) mice have provided greater insight into the role of estrogens *in vivo*. The phenotype of ERKO females includes hemorrhagic and cystic ovaries (Lubahn et al., 1993), and accelerated folliculogenesis likely due to elevated levels of circulating FSH and LH (Schomberg et al., 1999). Although follicles at all stages of development are present in $ER\beta^{-/-}$ ovaries, there are fewer corpora lutea and animals are sub-fertile (Krege et al., 1998). At puberty, ArKO females exhibit internal and external anomalies of the reproductive tract, and follicles contain an increased number of apoptotic GCs (Britt et al., 2000; Fisher et al., 1998). Although estrogens act on an array of extra-ovarian target tissues, its primary functions in the ovary are follicular growth and differentiation. It is well documented that E_2 stimulates granulosa cell proliferation, and regulates cyclin D2 and p27 expressions in a temporal fashion during folliculogenesis. However, whether other cell cycle-related genes are involved in this process and whether interactions between these molecules exist, are not known.

1.7. Mammalian cell cycle

In order to maintain tissue homeostasis, the cells maintain a fine balance between proliferation, differentiation and apoptosis. Apoptosis and mitosis share some morphological characteristics: loss of substrate attachment, cell shrinkage, chromatin condensation and membrane blebbing (King et al., 1998). However, mitotic cells do not undergo DNA fragmentation and fail to exhibit the other apoptotic hallmarks described in

Section 1.4.1. Mitosis involves the segregation of DNA, cytokinesis and the production of two identical diploid daughter cells.

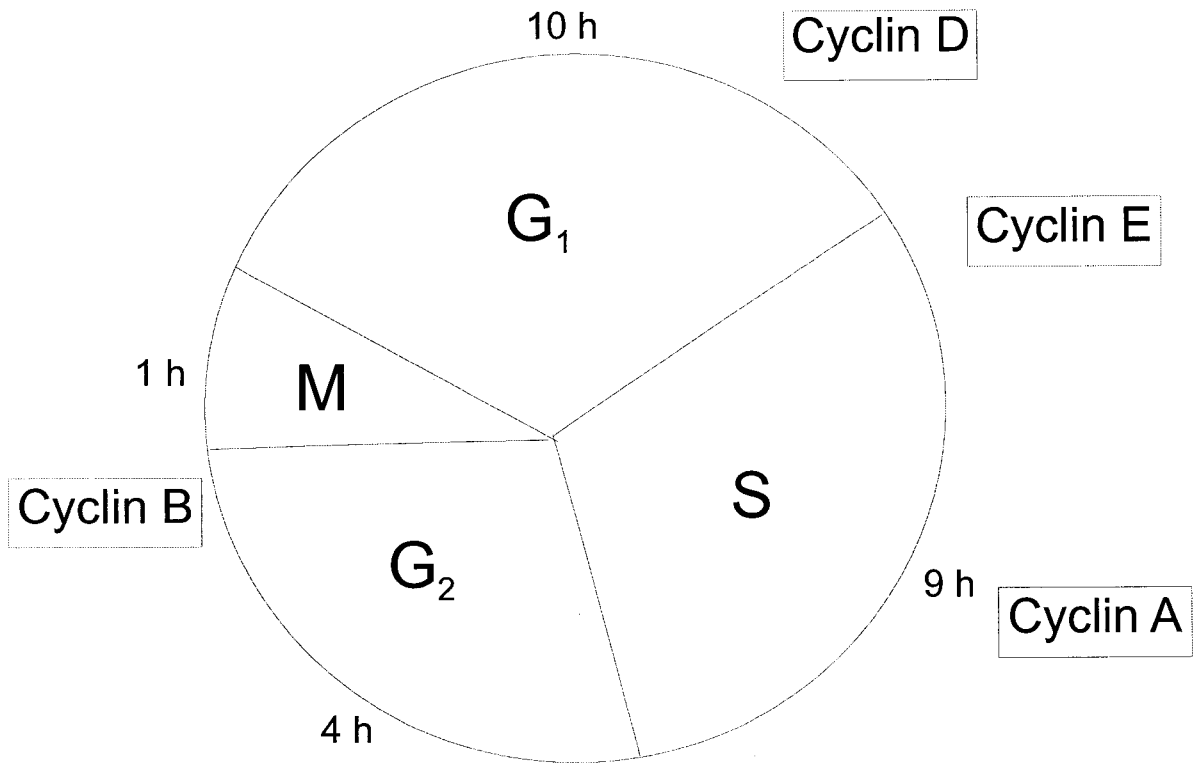
1.7.1. Overview of the cell cycle

In 1953, Howard and Pelc observed that DNA is replicated during a specific phase of cell growth (Howard et al., 1953). The cell cycle is divided into four phases: Gap 1 (G_1), DNA synthesis (S), Gap 2 (G_2) and mitosis (M) phases (Figure 4). During the G_1 phase, cells increase in cell mass and prepare for DNA replication through the synthesis of RNA and proteins. The onset of DNA synthesis marks the G_1-S transition and during S phase, the cell produces chromosomes comprised of two sister chromatids.

In the G_2 phase, the cell prepares for mitosis through additional protein synthesis. Lastly, during M phase or mitosis, the 2 sister chromatids separate and migrate to opposite poles of the cell, and undergo cytokinesis to produce 2 identical daughter cells (Lodish et al., 1996). Cell cycle checkpoints are in place prior to the G_1-S transition ("restriction point"), during the S phase and during the G_2-M transition to ensure appropriate cell cycle progression (Lodish et al., 1996). In response to stimuli such DNA damage, TGFβ ligand binding, cell-cell contact, growth factor withdrawal and replicative senescence, the cell exits cell cycle into G_0 and undergoes growth arrest (Lodish et al., 1996). At this point, the cell will attempt to repair the DNA and resume cell cycle; if DNA repair is not possible, the cell will undergo apoptosis.

Figure 4: Stages and features of the cell cycle.

One mammalian cell cycle lasts ~ 24 h and the G_1 and S phases have the longest duration at ~10 h and 9 h, respectively. Upon receipt of a growth stimulus, the cell exits G_0 and enters G_1 phase, where it prepares for DNA replication. The transition between the different phases is controlled by the activity of cyclin-cdk complexes, which, when activated, can phosphorylate an array proteins involved in cell cycle progression.


10 h
Cyclin D
G1
Cyclin E
1 h
M
S
Cyclin B
G2
9 h
Cyclin A
4 h

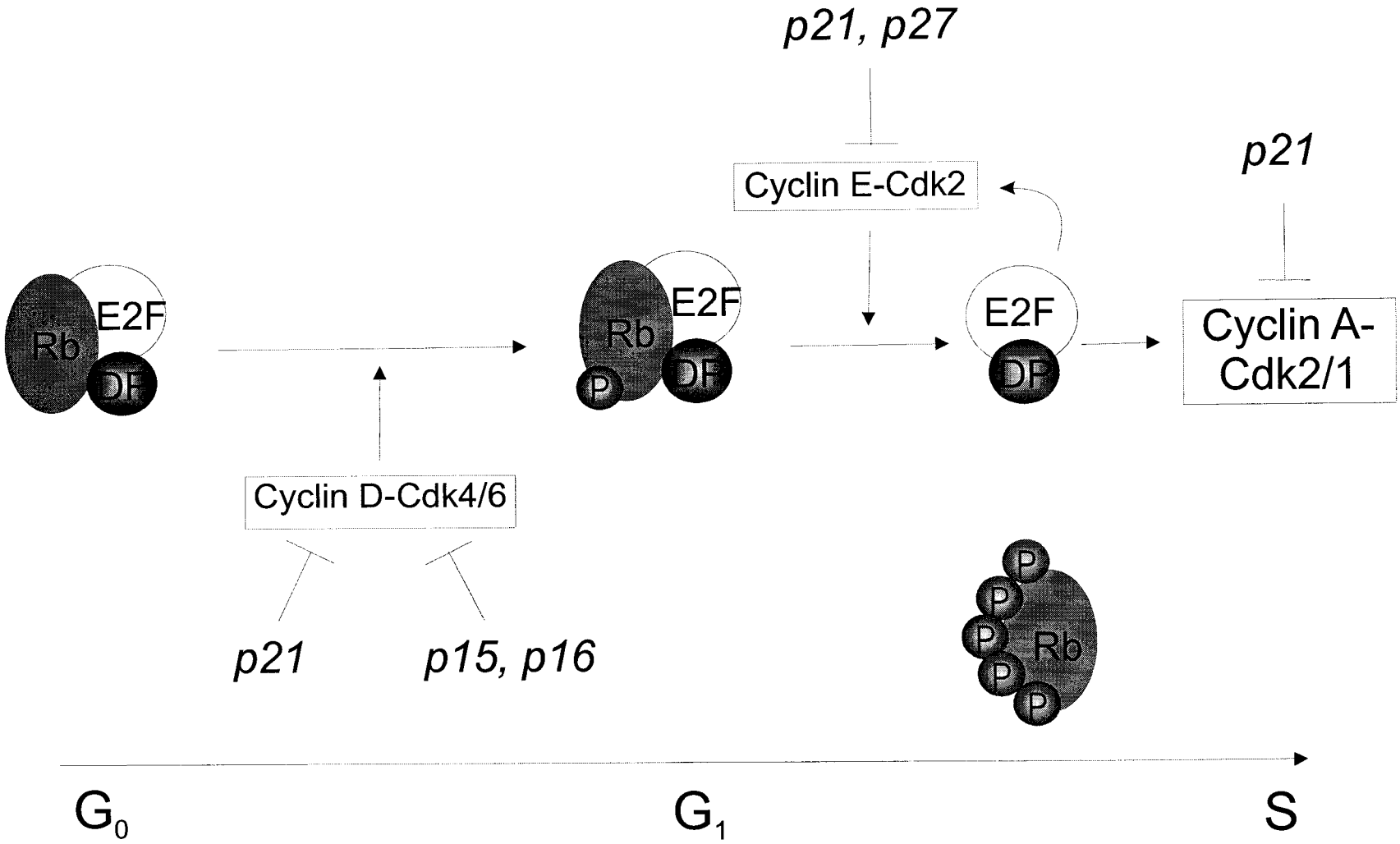
1.7.2. Regulation of the G_1-S transition

Cyclin-dependent kinases (cdk) and their activating subunits, the cyclins, phosphorylate substrates involved in cell cycle progression. Cyclin levels are relatively constant throughout cell cycle and the activity of cyclin-cdk kinase activity is regulated by post-translational modifications (Johnson et al., 1999; Yew, 2001). The D-type cyclins (D1, D2 and D3) are the first cyclins induced after exit from quiescent G_0 phase. Cyclin D-cdk4/6 complexes phosphorylate retinoblastoma protein (Rb) causing it to dissociate from a repressor complex containing the transcription factor E2F and DP protein (Figure 5). Dissociated E2F translocates to the nucleus and regulates the transcription of cell cycle-related genes such as cyclins A and E, and DNA polymerase α (Johnson et al., 1999). After E2F activation, cyclin E expression is upregulated and associates with cdk2 in mid- to late- G_1 phase. Cyclin E-cdk2 maintains phospho-Rb in a hyperphosphorylated state, prolongs E2F action (Hinds et al., 1992) and is required for S phase entry (Obaya et al., 2002). Cyclin A associates with cdk2 and then cdk1 (cdc2) in late S phase, and is active during the S-M transition (Johnson et al., 1999).

The Cip/Kip family of cyclin-dependent kinase inhibitors includes *p21* and *p27* (Figure 5). The *p21* promoter contains a p53-binding site and is upregulated in response to DNA-damaging agents (el Deiry et al., 1993). During proliferation, *p27* is expressed but is sequestered and inactive; subsequent binding to the cyclin E-cdk2 complex strongly inhibits its function (Polyak et al., 1994). In rat GCs, *p27* is a key negative regulator of cell cycle. Estradiol treatment increases cyclin D2 and E expressions and decreases *p27* levels (Robker et al., 1998b). After LH surge and during the peri-

Figure 5: Regulation of the G_1-S transition

In quiescent cells, Rb is hypophosphorylated and remains bound to an E2F (transcription factor) / DP protein complex. In response to mitogenic stimuli, cyclin D binds with cdk4/6 and phosphorylates and inactivates Rb. Inactivation of Rb releases the bound E2F-DP heterodimer complex, which, in turn, may regulate transcription. E2F target genes such as cyclins E and A are required for G_1-S transition, however evidence also suggests that E2F-DP can inhibit transcription through histone deacetylase activity and chromatin remodeling. Up-regulation of cyclin E expression causes phosphorylation of *p27* and facilitates its degradation. The G_1-S transition is negatively regulated by cdk-inhibitors including the Cip/Kip members, *p21* and *p27*, as well as the INK4 members, *p15* and *p16*. The INK4 family members inhibit monomeric cdk-4 activity through a binding site that overlaps the cyclin binding domain. In contrast, the Cip/Kip family members inhibit the activity of pre-formed cyclin-cdk complexes through obligate binding.



Adapted from Obaya and Sedivy, 2002

ovulatory period, *p27* expression is significantly increased (Robker et al., 1998b). The principal INK4 family members, *p16* and *p15* bind to Cdk4 and Cdk6 but not the other cdks (Johnson et al., 1999). At the G_1-S transition, they are released in response to cdk-mediated Rb phosphorylation, and inhibit cdk4 activity (Figure 5) (Serrano et al., 1993).

1.7.3. Regulation of granulosa cell cycle during follicular growth

The GCs of primordial follicles undergo a slow rate of proliferation (Hirshfield, 1991), while those of early antral follicles become progressively more responsive to circulating gonadotropins, produce more estrogens and rapidly proliferate (Gaytan et al., 1996; Hirshfield, 1991; Rao et al., 1978). During follicular growth in the rat, only 2-16 % of the GC nuclei are in S phase within an entire follicle (Hirshfield et al., 1988; van Weissenbruch et al., 1990), indicating that rat GCs have a relatively low replicative potential. Mural and cumulus GCs were originally identified according to size and differ in their degree of differentiation and steroidogenic capacity (Kerketze et al., 1996; Rao et al., 1991a; Rao et al., 1991b). Four hours after LH surge, the GCs exit cell cycle and undergo a program of terminal differentiation (Robker et al., 1998a).

Although FSH and E_2 induce GC proliferation *in vivo* (Rao et al., 1978), neither hormone alone has a significant effect on the mitotic capacity of GCs in culture (Bley et al., 1991; Hsueh et al., 1984). Therefore, the previously described intra-ovarian factors likely play a crucial role in the control of ovarian cell proliferation during early follicular growth. In contrast, during the later stages of follicular development, FSH is required for cellular differentiation, as evidenced by the lack of large antral follicles in FORKO mutants (Dierich et al., 1998). After FSH treatment, low levels of cAMP stimulate

cyclins D2 and E in granulosa cells *in vitro* (Robker et al., 1998b). However, in other cell types, high levels of cAMP have been shown to cause exit of cell cycle (Grieco et al., 1996; Kato et al., 1994). Therefore, FSH and appropriate levels of cAMP may dictate whether a cell progresses through or exits the cell cycle during follicle development.

1.7.4. Proliferating cell nuclear antigen (PCNA) and its role in follicular development

PCNA, a member of the DNA sliding clamp family of proteins, was originally discovered in the nuclei of dividing cells, and is highly expressed in the S and G_2 phases (Bravo et al., 1980; Liu et al., 1989; Maga et al., 2003; Miyachi et al., 1978). For many years, PCNA has been considered the ideal cellular marker for cell proliferation. However, more recent studies have demonstrated that PCNA is also an important DNA repair protein outside of S phase (Shivji et al., 1992). Classically, PCNA increases the activity of DNA polymerase δ through initiation of leading strand DNA replication and interaction with PCNA-binding proteins to fill the gaps between Okazaki fragments (Kelman et al., 1995; Maga et al., 2003). During DNA repair, PCNA interacts with mispair-binding proteins such as MutS homologue (MSH), and enhances binding specificity (Clark et al., 2000). Granulosa cell PCNA is expressed during follicular development beyond the primordial stage, and is present in both healthy and atretic follicles (Oktay et al., 1995). Theca cell PCNA expression is abundant throughout folliculogenesis and decreases only after the onset of atresia. Lastly, oocyte PCNA is highly expressed after the primary stage and displays a similar developmental pattern as TC PCNA expression. Based on the developmental pattern of PCNA, it is a sensitive marker of proliferation in rat GCs (Oktay et al., 1995). Although FSH alone cannot

stimulate PCNA expression in cultured rat GCs, co-treatment with activin markedly increases PCNA and cyclin D2 expressions in undifferentiated GCs, responses that are closely associated with the induction of P450 aromatase and LHR (El Hefnawy et al., 2001).

1.7.5. Role of cyclin D2 during GC proliferation

On postnatal days 5 and 10 in the mouse, cyclin D2 is expressed in follicles with 1-2 layers of GCs, despite their low proliferative capacity (Robker et al., 1998b). High levels of cyclin D2 are also expressed in the small follicles of FSHβ and GDF-9 knockout ovaries, suggesting that regulation of GC cyclin D2 expression is gonadotropin- and GDF-9-independent (Robker et al., 1998b). Therefore, during early follicular development, GC proliferation is likely mediated through molecules other than cyclin D2 alone. Treatment of hypophysectomized rats with E_2 increases cyclin D2 expression, promotes GC proliferation and preantral follicular growth (Robker et al., 1998b). Further treatment with FSH increased cyclin D2 mRNA in rapidly dividing GCs, enhanced proliferation and stimulated the formation of large antral follicles. An ovulatory dose of LH caused luteinization of GCs and down-regulation of cyclin D2 mRNA (Robker et al., 1998b). Interestingly, the chronology of these responses corresponded with the doubling time of GCs during exponential growth (Hirshfield, 1991), suggesting that these hormones may play a role in the control of cell cycle progression. Cyclin D2-deficient ovaries contain follicles with no more than 4 layers of GCs and the homozygote females are infertile (Sicinski et al., 1996). Mutants exhibited impaired FSH and LH response, defective GC proliferation and corpora lutea that contained trapped oocytes (Sicinski et

al., 1996). Taken together, these studies suggest that cyclin D2 plays an important role in GC proliferation and is positively regulated by estradiol and FSH.

1.8. Leucine-rich acidic nuclear protein (LANP) family

1.8.1. Structural characteristics

The LANP family members are characterized by regions called leucine rich repeats (LRR; 20-29 residue motifs), which were originally identified in leucine-rich α2-glycoprotein in human serum (Takahashi et al., 1985). LRRs are composed of unique β-α structural units on a common axis and an exposed β sheet formation along the inner circumference; the majority of proteins with LRR have a high surface area for potential interactions (Kobe et al., 1993). Proteins containing LRRs have been implicated in processes such as early embryonic development (Tong et al., 2000) and apoptosis signaling (Inohara et al., 1999). Other notable features of the LANPs include: a hydrophobic C-terminal acidic region abundant in aspartic and glutamic acids, an acidic region important in histone and non-histone chromosomal protein interaction (Bernues et al., 1986), and a C-terminal putative nuclear localization signal (NLS).

1.8.2. Key LANP family members

Rat LANP [also known as human phosphoprotein 32 (pp32) in its phosphorylated form, and as putative HLA class II associated protein I (PHAPI) (Vaesen et al., 1994)] has been implicated in rat cerebellar neuron morphogenesis (Matilla et al., 1997; Matsuoka et al., 1994). As in other LANP family members, LANP contains a LRR (10-128 aa), an acidic region (165-247 aa), a NLS (234-237 aa), and may be a tumour

suppressor and activator of caspase-9 in the mitochondrial cell death pathway (Jiang et al., 2003). LANP has also been implicated in various nuclear functions such as 1) the nuclear shuttling of HuR, an RNA-binding protein involved in RNA stability (Brennan et al., 2000; Ma et al., 1996) and 2) inhibition of histone acetylation or "histone masking" leading to transcriptional repression (Seo et al., 2001; Seo et al., 2002).

Acidic protein rich in leucines (APRIL; also known as rat PAL31) has ~68% and 65% sequence homology to human pp32 and rat LANP, respectively. APRIL contains the characteristic LRR and acidic regions of LANP family members, and is located on chromosome 15q5 (Mencinger et al., 1998). Like pp32, APRIL also has putative phosphorylation sites including one for cAMP-dependent protein kinase and four sites for casein kinase II (Mencinger et al., 1998). Noteworthy, is that human APRIL has over 70% homology to proliferation-associated leucine-rich protein 31 (PAL31; also known as rat APRIL) at the protein level (Mutai et al., 2000).

1.8.2.1. Generation and Characterization of LANP / pp32 Null Mice

Recently, LANP / pp32 null mice were developed by homologous recombination with genomic clones corresponding to the mouse *Lanp* locus (Opal et al., 2004). Surprisingly, these animals were both viable and fertile, which can possibly be explained by functional redundancy amongst LANP family members. LANP null mice displayed normal growth curves, behavioral profiles and neural electrophysiology compared to their normal and heterozygote counterparts (Opal et al., 2004). Although there is high homology between mouse LANP and APRIL, there was no compensatory up-regulation

of rat APRIL (PAL31) expression in LANP mutants (Opal et al., 2004). Although the LANP null mice are virtually indistinguishable from their wild-type counterparts, it is possible that other homologous family members may compensate for this loss-of-function. Currently, the role of LANP in the rat ovary and its function during follicular development has not been established.

1.8.3. Cloning and identification of PAL31

PAL31 was originally identified by differential display as a gene product predominantly expressed in fetal brain (Mutai et al., 2000). Subsequent Northern blot analysis with one of the cDNA fragments (4p16) recognized a 1.5 kb mRNA sequence. A cDNA containing 1225 nt, a single open reading frame (272 aa) and a poly(A)signal was isolated and subsequently named PAL31. It has a molecular weight of 31,064 Da and a p*I* value of 3.87 (Mutai et al., 2000).

1.8.4. PAL31 structure and homology with other proteins

Like other leucine-rich acidic nuclear proteins, PAL31 contains a LRR (xLxxLxLxxNxL), an acidic region and a putative NLS (Lys-Arg-Lys-Arg) (Figure 6) (Mutai et al., 2000). PAL31 differs from the other family members by the presence of a double repeat of ^{154}EAPDSD(G/V)EVD^{163}, and having the longest acidic region among all family members (Mutai et al., 2000). PAL31 also contains a putative caspase-3 cleavage site (DXXD) at 176-179 aa, however, whether it is a physiological substrate for caspase-3 in the ovary, is not known. Similar to LANP, the presence of an NLS in the C-terminus of PAL31 may facilitate its movement between cytoplasm to nucleus.

Figure 6: PAL31 structure

PAL31 is comprised of a leucine-rich repeat (xLxxLxLxxNxL), an acidic region, and a putative nuclear localization signal (Lys-Arg-Lys-Arg). PAL31 differs from other family members by the presence of a unique double repeat of ^{154}EAPDSD(G/V)EVD^{176} and having the longest acidic region among all family members. PAL31 also contains a putative caspase-3 cleavage site (DXXD) at 176-179 aa and a nuclear localization signal in the C-terminal.

Adapted from Mutai et al., 2000

1.8.5. PAL31 tissue expression and developmental regulation

Although PAL31 mRNA is highly expressed in the fetal rat brain, it is also found in other tissues containing highly-proliferating cells, including the spleen, thymus, ovary and testis (Mutai et al., 2000). In cell lysates from embryonic brain, two bands at a molecular weight of ~31 kDa were visible by Western blotting, with the intensity of the upper band being higher than that of the lower band (Mutai et al., 2000). As noted in APRIL and LANP, PAL31 contains potential phosphorylation sites at serines (Ser) 225 and 255, and threonine (Thr) 265; it was therefore hypothesized that the doublet may represent a phosphorylated and non-phosphorylated form of the protein (Mutai et al., 2000). The 31 kDa band was present at all stages of fetal brain development, with peak intensity on embryonic days 12 and 16 and gradual decline until adulthood, suggesting a possible role for PAL31 in brain development during embryogenesis and the postnatal period.

Analysis of PAL31 expression in rat brain by immunohistochemistry indicated high expression in the cells of the neural tube (Mutai et al., 2000). On postnatal day 5, the number of PAL31-positive cells were markedly decreased compared to the fetal brain (Mutai et al., 2000), suggesting that the temporal change in PAL31 expression is due to a decreased number of PAL31-positive cells in the brain. To evaluate the expression pattern of PAL31 during neuronal development, immunolocalization of PAL31, nestin (a marker of neural progenitor cells) and PCNA were performed. Nestin-positive neural progenitor cells expressed PAL31 and expression overlapped that of PCNA, suggesting that PAL31 may facilitate brain development through progenitor cell proliferation. This

phenomenon was further confirmed by the co-localization of PAL31 and PCNA in the perinuclear compartment of mitotic PC12 cells (Mutai et al., 2000).

1.8.6. PAL31 promotes cell cycle progression from the G_1 to the S phase

The Nb2 and BaF3 cell lines are derived from prolactin (PRL)-dependent pre-T lymphomas (Gout et al., 1980) and murine pro-B lymphoid cells (Palacios et al., 1985), respectively. Nb2 cells can be rendered quiescent by removal of lactogenic hormone from the media and induced into active proliferation by addition of lactogenic hormone [e.g. PRL, placental lactogen-I (PL-I)] (Schellenberg et al., 1982; Sun et al., 2001) Similarly, these hormones induced PAL31 mRNA expression in a dose- and time-dependent fashion in BaF3 cells, suggesting that PAL31 mRNA is highly abundant during proliferation (Sun et al., 2001). Although a small amount of PAL31 protein was present in resting Nb2 cells, PAL31 protein content increased in response to PRL and was associated with increased cyclin E expression and entry into S phase (Sun et al., 2001). Cell cycle-dependent expression of PAL31 during the G_1-S transition was further confirmed by the presence of a strong nuclear PAL31 signal in S phase cells (Sun et al., 2001). Down-regulation of PAL31 significantly inhibited cell proliferation and prevented cell cycle progression into S phase (Sun et al., 2001). These studies suggest that PAL31 is important for cell cycle progression from the G_1 into S phase.

1.8.7. PAL31 expression is down-regulated after cellular differentiation

The trophoblast giant cell (TGC) of the placenta contains more than 100-fold greater DNA content than a diploid cell, and undergoes multiple rounds of DNA replication (Ohgane et al., 1998). The Rcho-1 cell line, derived from a spontaneous

choriocarcinoma (Teshima et al., 1983), has TGC morphology and continues replication without mitosis after terminal differentiation. PAL31 mRNA is highly expressed in the cytotrophoblasts and syncytiotrophoblasts of the placenta (Oda et al., 2001). In contrast, low levels of PAL31 mRNA were detected in the peripheral layer of the junctional zone comprising TGCs (Oda et al., 2001). Although high levels of PAL31 mRNA were present in proliferative in Rcho-1 cells, PAL31 expression significantly decreased after the onset of differentiation (Oda et al., 2001). PAL31 immunoreactivity in TGCs was quite low despite a strong signal for PCNA, suggesting that expression of PAL31 does not overlap that of PCNA in TGCs (Oda et al., 2001).

1.8.8. Putative role for PAL31 in apoptosis

Etoposide, a topoisomerase II inhibitor, is a chemotherapeutic agent and an inducer of cellular apoptosis (Li et al., 2001). To evaluate the role of PAL31 in the regulation of apoptosis, Rat1 cells expressing PAL31 were constructed using a Tetracyclin (Tet)-transactivator system (Sun et al., 2005). Cells expressing high levels of PAL31 exhibited lower apoptotic response after exposure to etoposide (Sun et al., 2005). In addition, over-expression of PAL31 conferred a similar level of cytoprotection to UV, as did the cell survival factor XIAP(Sun et al., 2005). On the other hand, down-regulation of PAL31 induced G_1 arrest and DNA fragmentation, suggesting that PAL31 is a physiological inhibitor of apoptosis (Sun et al., 2005). During etoposide-induced apoptosis, there was a decrease in endogenous PAL31 content, and the appearance of a 19 kDa immunoreactive band in Rat1 cells. Since PAL31 contains a putative DXXD motif, it was hypothesized that PAL31 may be a substrate for caspase-3. Incubation of PAL31 protein with purified caspase-3 resulted in a concentration-dependent increase in

the 19 kDa band detected by the PAL31 antibody (Sun et al., 2005). Transient transfection of the C-terminal (12 kDa) but not the N-terminal (19 kDa) PAL31 fragment induced cell death, suggesting that the 12 kDa fragment may be pro-apoptotic. Together, these data suggest that PAL31 is a substrate for caspase-3 and that PAL31 processing may be a determinant of cell survival or apoptosis. However, the mechanism by which PAL31 functions in these capacities is not known.

1.9. Goals of this thesis

1.9.1. Rationale for the proposed studies

Mutai and colleagues demonstrated that PAL31 is highly expressed in adult rat tissues containing proliferating cells, including the ovary (Mutai et al., 2000). Subsequent studies have shown that PAL31 is highly abundant during cellular proliferation and is immediately down-regulated after cytodifferentiation (Oda et al., 2001; Sun et al., 2001). Granulosa cell proliferation and subsequent differentiation in response to gonadotropins is crucial for follicular growth and maturation. To date, the role and regulation of PAL31 has only been examined in neuronal and placental cells. However, whether PAL31 is expressed in the ovarian follicle and whether its expression is modulated by gonadotropins during follicular development, remains unknown.

In Rat1 cells, PAL31 is a physiological inhibitor of apoptosis in its intact form and during etoposide-induced apoptosis, is processed to generate an immunoreactive band at a molecular weight of 19 kDa (Sun et al., 2005). Incubation of purified PAL31 protein with active caspase-3 *in vitro* produced a product consistent with the fragment size detected *in vivo* (Sun et al., 2005). Over-expression of the N-terminal (19 kDa)

PAL31 fragment had no effect on apoptotic response, while transient expression of the C-terminal (12 kDa) cleavage fragment caused cell death *in vivo* (Sun et al., 2005). These results suggest that the intact form of PAL31 and its C-terminal (12 kDa) cleavage product may possess opposed roles during cell survival and apoptosis, respectively (Sun et al., 2005). Gonadotropin withdrawal in eCG-primed immature rats using a neutralizing anti-eCG antiserum causes GC apoptosis in small- and medium-sized antral follicles (Boone et al., 1997a). During induction of apoptosis, caspase-3 has been shown to cleave an array of substrates including essential nuclear proteins (e.g. PARP) and cell survival proteins (e.g. XIAP). To our knowledge, the role and regulation of PAL31 in the rat ovary has not been examined. Moreover, whether the intact form of PAL31 and its cleavage product play any role in granulosa cell survival and apoptosis, respectively, remains to be determined. Although the PAL31 protein sequence contains a classical caspase-3 consensus sequence (DXXD), whether PAL31 is a physiological substrate for caspase-3 during follicular atresia, is not known.

FSH-stimulated folliculogenesis is driven, in part, by the action of estrogens, which are potent mitogens of rat GCs *in vivo* (Britt et al., 2002; Robker et al., 1998a). During early follicular growth, small preantral follicles gradually acquire FSH and E_2 responsiveness, and rapidly proliferate in response to these hormones (Rao et al., 1978; Richards, 1975). Thus, functional integrity of FSH and E_2 signaling pathways is necessary for appropriate regulation of GC proliferation and differentiation during folliculogenesis. This is supported by the pathophysiology observed in cyclin D2- and estrogen-deficient animals, which exhibit impaired GC proliferation and follicular arrest,

or inefficient ovulation (Palter et al., 2001; Sicinski et al., 1996). In neuronal cells, PAL31 is highly expressed during cellular proliferation and is required for G_1-S transition of cell cycle (Mutai et al., 2000; Sun et al., 2001). However, the precise role of PAL31 during proliferation has not yet been elucidated. Moreover, whether PAL31 is regulated by estrogens, is not known. The PAL31 promoter does not contain a classical estrogen responsive element (ERE; GGTCAxxxTGACC sequence), however, estrogens can regulate gene expression without binding to DNA through non-genomic pathways and protein-protein interactions at the promoter (Bjornstrom et al., 2005). Therefore, estrogens may regulate GC PAL31 expression through a non-classical mechanism.

1.9.2. Objective and hypotheses

1.9.2.1. Overall Objective

To study the regulation of ovarian PAL31 expression by gonadotropins and estradiol during follicular development *in vivo* and *in vitro*, using immature rat models and a granulosa cell culture system.

1.9.2.2. Specific Objectives

1. To determine which follicular cell type(s) express PAL31 in the rat ovary and whether PAL31 expression is developmentally regulated;

2. To compare PAL31 expression in 14-day and 22-day rat granulosa cells; To examine the influence of gonadotropins on ovarian follicular PAL31 content *in vivo*;

3. To study the changes in GC PAL31 contents and cleavage following induction of follicular atresia in gonadotropin-primed immature rats *in vivo*;

4. To investigate the regulation of PAL31 content in granulosa cells by FSH *in vitro*;

5. To examine the effect of estradiol in GC PAL31 content in immature rats *in vivo*.

1.9.2.3. Overall Hypothesis

FSH modulates PAL31 expression in a biphasic manner. During early follicular development when GCs are highly proliferative, FSH and estradiol increase PAL31 expression, while PAL31 expression is down-regulated as GCs differentiate during late stage follicular development.

1.9.2.4. Specific Hypotheses

1. PAL31 expression is increased during GC proliferation and down-regulated after the onset of differentiation;

2. Granulosa cell PAL31 protein is cleaved during induction of apoptosis during follicular atresia;

3. FSH increases GC PAL31 content at low concentrations, but causes premature GC differentiation and decreased PAL31 content at high concentrations *in vitro*;

4. Estradiol-stimulated GC proliferation in immature rats is associated with increases in GC PAL31 contents *in vivo*;

CHAPTER 2: MATERIALS AND METHODS

2.1. Materials

All reagents were obtained from Sigma Chemical Co. (St. Louis, MO, USA), unless otherwise stated. Culture media (RPMI-1640, L15), fetal bovine serum, penicillin-streptomycin, trypsin-EDTA, TEMED, Alexa-Fluor® 350, goat anti-mouse IgG, M-MLV reverse transcriptase and all primer sets used for RT-PCR were purchased from Invitrogen (Burlington, ON, Canada). Diethylstilbestrol was obtained from Steraloids Inc (Newport, RI, USA). Ovine FSH (NIAMDD oFSH-14) and human recombinant FSH were from the National Hormone and Pituitary Program of the National Institute of Diabetes and Digestive and Kidney Disease (Baltimore, MD, USA). The PAL31 antibody (rabbit polyclonal IgG) and mouse recombinant protein were graciously provided by Professor Kunio Shiota (Laboratory of Cellular Biochemistry, Animal Resource Sciences/Veterinary Medical Sciences, University of Tokyo). Mouse monoclonal anti-GAPDH and goat polyclonal anti-LDH antibodies were purchased from Abcam (Cambridge, MA, USA). Rabbit polyclonal anti-P450 aromatase and mouse monoclonal anti-P450 aromatase IgG were purchased from Acris (Hiddenhausen, Germany) and Serotec (Raleigh, NC, USA), respectively. Antibodies for PCNA (rabbit polyclonal IgG) and cyclin D2 (rabbit polyclonal IgG) were obtained from Santa Cruz Biotechnology (Santa Cruz, CA, USA). Antibody for caspase-3 (rabbit polyclonal IgG) and Falcon culture wares were purchased from BD Biosciences (Mississauga, ON, Canada). Goat anti-rabbit and goat anti-mouse IgG HRP conjugate, acrylamide (electrophoresis grade), *N, N'*-methylene bis-acrylamide, DC Protein Assay kit, sodium dodecyl sulphate (SDS), Tris, nitrocellulose membranes and SDS polyacrylamide gel

electrophoresis (SDS-PAGE) prestained molecular weight standards (low range) were purchased from Bio-Rad Laboratories (Mississauga, ON, Canada). Prestained wide range molecular weight protein standards and MassRuler™ DNA ladder were purchased from MBI Fermentas (Newington, NH, USA). Dithiothreital (DTT) was purchased from Boehringer Mannhein (Montreal, QC, Canada). Enhanced chemiluminescence (ECL™) kit and Hyperfilm ECL™ high performance chemiluminescence film were purchased from Amersham Life Sciences (Oakville, ON, Canada). Glycine, 3'-3' diaminobenzidine (DAB) and DAB Substrate solution were purchased from Roche Diagnostic (Laval, QC, Canada). Xylene and ethanol were purchased from BDH (Toronto, ON, Canada). Methanol was purchased from EM Sciences (Gibbstown, NJ, USA). Cytoseal mounting medium for immunohistochemistry was purchased from Richard-Allan Scientific (Kalamazoo, MI, USA). LSAB2 kit was obtained from DAKO Cytomation Inc. (Mississauga, ON, Canada). Perhydrol 30 % hydrogen peroxide was obtained from EM Industries (Darmstadt, Germany). Gill's hematoxylin stain and bromophenol blue were purchased from Fisher Scientific (Ottawa, ON, Canada). Quantitect SYBR Green PCR Kit, HotStarTaq DNA polymerase and the RNeasy Micro kit were from QIAGEN (Mississauga, ON, Canada). Anti-eCG antiserum was generated in our laboratory, as previously described (Kim et al., 1998).

2.2. Methods

2.2.1. Animal preparation and hormone treatment

All animal work was performed in compliance with the Guide to Care and Use of Experimental Animals of the Canadian Council on Animal Care, and was approved by the Animal Care Committee of the Ottawa Health Research Institute. Sprague-Dawley rats (13- to 24-days) were obtained from Charles River Laboratories Inc. (St. Constant, QC, Canada), housed in polycarbonated cages at 21°C, 50 % humidity and in 12 h day-night cycles (light at 0700 h – 1700 h), and given bullet-type rat chow and tap water *ad libitum*. Infantile animals were housed with a lactating female for the duration of the experiments. Hormone treatments for experiments contained in this thesis are outlined in Table 1. Animals were anesthetized by inhalation of isoflurane (Abbott Laboratories Ltd., St. Laurent, QC, Canada) and sacrificed by cervical dislocation. Whole ovaries were excised, cleared of the ovarian bursa and adipose tissue, weighed and kept in PBS or L15 + 0.1 % BSA (cells used for culture) at 37°C. Ovaries of non-responders or high-responders (extreme ovarian weights relative to the other animals in the same group) were excluded. Ovaries were then used for: 1) GC isolation for Western blotting, or 2) paraffin section for immunohistochemistry.

2.2.2. Rat granulosa cell isolation and culture

Granulosa cells were harvested by follicle puncture with a 28 1/2-gauge needle and under a light microscope. Large ovarian pieces and whole follicles were allowed to settle by gravity for 2-5 min and the supernatant containing GCs was collected.

For *in vivo* studies, the cell preparation was centrifuged at ~1100g (20 min, 4°C) and the cell pellets were frozen at -20°C for future analysis or were immediately lysed for Western analysis. For *in vitro* studies, oocytes and small follicles were removed by aspiration with a glass pipette. Granulosa cell number was determined by cell counting with a hemocytometer. Viable cells (determined by Trypan Blue exclusion; $0.48x10^6$ cells/mL) were plated overnight (18-24 h) at 40 % confluence in 6-well plates containing RPMI-1640 with FBS (10 %) and under a humidified atmosphere of 95 % air and 5 %

Table 1: Hormone treatments in immature rats

Hormonal Treatment	Age of Animals	Treatment Dosages	Treatment Duration	Subsequent analyses
eCG	13- to 19-days	0.2 IU/g body weight (in 100 µL PBS; s.c)	48 h	WB, H&E
eCG	21- to 24-days	4 IU or 10 IU per rat (in 100 µL PBS; s.c)	0, 12, 24, 36, 48 h	WB, IHC, H&E
β-estradiol	21- to 24-days	3 mg per rat (in 100 µL corn oil; s.c.)	0, 24, 48 h	WB, H&E
eCG + anti-eCG (gonadotropin withdrawal)	21- to 24-days	10 IU eCG per rat (in 100 µL PBS; s.c.) + anti-eCG (1:20, 1:10 or 1:5 dilution in 100 µL saline; i.p)	eCG (24 h) + anti-eCG (24 h thereafter)	WB
DES	21- to 24-days	1 mg/day for 3 days (in 100 µL sesame oil; s.c.)	3 days	GC Culture, WB

$C0_2$. Next, the media was removed, cells were washed in sterile PBS (37°C) and treated with increasing concentrations of ovine FSH or human recombinant FSH in 2 mL serum-free RPMI-1640. At the end of the culture period, cells were harvested by the addition of 500 µL trypsin-EDTA (3-5 min, 37°C) and the reaction was terminated by the addition of

2 mL RPMI-1640 with FBS (10 %). Cells were pelleted by centrifugation at ~1100g (20 min, 4°C), and frozen at -20°C or used immediately for protein extraction.

2.2.3. Evaluation of protein content

2.2.3.1. Protein extraction

The cell pellet was re-suspended in cell lysis buffer [50 mM HEPES, 150 mM NaCl, 1.5 mM $MgCl_2$, 1 mM EDTA, 100 mM NaF, 10 mM sodium pyrophosphate, 10 % (v/v) glycerol, 1 % (v/v) Triton X-100, pH 7.4) supplemented with freshly-added protease inhibitors (1 mM PMSF, 10 μg/μL aprotinin, 1 mM Na_3V0_4), sonicated (3 x 5 s pulses) and the proteins were allowed to solubilize for 1 h at 4°C. Cell lysates were centrifuged at 14,000g (20 min, 4°C), and the supernatant containing membrane and cytosolic proteins were transferred to fresh centrifuge tubes. Protein concentration for each sample was quantitated using the Bio-Rad DC Protein Assay kit (Bradford method) and absorbance at a 750 nm wavelength was determined spectrophotometrically, using BSA as standard (0.19 – 3.0 μg/μL). One-third of the total volume of 4X-sample buffer (200 mM Tris-HCl, 400 mM DTT, 8 % (w/v) SDS, 40 % (v/v) glycerol and 0.4 % (w/v) bromophenol blue; pH 6.8) was added and samples were boiled for 5 min prior to denaturing SDS-PAGE.

2.2.3.2. Protein separation and Western analysis

Proteins (30-80 μg) in samples were separated by 10-15 % SDS-PAGE and run at 80 volts (V) until the dye-front passed the stacking gel, and then at 110 V for the remainder of the gel. Proteins were then transferred onto nitrocellulose membranes for 2 h at 80 V or overnight (18-20 h) at 30 V. Membranes were stained with Ponceau S stain

to visually compare crude protein amounts per lane and to ensure that the transfer was successful. Membranes were then blocked in tris-buffered saline containing Tween-20 (TBS-T) containing 5 % skim milk powder [1 h, room temperature (RT)] and incubated overnight (18-24 h; 4°C) with the appropriate primary antibody. After 3 washes (10 min each) in TBS-T, membranes were incubated with horseradish peroxidase-conjugated secondary antibodies in TBS-T with 5 % skim milk powder (1 h, RT). Recently, detection of GADPH was accomplished using a fluorescent-labeled secondary antibody (Alexa-Fluor®350 anti-mouse IgG; Molecular Probes; 1 h, RT). After 3 washes (30 min each for anti-PAL31; 10 min each for other primary antibodies) in TBS-T and 1 wash (5 min) in TBS, the proteins of interest were visualized with: 1) ECL chemiluminescence reagents and signal was captured on Hyperfilm ECL film, or 2) Phosphoimager Typhoon 8600 (Amersham Life Sciences; for detection of fluorescent-labeled secondary antibody). To re-probe a membrane with a different antibody, membranes were incubated in stripping buffer (1 M Tris-HCl, 10 % SDS, 14.3 M β-ME) for 15-20 min at 56°C, and washed in water (3 x 5 s) and TBS-T (5 min) before addition of the primary antibody (overnight, 4°C).

2.2.3.3. PAL31 antibody

The rabbit polyclonal PAL31 antibody (0.1 μg/μL) used in the present studies was generously provided by Professor Kunio Shiota (University of Tokyo). It recognizes the coding region of rat and mouse PAL31 protein (1-171 aa). Briefly, a GST fusion protein containing the PAL31 cDNA was generated in *E. Coli* and affinity purified using a nickel column. Five hundred μg of the denatured recombinant protein was injected into New Zealand white rabbits until appropriate immune signal was detected. The rabbit

polyclonal antiserum was then purified with a GST-Sepharose column to remove anti-GST antibodies.

2.2.3.4. Immunohistochemistry

Ovaries were fixed in 10 % buffered formalin. Sections of paraffin-embedded tissues and H and E staining were prepared by the Department of Pathology and Laboratory Medicine, The Ottawa Hospital or within our laboratory. Slides were deparaffinized in xylene (3 x 10 min) and re-hydrated in decreasing grades of ethanol (100 %, 95 %, 80 %, 70 %, 50 %; 5 min each). Endogenous peroxidase activity was inhibited by incubation of slides with 1 % (v/v) hydrogen peroxide in methanol (30 min, RT). To reduce non-specific binding, sections were blocked in 10 % normal rat serum (40 min, RT), and incubated with: 1) anti-PAL31 (1:200), 2) negative control rabbit IgG (1:200) or 3) anti-PAL31 pre-absorbed with an excess amount (12 μg) of mouse recombinant protein overnight (18-24 h, 4°C). After incubation with the primary antibody, slides were washed in PBS and subjected to secondary antibody-linked biotinylated anti-rabbit IgG (40 min, 37°C) and subsequently with a streptavidin complex (40 min, 37°C). Immunostaining was completed by the addition of substrate-chromagen DAB working solution (1:20; < 5 min) and the reaction was stopped by inserting the slide in PBS. Sections were covered in 50 % (v/v) glycerol (plus coverslip), and images were captured using the Empix® Northern Eclipse software imaging system. Some sections were counter-stained with Gill's hematoxylin (15-30 s, RT) to demonstrate nuclear staining. Immunostaining was preserved by the addition of Cytoseal mounting medium (plus coverslip) over the section.

2.2.3.4.1. Qualitative evaluation of PAL31 immunoreactivity

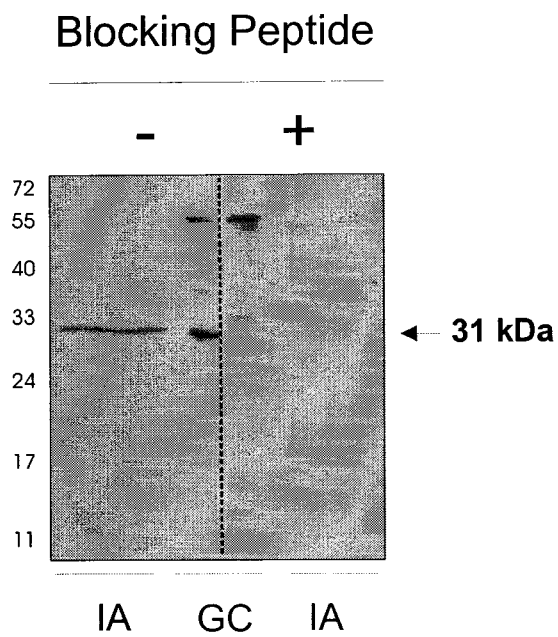
Relative levels of PAL31 immunostaining between experimental groups, ovarian cell types or time of treatment were assessed by numerical evaluation of immunosignal strength on a discrete scale, with 0 and 3 being the weakest and highest, respectively. The data presented herein were collected from four different ovaries per treatment group, and numerical values were obtained from all intact follicles in each section. For each replicate, the signal intensity of the ovarian cells was obtained at one sitting by a single observer to ensure consistency in observation, and the evaluator was unaware of the experimental group. Final results are expressed as the mean ± SEM of immuno-signal strength across all observations.

2.2.3.5. Antigen absorption / blocking peptide studies

To confirm the specificity of the PAL31 antibody for Western analysis and for immunohistochemistry, anti-PAL31 antibody (0.1 μg/μL) was incubated with an excess amount (12 μg; overnight, 4°C) of mouse recombinant PAL31 protein on a shaker. For Western blotting, a single lane of GC lysate from 24 h eCG-treated animals was subjected to SDS-PAGE, electrotransferred onto a nitrocellulose membrane, and the lane was cut in half after Ponceau S staining. Half of the membrane was incubated with anti-PAL31 and the other half with anti-PAL31 pre-absorbed (overnight, 4°C) with the blocking peptide. Secondary antibody incubation and detection of the signal was performed as described previously. As shown in Figure 7, pre-absorption of the PAL31 antibody with an excess amount of recombinant PAL31 protein, blocked the band detected at 31 kDa, suggesting that the PAL31 antibody is specific for rat PAL31. For

Figure 7: Specificity of the PAL31 antibody by antigen absorption studies using Western blotting

Granulosa cell lysate from eCG-treated immature rat (24 h; 50 μg total protein) was subjected to SDS-PAGE, electro-transferred to nitrocellulose membrane and the lane was cut in half. Half of the membrane was incubated with anti-PAL31 and the other half with anti-PAL31 pre-absorbed (overnight, 4°C) with the blocking peptide. IA, interassay pool from homogenized whole rat ovary (positive control); GC, GC lysate from 24 h eCG-primed ovaries.


Blocking Peptide
-
+
72
55
40
33
24
17
11
31 kDa
IA
GC
IA

immunohistochemistry, the pre-absorbed anti-PAL31 preparation was applied directly onto the tissue sections, which were incubated overnight at 4°C. Similar to the Western blot findings, PAL31 immunoreactivity in the follicular cells detected by the PAL31 antibody was indeed specific for PAL31 (Figure 8).

2.2.3.6. Subcellular microsomal extraction for aromatase proteins

Rat ovaries (~200 mg tissue) were snap-frozen in liquid nitrogen, homogenized by mortar and pestle in 4 mL homogenization buffer [100 mM Tris-HCl, pH 7.4, 20 % glycerol (v/v), 150 mM KCl, 1 mM dithiothreitol (DTT), 1 mM EDTA, and protease inhibitor (2 tablets per 50 mL buffer), and cells were allowed to swell for 10 min at RT. Samples were homogenized briefly and centrifuged at 10,000g (30 min; 4°C) to remove nuclei and cellular debris. The supernatant was removed and centrifuged (100,000g, 1 h; 4°C) to extract the microsomal fraction. The pellet was then washed and re-suspended in 200 μL solubilization buffer [50 mM potassium phosphate buffer (pH 7.4), containing 20 % (v/v) glycerol, 0.5 % (w/v) sodium cholate, 0.1 mM EDTA, 1 mM DTT, 0.2 % (w/v) Igepal CO-990, 2 protease tablets per 50 mL buffer). The microsomal fraction of GC lysate (50 μg) from 48 h eCG-primed ovaries was also isolated by this method. Aromatase protein content was subsequently examined by Western blotting (Figure 9).

2.2.3.7. Specificity of monoclonal anti-aromatase antibody

The specificity of the Serotec® human anti-aromatase antibody used in the present studies could not be confirmed in our hands, as no blocking peptide or recombinant protein was available for this purpose. However, prior to

Figure 8: Specificity of the PAL31 antibody by antigen absorption studies using immunohistochemistry

21- to 24-day animals were injected with 10 IU eCG and sacrificed 24 and 48 h thereafter. Ovaries were excised, fixed in 10 % formalin, and paraffin sections were prepared by the Department of Pathology and Laboratory Medicine, The Ottawa Hospital. The anti-PAL31 antibody (1:200 dilution) was preabsorbed with an excess amount of antigen (12 μg, mouse recombinant PAL31 protein) overnight at 4°C and subsequently used for immunohistochemical analysis with anti-PAL31 antibody (1:200 dilution) as a positive control. Representative images of preantral **(A)** and multiple stages of follicles **(B)** from both experimental groups. Bar = 50 μm.

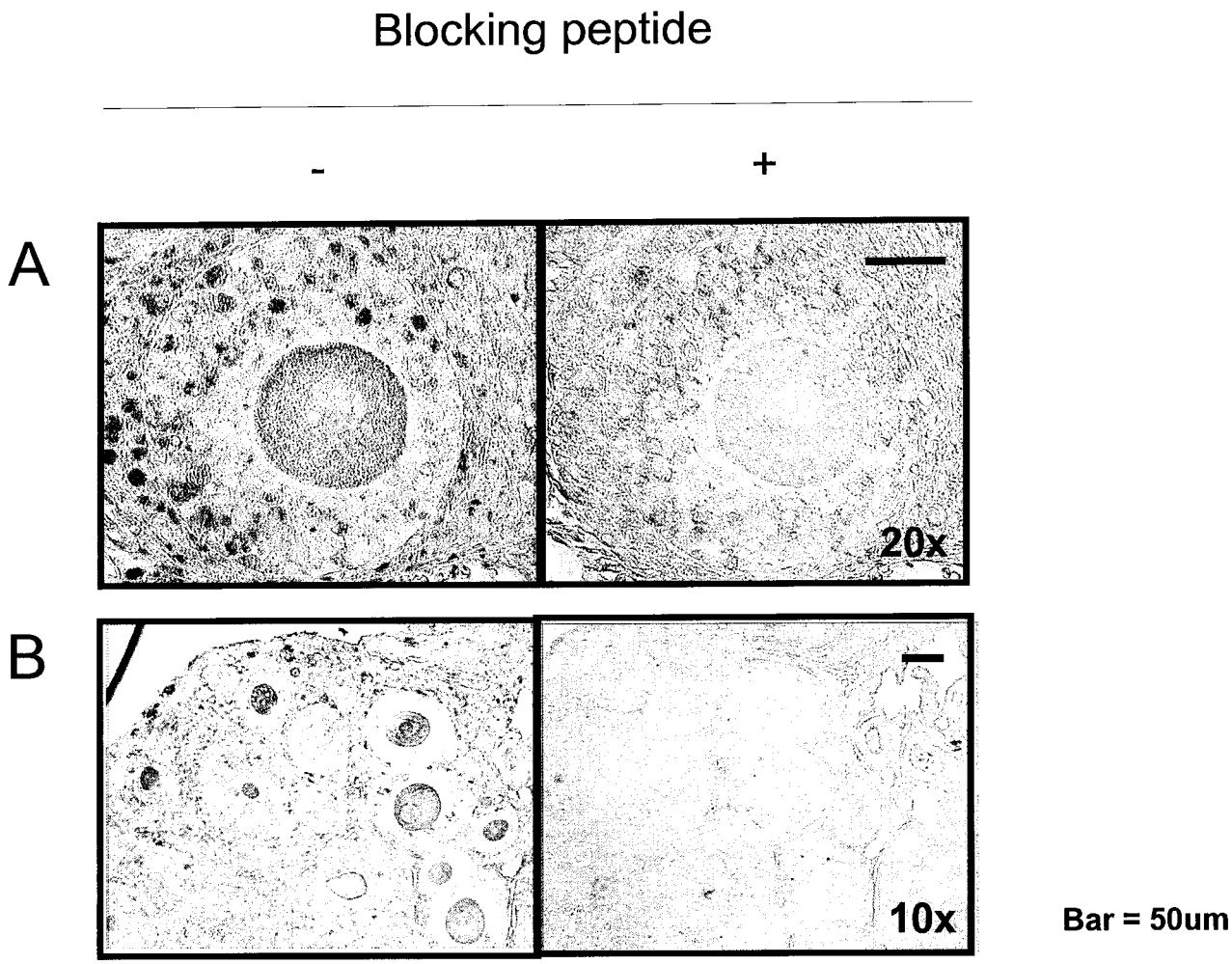
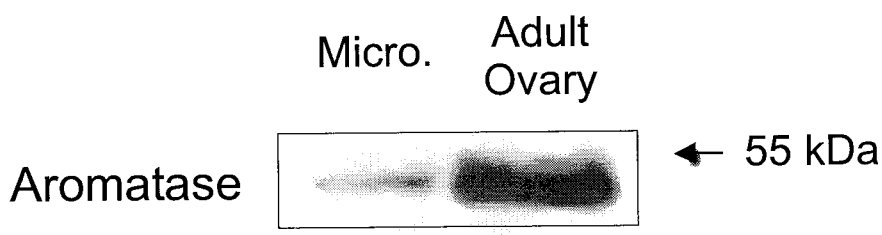

Blocking peptide
-
+
A
20x
B
10x
Bar = 50um

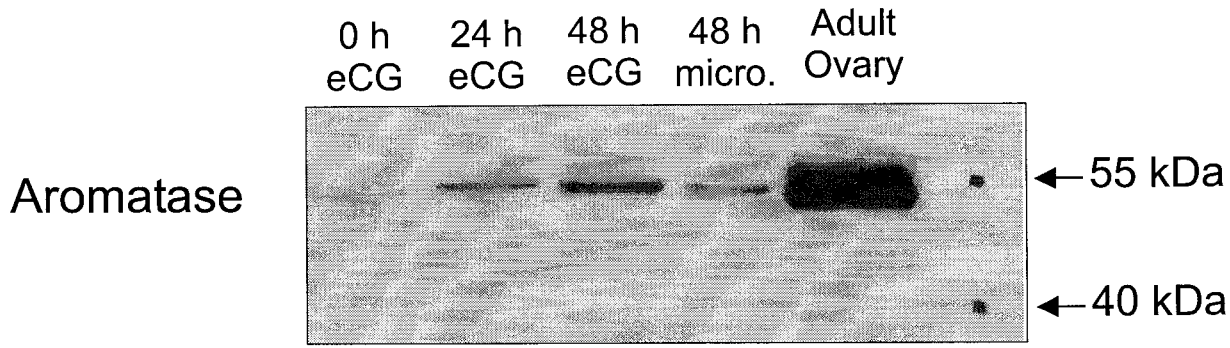
Figure 9: Microsomal extraction of aromatase proteins from rat granulosa cells

Rat ovaries (~200 mg tissue) were snap-frozen in liquid nitrogen, homogenized by mortar and pestle in homogenization buffer, and cells were allowed to swell. Samples were homogenized briefly and centrifuged at 10,000g (30 min; 4°C) to remove nuclei and cellular debris. The supernatant was removed and centrifuged (100,000g, 1 h; 4°C) to extract the microsomal fraction. The pellet was then washed and re-suspended in 200 μL solubilization buffer. The microsomal fraction of GC lysate from 48 h eCG-primed ovaries was also isolated by this method. Aromatase protein levels were subsequently examined by Western blotting. **A.** Double bands at ~55 kDa detected by α-aromatase antibody (1:400 dilution; Serotec) in adult rat ovary; Micro = microsomal fraction of the same sample. [50 μg protein]. **B.** Single aromatase band detected at ~55 kDa in granulosa cell lysates from eCG-primed immature rats (0-48 h) and in adult rat ovary [50 μg protein]. 48 h micro. = microsomal fraction of the same 48 h GC lysate.

A


Micro.
Adult Ovary
Aromatase
55 kDa

B


0 h eCG
24 h eCG
48 h eCG
48 h micro.
Adult Ovary
Aromatase
55 kDa
40 kDa

commercialization, the same antibody was validated by Turner and colleagues (Turner et al., 2002). In brief, this antibody was raised in mouse against a synthetic peptide encoding a highly conserved region of human aromatase from 376-390 aa. Its specificity was determined with the immunizing peptide in antigen absorption studies (Western blot and immunohistochemistry). Western analysis demonstrated the detection of a single 55 kDa band in microsomal extracts from rat, marmoset and human tissues, which could be reabsorbed with the antigen (Turner et al., 2002). Similar results were observed in positive control human placenta sections incubated with the pre-absorbed antibody.

2.2.4. Evaluation of mRNA abundance

2.2.4.1. RNA extraction

Granulosa cells were collected as previously described and the cell pellet was frozen at -20°C for subsequent analysis or used immediately for RNA extraction. Total RNA from GCs was isolated using the QIAGEN RNeasy Micro kit, according to the manufacturer's instructions. Briefly, cells were disrupted using a buffer containing guandidine isothiocyanate, sonicated, and ethanol was added to optimize binding of RNA to the silica-gel spin column. High quality RNA remains bound to the column, while contaminants are removed in several washing steps. RNA was eluted in RNase-free water, and its concentration and purity were determined using a spectrophotometer (absorbance at 260 nm). The relative purity was expressed as a ratio of the readings at 260 nm and 280 nm (1 unit of absorbance = 40 μg/mL).

2.2.4.2. Reverse transcription and real-time RT-PCR

One μg of total RNA was incubated with random decamer primers (0.2 μg, Ambion) and incubated at 70°C (5 min). A master mix containing 5x reaction buffer, 10 mM dNTP, RNase inhibitor (20 IU) was added to the mixture, and incubated at 37°C (5 min). One μL of RevertAid Enzyme (H Minus M-MuLV RT; Fermentas) reverse transcriptase was added and reverse transcribed under the following conditions: 42°C (60 min) and 70°C (10 min).

Prior to real-time RT-PCR, cDNA were diluted 5x in RNase-free water. Standards for PCR were prepared using samples containing high PAL31 or β-actin mRNA expressions from a 40 μg stock, and working dilutions were prepared using 10x serial dilutions (range: 0.1 μg - $1x10^{-7}$ μg). A master mix containing 2X Quantitect SYBR Green PCR [5 mM $MgCl_2$, dNTP mix, Quantitect SYBR Green PCR buffer, HotStarTaq® DNA polymerase, ROX (passive reference dye), SYBR Green I] and 5 μM forward + reverse primers was added to 5 μL cDNA / standard. Three primer sets (Invitrogen; Table 2) were employed in optimization studies performed by Jin-Yi Jiang, Ph.D., and he demonstrated that the PAL31(3) primers were suitable for real-time RT-PCR. Two μg of resultant cDNA was used for real-time PCR under the following conditions: 95°C (15 min; denaturation); 95°C (15 s), 56°C (20 s), 72°C (30 s) for 40 cycles; 40°C hold (PAL31, β-actin). Eight μL of PCR product was added to 2 μL 6x loading buffer (Fermentas) and separated on a 1.5 % agarose gel (100 V, 1 h). Bands were visualized by ethidium bromide under UV light (Molecular Analyst, Bio-Rad Laboratories).

Table 2: Primer sets used for RT-PCR

Gene	Forward Primer Sequence (5'→3')	Reverse Primer Sequence (5'→3')	Product Region	Predicted size (bp)
RAT PAL31 (1)	TTTCAGACCTCCCGAAGCTA	CCCACTTTCTTCCTCCTCTTC	161-759 aa	599
RAT PAL31 (2)	GAGGATGAGGATGGTGAGGA	AATCCATGAGCAGTCCAACC	591-889 aa	299
RAT PAL31 (3)	GACCAGGAAGCACCTGACTC	TCACCATCCTCATCCTCCTC	465-607 aa	143
RAT β-actin	AGCCATGTACGTAGCCATCC	CTCTCAGCTGTGGTGGTGAA	471-698 aa	228

2.2.5. Quantitation of protein contents and mRNA abundance

Band intensities obtained from Western analysis were quantitated using software from Scion Corporation Inc. (Frederick, Maryland, USA). Background values for each band were also determined, and were subtracted from the band intensities. For select GAPDH Western blots developed using the Phosphoimager, quantitation was also determined using ImageQuant software (Amersham Life Sciences). Results from both methods were normalized to an internal control (GAPDH or LDH for Western blots) and subsequently expressed as a fold change compared to a control arbitrarily set to a value of 1. Lightcycler real-time RT-PCR technology employs SYBR Green I dye that binds to the minor groove of newly synthesized double-stranded DNA, and emits light upon excitation (http://www.roche-applied-science.com/lightcycler-online/). Fluorescence data were acquired after the extension step of each cycle and quantification of mRNA abundance was determined using a standard curve (0.1 μg - $1x10^{-7}$ μg) during the log-linear phase of the PCR. Data were expressed as a relative ratio of PAL31 to β-actin, and as fold of interassay control.

CHAPTER 3: RESULTS

3.1. PAL31 expression in the rat ovarian follicle and possible regulation by FSH

3.1.1. Immunolocalization of PAL31 in the follicle

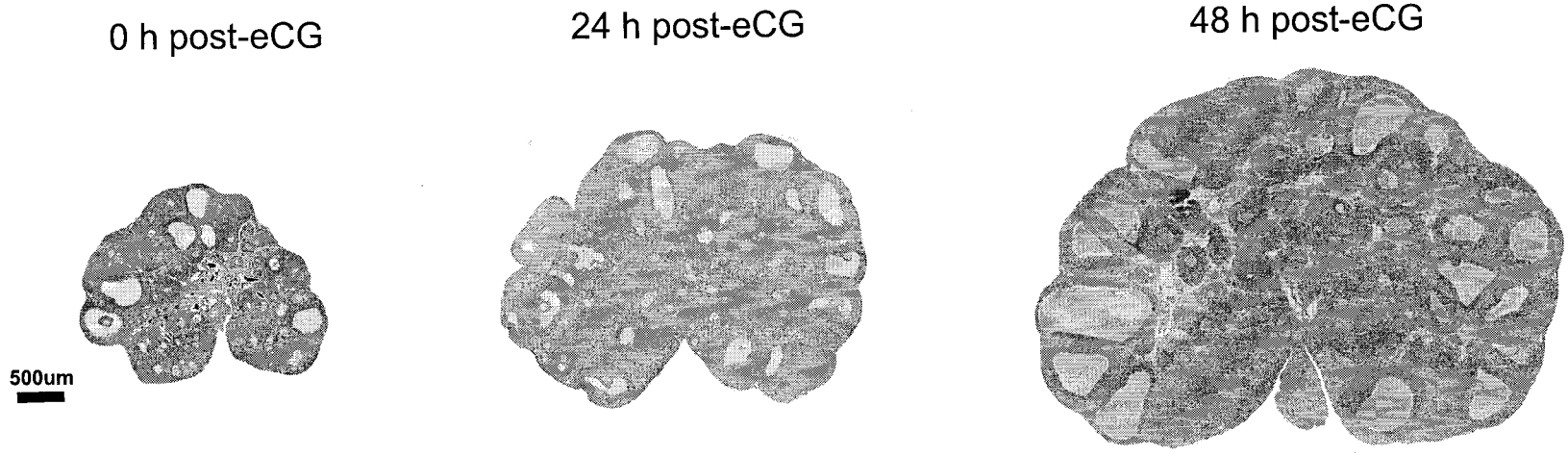
Although previous studies have shown the presence of PAL31 mRNA in the adult rat ovary (Mutai et al., 2000), which ovarian cell type(s) expresses PAL31 is not known. Therefore, our first objective was to immunolocalize PAL31 in the immature rat ovary. The gonadotropin-treated immature rat model has been used extensively to study folliculogenesis without the cyclicity associated with estrous. During the infantile and juvenile periods, endogenous gonadotropins are present in low and moderate levels, respectively. By 19-days of age, the juvenile rat ovary contains well-formed antral follicles, which eventually undergo atresia due to lack of gonadotropic support from the anterior pituitary (Hirshfield, 1991). Since the immature rat ovary contains a relatively homogeneous first wave of follicles that are gonadotropin-responsive, this model has often been used to evaluate the *in vivo* effects of gonadotropins. As such, we were interested in determining whether PAL31 protein is developmentally regulated during follicular growth and whether its expression is modulated by eCG treatment *in vivo*.

In 21- to 24-day immature rats (moderate levels of endogenous gonadotropins), eCG promoted follicular development and caused a significant increase in ovarian weight (Figure 10). PAL31 protein was ubiquitously expressed in the follicle and high immunosignals were present in the oocyte, the granulosa cells and the theca cells (Figure 11). It is worth noting that PAL31 is highly expressed in a small subset of granulosa cells

Figure 10: eCG-induced follicular growth in the immature rat ovary

21- to 24-day old immature rats were treated with 10 IU eCG and sacrificed 24 and 48 h thereafter. **A.** Cross-sections of eCG-induced rat ovary indicating a shift in the predominant follicle population from preantral/early antral (untreated) to antral/preovulatory follicles 48 h post-eCG. Hematoxylin and eosin staining, Bar = 500μm. **B.** Ovarian weights at different periods after eCG injection. Data are the mean ± SEM (N = 5) and were analyzed by one-way ANOVA and the Tukey's multiple comparison post-test to determine differences between time-points. Different letters between columns indicate significant differences ($P<0.01$).

A


0 h post-eCG
24 h post-eCG
48 h post-eCG
500um

B

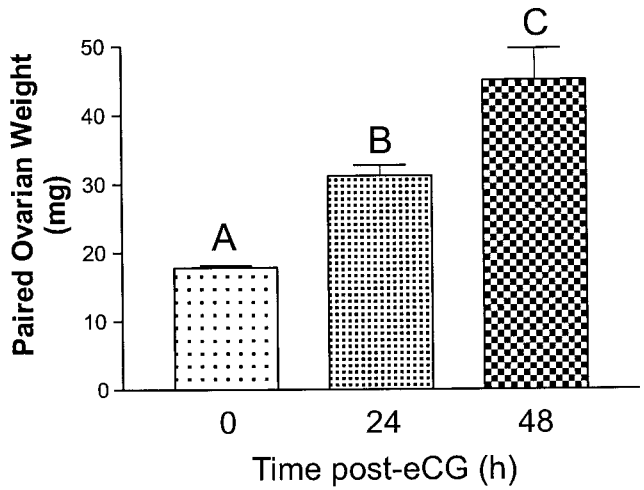
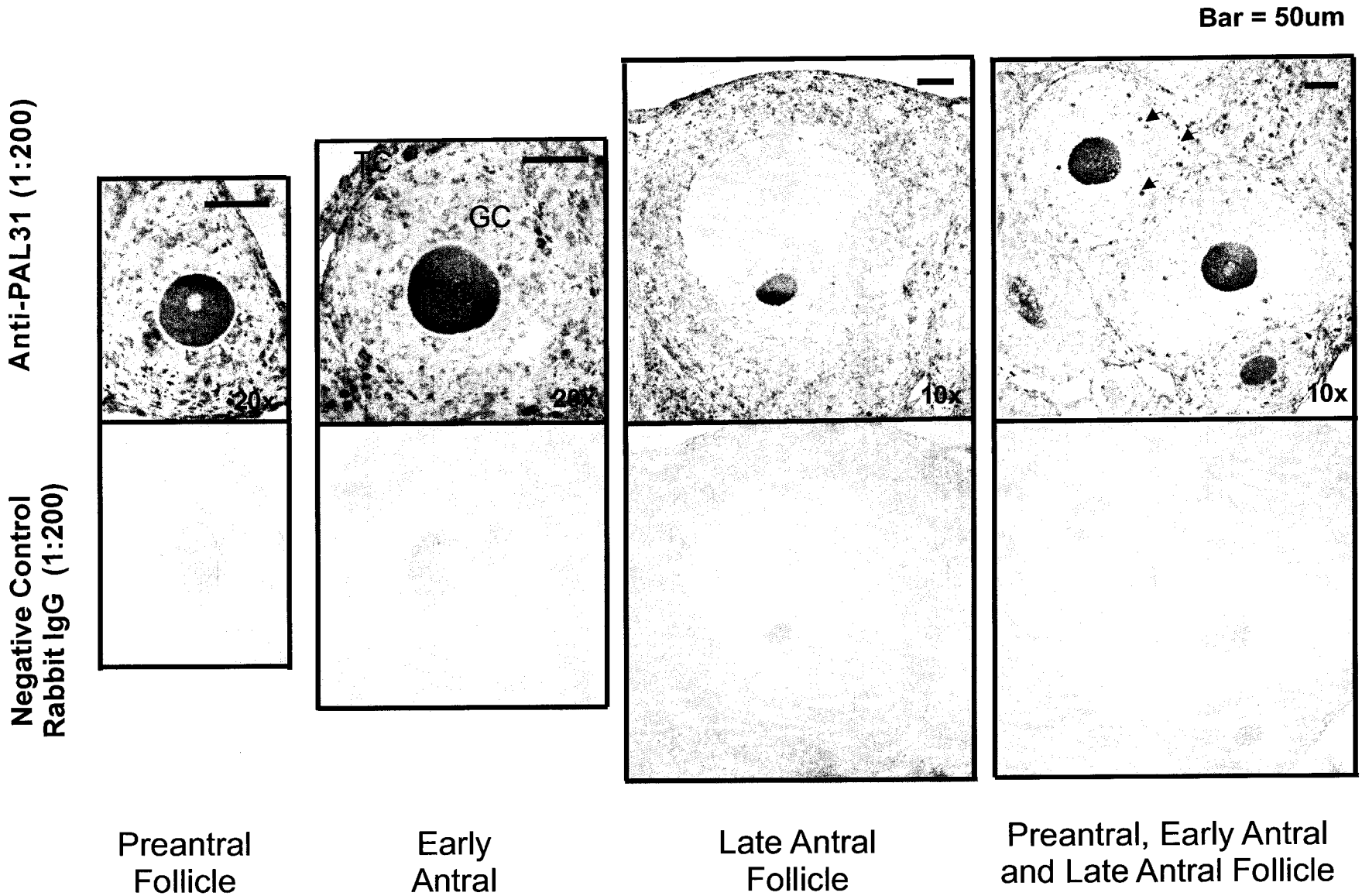

Paired Ovarian Weight (mg)
50
40
30
20
10
0
A
B
C
0
24
48
Time post-eCG (h)

Figure 11: PAL31 protein is ubiquitously expressed in the immature rat ovary

Immunohistochemical analysis of PAL31 expression during follicular growth *in vivo*. Ovaries were excised and fixed in 10 % formalin. Paraffin sections were incubated with rabbit IgG (1:200 dilution; negative control) or rabbit polyclonal anti-PAL31 antibody (1:200 dilution). Bar = 50 μm. TC = theca cells, GC = granulosa cells, OC = oocyte. Arrows indicate immunopositive signal in the follicular cells.


Bar = 50um
Anti-PAL31 (1:200)
Negative Control
Rabbit IgG (1:200)
TC
GC
20x
10x
10x
Preantral
Follicle
Early
Antral
Late Antral
Follicle
Preantral, Early Antral
and Late Antral Follicle

at various stages of follicular development, an observation consistent with the concept that PAL31 is involved in cell cycle progression (at the G_1-S transition) and proliferation and that only a small number of the GCs within an entire follicle are in the S phase of the cell cycle (Hirshfield et al., 1988). High PAL31 expression was also present in the TCs and the oocyte, but the functional role of PAL31 in these cell types is not known.

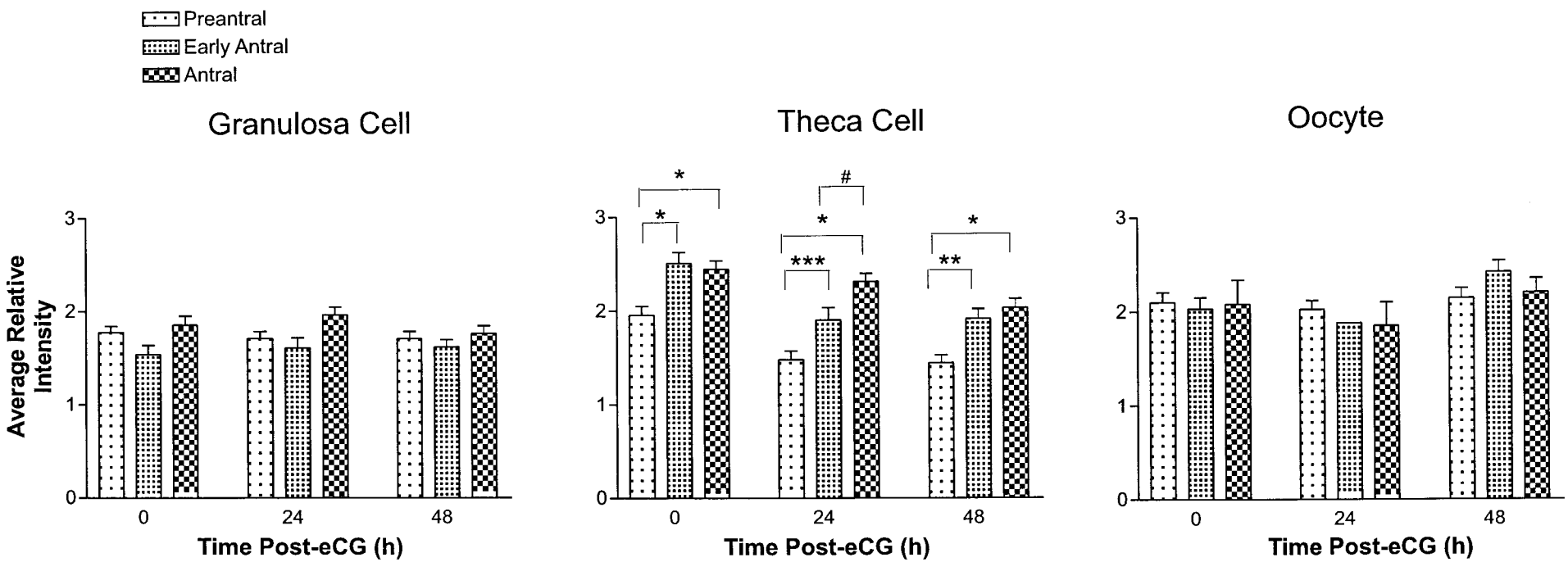
To examine the developmental regulation of PAL31 expression in the follicle, PAL31 immunosignal intensity was qualitatively compared between follicular cell types, stages of follicular development and time after eCG treatment (Figure 12). In untreated rat ovaries, follicular cell PAL31 levels differed ($P<0.05$), although significant effects in follicle stage and treatment time were not observed. Moreover, there was no significant interaction between follicle stage and cell type in terms of relative PAL31 intensity. It is worth noting that theca cell PAL31 expression increased during follicular development ($P<0.05$). Antral follicles expressed higher levels of TC PAL31 than preantral follicles at all time-points after eCG treatment.

3.2. Comparison of PAL31 expression in 14-day and 22-day rat granulosa cells

PAL31 mRNA expression has been detected in neuronal and placental cell lines, and numerous adult rat tissues by Northern analysis (Mutai et al., 2000). In addition, the present studies have demonstrated the presence of PAL31 protein in the rat ovarian follicle. In the infantile period, the ovary contains predominantly preantral follicles, and has a number of early antral and antral follicles by day 19. Studies in infantile and juvenile rats suggest that granulosa cell PAL31 protein contents decreased as the cells

Figure 12: Relative PAL31 immunoreactivity during different stages of follicular development and after eCG treatment

Relative intensity of PAL31 immunoreactivity was assessed on a discrete scale, with 0 and 3 being the lowest and highest, respectively. Data were expressed as a mean ± SEM and were analyzed by ANOVA for: 1) treatment (0-48 h eCG) using all follicle stage-cell type combinations, and 2) all follicle stage-cell type combinations at 0 h eCG with a Tukey Honest Significant Differences post-test to determine individual differences between cell types [249 follicles observed for GCs, 248 follicles observed for TCs and 98 follicles observed for OCs; 4 different ovaries for each treatment time]. Overall cell type effect at 0 h eCG, $P<0.01$. Theca cell observations were subsequently analyzed by a two-way ANOVA with a Bonferonni post-test. Asterisks (*) indicate means significantly different from control values, and # symbols indicate significant differences between selected means. TC PAL31 relative intensity, overall treatment and follicle stage effect ($P<0.001$); *, $P<0.001$; **, $P<0.01$, ***, $P<0.05$; #, $P<0.05$.


Preantral
Early Antral
Antral
Granulosa Cell
Theca Cell
Oocyte
Average Relative Intensity
0
1
2
3
0
24
48
Time Post-eCG (h)
*
#
***
**

undergo differentiation. Based on this developmental change, we were interested in comparing the basal levels of PAL31 mRNA and protein contents during the infantile and juvenile periods, and to determine by real-time RT-PCR and Western blotting whether a similar pattern of expression may be observed in GCs from 14-day and 22-day animals. Granulosa cell PAL31 mRNA abundance was not significantly different between 14-day and 22-day immature rats, although PAL31 mRNA was slightly higher as the rats matured (Figure 13A; $P>0.05$). Granulosa cell PAL31 protein levels were higher in the 22-day animals compared to the 14-day animals (Figure 13B; $P<0.05$).

3.3. Changes in granulosa cell PAL31 content during follicular development *in vivo*

To determine the changes in PAL31 content in granulosa cells during follicular development *in vivo*, granulosa cell lysates from 21- to 24-day old immature rats treated for 24 and 48 h of eCG were analyzed for PAL31 protein contents by Western blotting. Since aromatase is localized to the GCs of pre-ovulatory follicles and its expression is increases during FSH-induced differentiation (Richards et al., 1995), it was used as a marker of GC differentiation in the present studies. Forty-eight hours of eCG treatment caused a significant increase in aromatase content ($P<0.05$; Figure 14A), a decrease in intact PAL31 content ($P<0.05$; Figure 14B) and in the level of the 19 kDa immunoreactive band detected by the PAL31 antibody ($P<0.05$; Figure 14C). These data suggest that granulosa cell PAL31 contents may be decreased as the GCs differentiate during late stage follicular growth.

Figure 13: Comparison of PAL31 mRNA and protein expressions in 14-day and 22-day immature rats

A. Total RNA (1 μg) from the GCs of 14-day and 22-day immature rats were reverse-transcribed and subjected to real-time PCR using the following conditions: 95°C (15 min; denaturation); 95°C (15 s), 56°C (20 s), 72°C (30 s) for 40 cycles; 40°C hold (PAL31, β-actin). Fluorescence data were acquired after the extension step of each cycle, and quantification of mRNA abundance was determined using a standard curve from serial dilutions (10x) of external standards (0.1 μg to $1x10^{-7}$ μg) during the log-linear phase of the PCR. Data were expressed as a relative ratio of PAL31 to β-actin, and as fold of interassay control. **A.** Data are the mean ± SEM of 4 independent experiments and data were analyzed by a Student's t-test ($P>0.05$). **B.** Granulosa cells from 22-day animals were harvested by follicle puncture with a 28 1/2-gauge needle. Large ovarian pieces and ovarian debris were allowed to settle by gravity, and supernatant was collected as the GC preparation. Cells were sonicated and centrifuged, and protein concentration was determined. Total protein between treatment groups were normalized, subjected to SDS-PAGE, electro-transferred onto nitrocellulose membrane and probed with the respective antibodies in the indicated dilution [rabbit polyclonal α-PAL31 (1:1000; provided by K. Shiota, University of Tokyo), mouse monoclonal α-GAPDH (1:30,000 - 40:000; Abcam)]. Data are the mean ± SEM of 4 independent experiments and were analyzed by an unpaired Student's t-test ($P<0.05$).

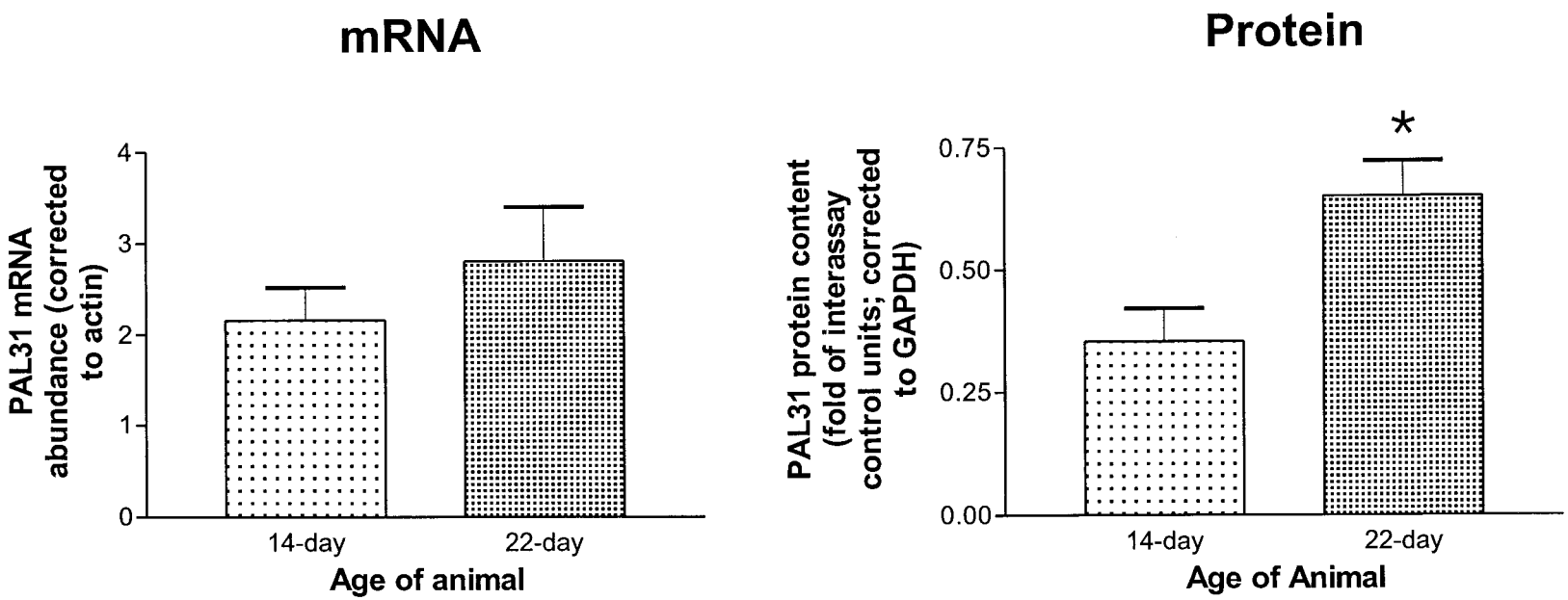
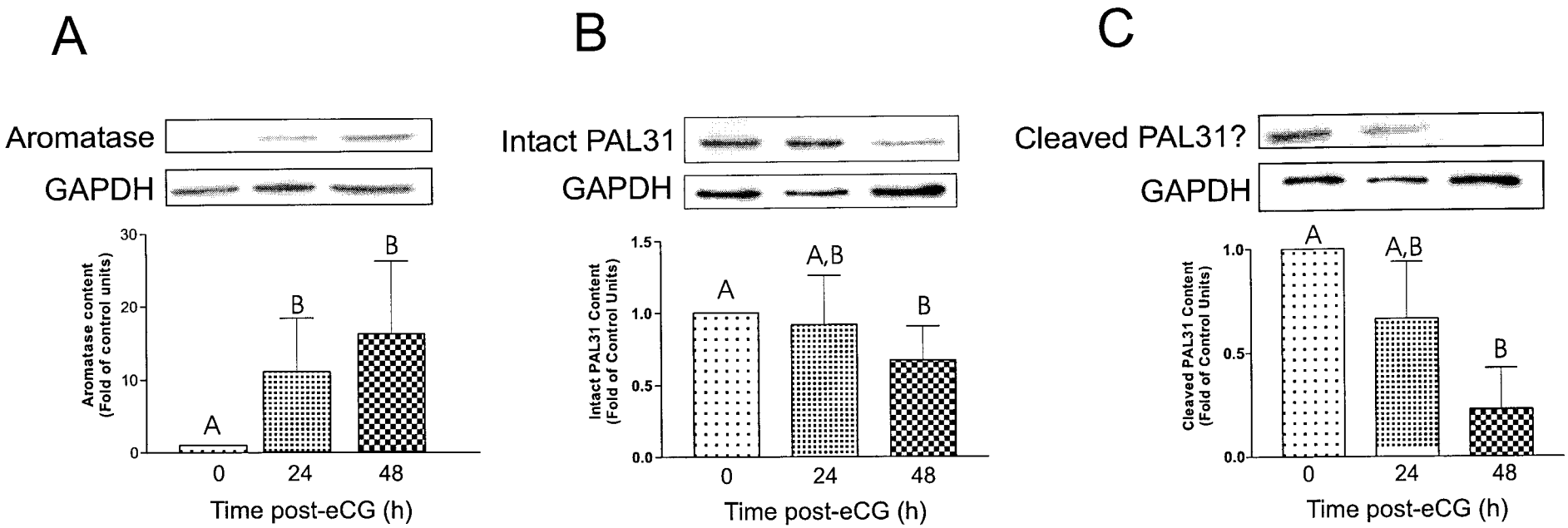

mRNA
PAL31 mRNA abundance (corrected to actin)
4
3
2
1
0
14-day
22-day
Age of animal
Protein
PAL31 protein content (fold of interassay control units; corrected to GAPDH)
*
0.75
0.50
0.25
0.00
14-day
22-day
Age of Animal

Figure 14: eCG causes granulosa cell differentiation and decreased PAL31 content *in vivo*

21- to 24-day old immature rats were treated with 10 IU eCG and sacrificed 24 and 48 h thereafter. Granulosa cells were isolated by follicle puncture and PAL31 proteins were analyzed by Western blotting. Forty-eight hours of eCG treatment caused granulosa cell differentiation, as indicated by increased aromatase protein content **(A),** and decreased levels of intact **(B)** and cleaved PAL31 **(C).** Data are the mean ± SEM of 3-5 independent experiments and were analyzed by a one-way ANOVA and a Tukey's multiple comparison post-test. Aromatase data were logarithmically transformed prior to analysis. Different letters between columns indicates significance ($P<0.05$).


A
Aromatase
GAPDH
Aromatase content (Fold of control units)
30
20
10
0
A
B
B
0
24
48
Time post-eCG (h)
B
Intact PAL31
GAPDH
Intact PAL31 Content (Fold of Control Units)
1.5
1.0
0.5
0.0
A
A,B
B
0
24
48
Time post-eCG (h)
C
Cleaved PAL31?
GAPDH
Cleaved PAL31 Content (Fold of Control Units)
1.0
0.5
0.0
A
A,B
B
0
24
48
Time post-eCG (h)

3.3.1. Determination of oocyte contamination in the granulosa cell preparation

Granulosa cell isolation by follicle puncture is a widely used method and produces a GC preparation that is >90 % pure (Campbell, 1979). Since PAL31 is also expressed in the oocyte and the theca cells, it was possible that the observed changes in PAL31 expression in the GC preparations may be due to contamination by other cell type (s). Theca cell contamination of GCs is negligible during follicle puncture since the TCs are difficult to dissociate from the surrounding interstitium. To determine the extent of oocyte contamination, PAL31 contents in GC preparations from eCG-primed animals were analyzed after removal of oocytes and compared to those without oocytes removed. No significant difference in GC PAL31 content was observed between the two groups at each experimental time-point ($P>0.05$; Figure 15), suggesting that oocyte contamination of the GC preparations was minimal or that PAL31 signal detected in the oocyte by immunohistochemistry is not specific to PAL31.

3.3.2. Dose- and time-dependent effects of eCG on GC PAL31 content *in vivo*

Our next objective was to examine the effect of lower dosages of eCG and shorter treatment durations on GC PAL31 content *in vivo*. As shown in Figure 16, 10 IU eCG caused a slight but a non-significant decrease in PAL31 content 36 h post-eCG ($P>0.05$). A significant treatment effect was detected across all time-points, suggesting that mean PAL31 levels are different between treatment groups ($P<0.05$), but individual differences fail to reach significance due to high levels of standard error between replicates.

Figure 15: Oocyte contamination did not account for the differences in granulosa cell PAL31 protein contents after eCG treatment

21- to 24-day rats injected with 10 IU eCG and sacrificed 24 and 48 h thereafter. The granulosa cell preparation were analyzed after removal of oocytes with a glass pipette *(Without Oocytes)* and compared to those without oocytes removed (*With Oocytes).* Cells were lysed and total protein was analyzed by Western blotting. There was no significant difference in GC PAL31 content between the two groups. Data are the mean ± SEM from five independent experiments and were analyzed by two-way ANOVA and a Bonferonni post-test ($P>0.05$).

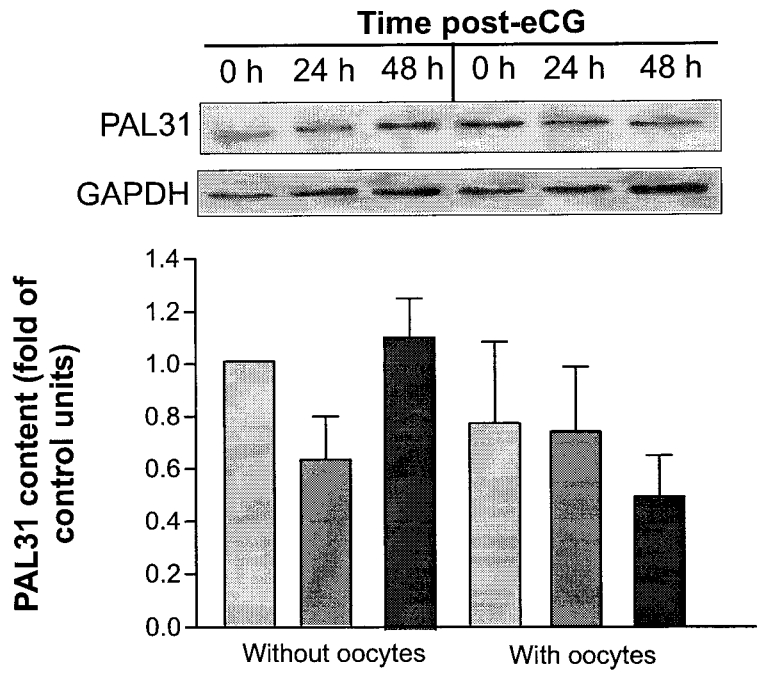
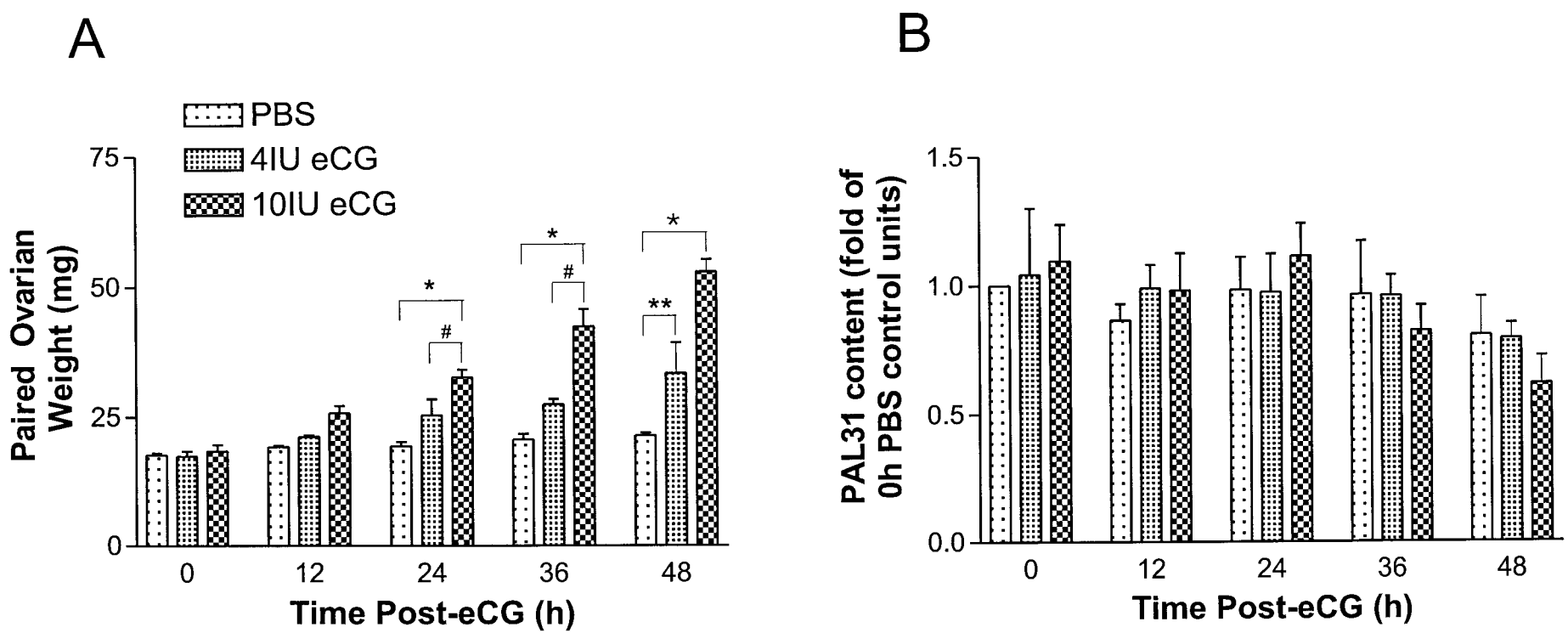

Time post-eCG
0 h
24 h
48 h
0 h
24 h
48 h
PAL31
GAPDH
PAL31 content (fold of control units)
1.4
1.2
1.0
0.8
0.6
0.4
0.2
0.0
Without oocytes
With oocytes

Figure 16: Effect of lower eCG dosage (4 IU) and shorter treatment durations on intact PAL31 content *in vivo*

21- to 24-day animals were injected with PBS, 4 IU or 10 IU eCG and sacrificed 12, 24, 36 and 48 h thereafter. Ovaries were excised, weighed and GCs were harvested by follicle puncture with a 28 1/2-gauge needle. Large ovarian pieces and ovarian debris were allowed to settle by gravity, and supernatant was collected as the GC preparation. Total protein between treatment groups were normalized, subjected to SDS-PAGE, electro-transferred to nitrocellulose membrane and probed with the respective antibodies [rabbit polyclonal α-PAL31 (1:1000), mouse monoclonal α-GAPDH (1:30,000-40:000; Abcam)]. Data are the mean ± SEM from 4 (control) to 11 (treatments) independent experiments and were analyzed by two-way ANOVA and a Bonferonni post-test. Asterisks (*) indicate means significantly different from control values, and # symbols indicate significant differences between selected means. **A.** Paired ovarian weight, $P<0.001$ (overall treatment effect); *, $P<0.001$; **, $P<0.01$; #, $P<0.001$. **B.** PAL31 content: $P<0.05$ (overall treatment effect).


A
PBS
4IU eCG
10IU eCG
Paired Ovarian Weight (mg)
75
50
25
0
0
12
24
36
48
Time Post-eCG (h)
*
#
*
#
*
**
B
PAL31 content (fold of 0h PBS control units)
1.5
1.0
0.5
0.0
0
12
24
36
48
Time Post-eCG (h)

Granulosa cell PAL31 content was not significantly affected by 4 IU eCG at all time-points examined ($P>0.05$). In addition, there were also no observable differences in GC PAL31 content in the control animals over the course of treatment, indicating that endogenous gonadotropins had minimal effect on PAL31 contents at 21- to 24-days of age.

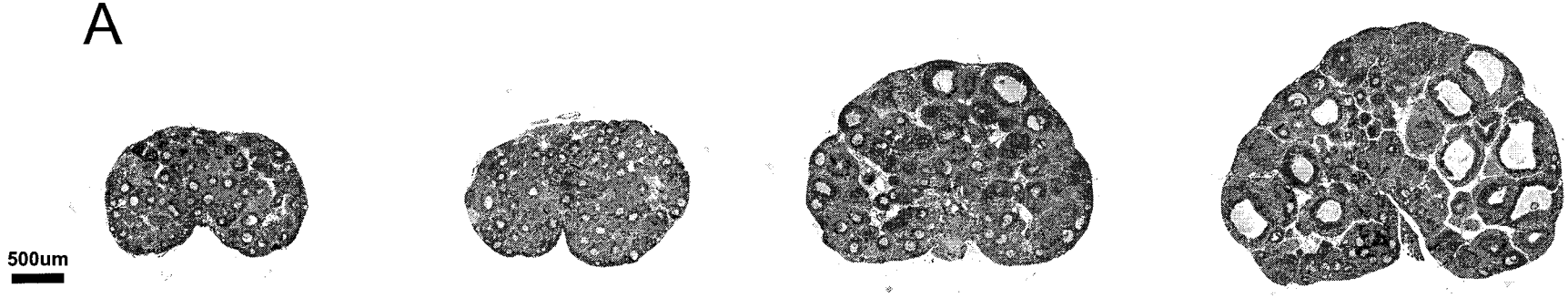
3.3.3. Effect of eCG on GC PAL31 content in infantile rats *in vivo*

To reduce the influence of endogenous gonadotropins on PAL31 expression during follicular development and to examine PAL31 expression during early stage follicular development, younger infantile animals (13-19 days) were treated with eCG, and GC lysates were examined by Western blotting. Forty-eight hours of eCG treatment promoted follicular growth and increased ovarian weight (Figure 17; $P<0.05$). These responses were associated with a slight but non-significant decrease in PAL31 (Figure 18A; $P>0.05$), and decreases in the proliferation markers, PCNA (Figure 18B; $P<0.05$) and Cyclin D2 (Figure 18C; $P<0.05$). Granulosa cell status was further examined by probing membranes with P450 aromatase antibody but after multiple attempts, the Serotec antibody was unable to detect aromatase protein in these samples *(data not shown)*, and the precise reason for the lack of aromatase in infantile GCs is not known. Interestingly, there was a marginal increase in PAL31 content in the GCs of control animals over time (Figure 18), however the physiological significance of this phenomenon is not known.

Figure 17: Effect of eCG on follicular development in infantile rats *in vivo*

13- to 19-day infantile rats were injected with 0.2 IU eCG/g body weight and sacrificed 48 h thereafter. **A.** Follicular development in the infantile rat ovary. Sections were subjected to hematoxylin and eosin staining to illustrate ovarian morphology. Bar = 500 μm. **B.** Ovarian weight increase after eCG treatment. Asterisks (*) indicate means significantly different from control values: overall treatment and time effect, $P<0.001$; *, $P<0.01$; **, $P<0.001$ ($N = 8$).

A


500um
13-day eCG
15-day eCG
17-day eCG
19-day eCG

B

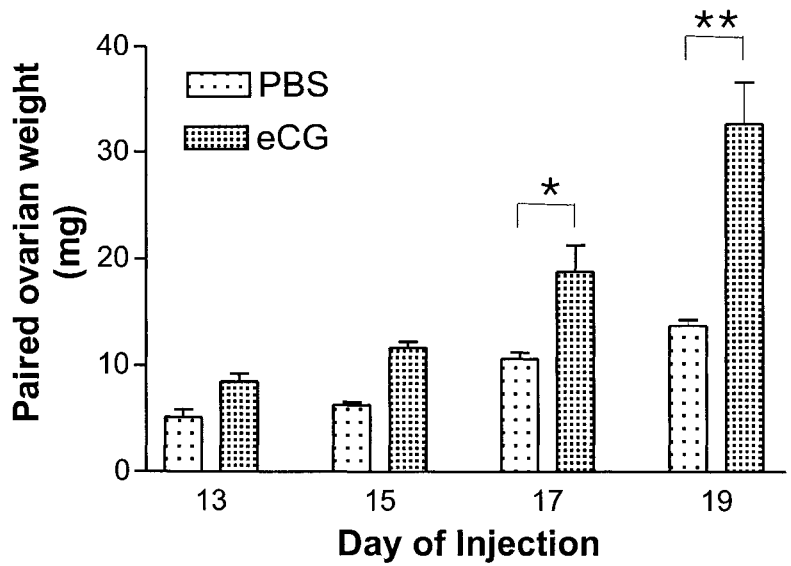
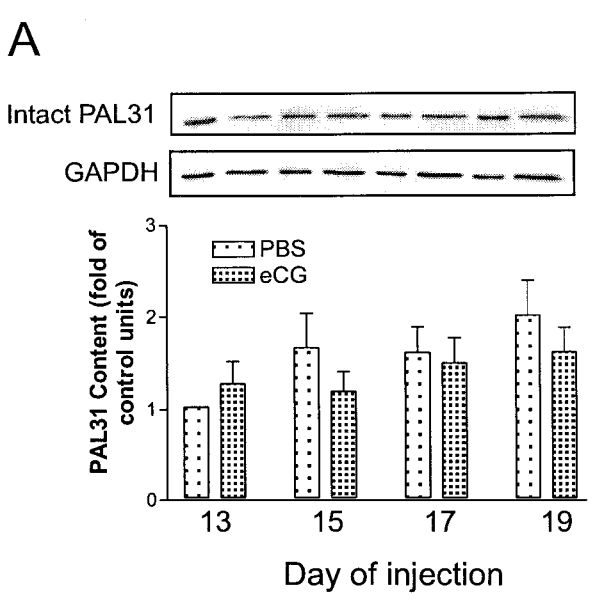
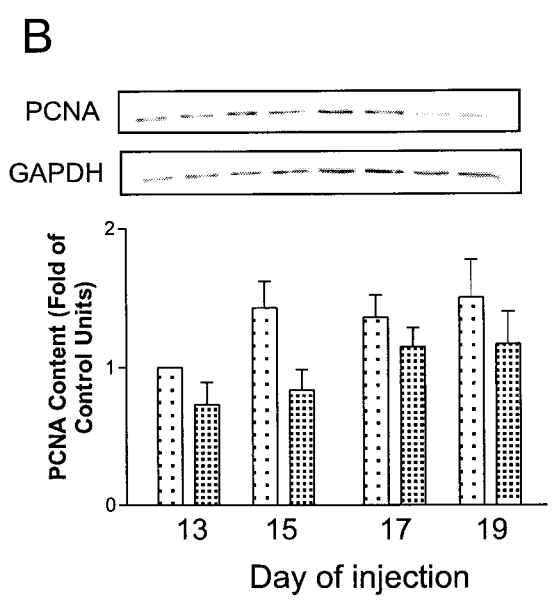

Paired ovarian weight (mg)
40
30
20
10
0
PBS
eCG
*
**
13
15
17
19
Day of Injection

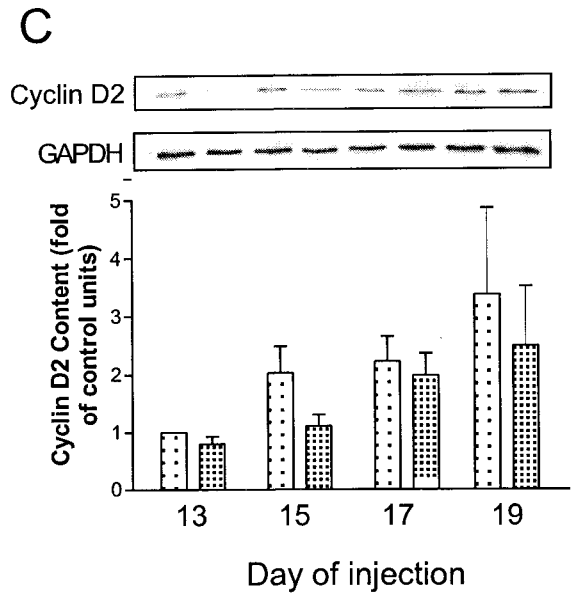
Figure 18: Effect of eCG on granulosa cell PAL31, PCNA and cyclin D2 contents in infantile rats

13- to 19-day infantile rats were injected with 0.2 IU eCG/g body weight and sacrificed 48 h thereafter. Ovaries were excised and weighed, and GCs were harvested by follicle puncture with a 28 1/2-gauge needle. Large ovarian pieces and ovarian debris were allowed to settle by gravity, and supernatant was collected as the GC preparation. Cells were sonicated and centrifuged, and protein concentration was determined. Total protein between treatment groups were normalized, subjected to SDS-PAGE, electro-transferred onto nitrocellulose membrane and probed with the respective antibodies with the indicated dilution [rabbit polyclonal α-PAL31 (1:1000), mouse monoclonal α-GAPDH (1:30,000-40:000; Abcam), mouse monoclonal α-PCNA (1:1000; Santa Cruz Biotechnology) and rabbit polyclonal α-cyclin D2 (1:200; Santa Cruz Biotechnology)].

Data are the mean ± SEM from 8 independent experiments and were analyzed by two-way ANOVA and a Bonferonni post-test. **A.** PAL31 contents: $P>0.05$. **B. & C.** Proliferation marker data were log transformed prior to analysis. PCNA: treatment and time effects ($P<0.05$). Cyclin D2: treatment and time effects ($P<0.05$).


A
Intact PAL31
GAPDH
PAL31 Content (fold of control units)
PBS
eCG
0
1
2
3
13
15
17
19
Day of injection


B
PCNA
GAPDH
PCNA Content (Fold of Control Units)
0
1
2
13
15
17
19
Day of injection


C
Cyclin D2
GAPDH
Cyclin D2 Content (fold of control units)
0
1
2
3
4
5
13
15
17
19
Day of injection

3.4. Effect of gonadotropin withdrawal on PAL31 cleavage *in vivo*

Previous studies in our laboratory have demonstrated that gonadotropin withdrawal *in vivo* using a neutralizing anti-eCG antiserum injection after eCG treatment rapidly induces GC apoptosis and follicular atresia in small- to medium-sized follicles (Boone et al., 1997a). More recently, studies in neuronal cell lines suggest that PAL31 is a substrate for caspase-3 *in vitro* (Sun et al., 2005). Therefore, our next objectives were to further examine the regulation of PAL31 by gonadotropins and to determine whether PAL31 is cleaved by caspase-3 during induction of GC apoptosis *in vivo*. 21- to 24-day old eCG-primed immature rats were treated with increasing concentrations of anti-eCG antiserum (or rabbit IgG control) for 24 h, and GC lysates were analyzed by Western analysis. Gonadotropin withdrawal prevented the increase in ovarian weight due to eCG treatment as previously reported (Figure 19A)(Boone et al., 1997a), but failed to induce caspase-3 activation [as indicated by an increase in cleaved caspase-3 (Figure 19B), and no significant effect on intact PAL31 (31 kDa; Figure 19C) and the 19 kDa band detected by the PAL31 antibody (Figure 19D)]. These results suggest that gonadotropin withdrawal hindered follicular growth, but not to the extent of caspase-3 activation, and these events were not associated with PAL31 processing.

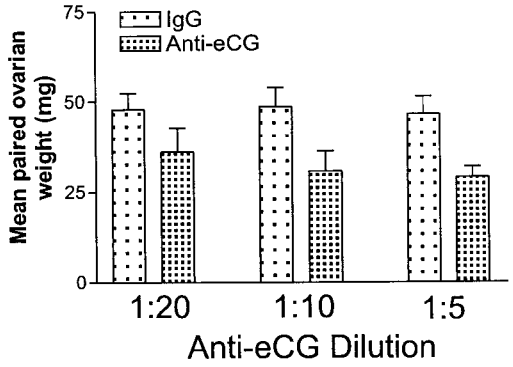
3.5. Effect of FSH on granulosa cell PAL31 content *in vitro*

To further investigate the regulation of GC PAL31 content by FSH and to exclude the possible confounding effects of oocyte- and theca-secreted factors observed *in vivo*, the present studies were extended to a well-established GC culture model. Granulosa

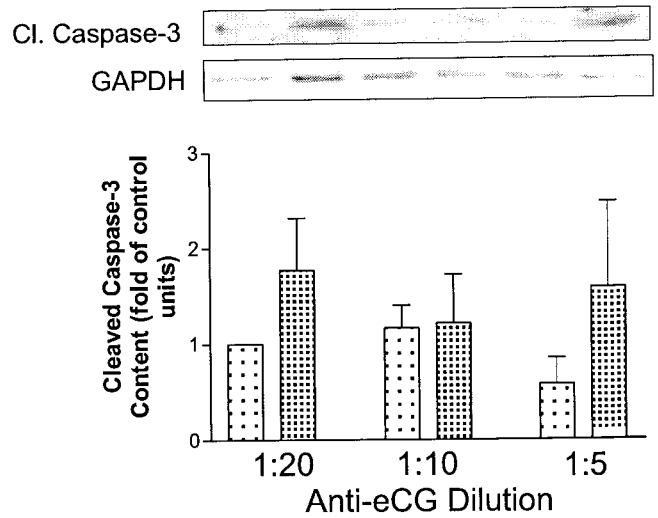
Figure 19: Effect of gonadotropin withdrawal on GC PAL31 contents *in vivo*.

21- to 24-day old rats were treated with 10 IU eCG for 24 h, and increasing dilutions of anti-eCG antibody or control rabbit IgG for 24 h thereafter. Ovaries were excised and weighed, and GCs were harvested by follicle puncture with a 28 1/2-gauge needle. Large ovarian pieces and ovarian debris were allowed to settle by gravity, and supernatant was collected as the GC preparation. Cells were sonicated and centrifuged, and protein concentration was determined. Total protein between treatment groups were normalized, subjected to SDS-PAGE, electro-transferred onto nitrocellulose membrane and probed with the respective antibodies in the indicated dilution [rabbit polyclonal α-PAL31 (1:1000; provided by K. Shiota, University of Tokyo), mouse monoclonal α-GAPDH (1:30,000-40:000; Abcam), rabbit polyclonal α-caspase-3 (1:2000; BD Pharmingen)]. PAL31 and caspase-3 cleavage products were quantitated by lengthening exposure of the x-ray film (densitometric units). **A.** Data are the mean ± SEM from 5 independent experiments and were analyzed by two-way ANOVA and a Bonferonni post-test. Treatment effect, $P<0.05$. **B & C.** Data are the mean ± SEM from 3-6 independent experiments ($P>0.05$). **D.** Only 2 of 8 experiments produced a cleavage band at 19 kDa that was quantifiable. Data for each replicate were obtained by quantification of the 19 kDa immunoreactive band, correction to GAPDH and expression as fold of 1:20 IgG. Due to high variation between the two replicates, the final values have been expressed as individual experimental values rather than a mean ± SD.

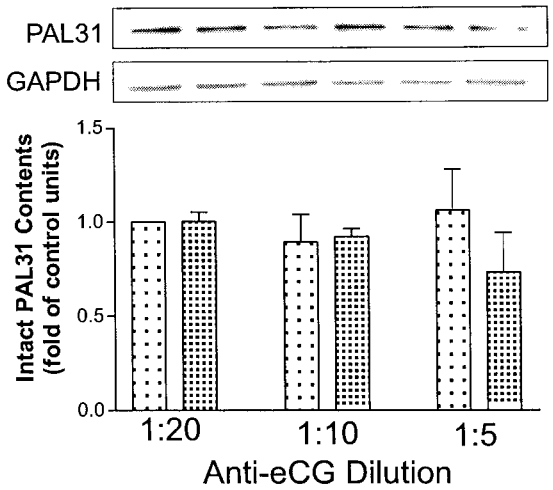
A



B



C



D

Raw Values Cleaved PAL31?

Experiment 1	**IgG**	**Anti-eCG**
1:20	1.000	0.255
1:10	0.878	0.518
1:5	0.711	0.291
Experiment 2	**IgG**	**Anti-eCG**
1:20	1.000	49.997
1:10	29.559	28.275
1:5	2.714	13.629

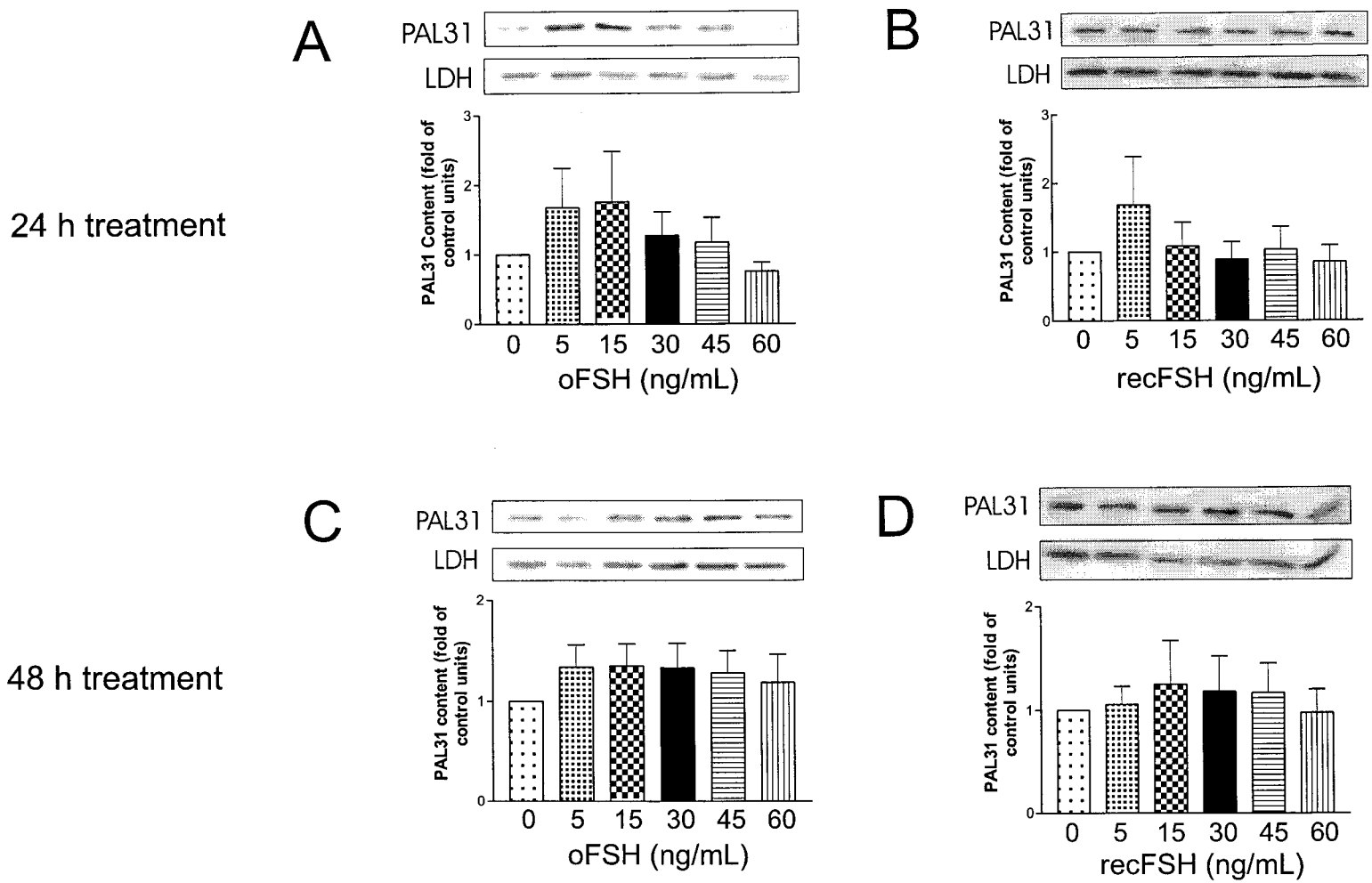
cells from DES-primed immature rats were cultured for various durations in the presence or absence of FSH, and PAL31 protein content was assessed by Western analysis. A preliminary concentration-response study (0 - 150 ng/mL) demonstrated that ovine FSH (oFSH; 30 ng/mL) increases PAL31 content, an effect that plateaus at higher concentrations (*data not shown*). Therefore, lower concentrations of oFSH (0 - 60 ng/mL) were used in subsequent GC culture experiments. After 24 h of treatment, lower concentrations of oFSH (5, 15 ng/mL) caused a slight but non-significant increase in intact PAL31 content whereas higher concentrations (30 - 60 ng/mL) were ineffective (Figure 20A). Similarly, human recombinant FSH (recFSH; 5 - 60 ng/ml) had a negligible effect in GC PAL31 (Figure 20B). Longer treatment duration (48 h) with either of the FSH preparations was also ineffective (Figures 20C, D).

3.6. Effect of estradiol on granulosa cell PAL31 content *in vivo*

FSH-stimulated folliculogenesis is driven, in part, by the action of estrogens, which are potent mitogens in rat GCs *in vivo* (Britt et al., 2002; Robker et al., 1998a). As such, it was hypothesized that treatment with E_2 may increase PAL31 expression. Analysis of the predicted PAL31 promoter region (Prestridge, 1995) failed to demonstrate the presence of a classical estrogen responsive element (ERE) sequence (GGTCAxxxTGACC) (Bajic et al., 2003). Therefore, regulation of the PAL31 gene is likely not regulated through a classical ERE-dependent pathway. However, since the PAL31 promoter region contains AP-2 and Sp-1 responsive elements (Prestridge, 1995), it is possible that GC PAL31 may be regulated by estrogens in an ERE-independent

Figure 20: Lack of FSH effect on granulosa cell PAL31 content *in vitro*

21- to 24-day old rats were treated with 1 mg/day diethylstilbestrol (DES; 3 days) to synchronize ovarian follicular development to the preantral stage and sacrificed 24h after the last injection. Ovaries were excised and GCs were harvested by follicle puncture with a 28 1/2-gauge needle. Large ovarian pieces and ovarian debris were allowed to settle by gravity, and supernatant was collected as the GC preparation. Oocytes were removed by aspiration with a glass pipette. Viable cells were then counted by Trypan blue exclusion with a hemocytometer and plated in 6-well plates at 40 % confluence overnight (18-24 h) in serum-containing RPMI-1640. Media was removed, wells were washed 2x in sterile PBS, and cells were treated with increasing concentrations of ovine FSH **(A, C)** or human recombinant FSH **(B, D)** for 24 and 48 h. Cells were harvested by trypsin-EDTA treatment (37°C, 3-5 min) and GC lysate was used for Western blotting. Total protein between treatment groups were normalized, subjected to SDS-PAGE, electro-transferred onto nitrocellulose membrane and probed with the respective antibodies in the indicated dilution [rabbit polyclonal α-PAL31 (1:1000; provided by K. Shiota, University of Tokyo), mouse monoclonal α-lactate dehydrogenase (LDH; 1:500; Abcam)]. In this experiment, LDH was used as a loading control since GAPDH was not constant under these culture conditions. Data are the mean ± SEM of 7 independent experiments for oFSH and of 3 independent experiments for recFSH, and were analyzed by one-way ANOVA and a Tukey's multiple comparison post-test ($P>0.05$).


A
PAL31
LDH
PAL31 Content (fold of control units)
0 5 15 30 45 60
oFSH (ng/mL)
B
PAL31
LDH
PAL31 Content (fold of control units)
0 5 15 30 45 60
recFSH (ng/mL)
24 h treatment
C
PAL31
LDH
PAL31 content (fold of control units)
0 5 15 30 45 60
oFSH (ng/mL)
D
PAL31
LDH
PAL31 content (fold of control units)
0 5 15 30 45 60
recFSH (ng/mL)
48 h treatment

fashion. Since estrogen stimulates cyclin D2 expression(Robker et al., 1998b), it was used in the present studies as a marker of estrogen responsiveness in the granulosa cells.

It is well established that chronic estradiol treatment inhibits FSH secretion from the anterior pituitary and hinders the development of smaller follicles. Therefore, the 48 h estrogen-treated ovary contains fewer antral follicles and a larger subset of smaller follicles that contain proliferating granulosa cells (Figure 21). To determine whether estradiol modulates GC PAL31 protein content *in vivo,* 21- to 24-day immature rats were treated with estradiol benzoate, and sacrificed 24 and 48 h thereafter. PAL31 and cyclin D2 contents in GC lysates were subjected to Western analysis. Forty-eight hours of estradiol treatment had no effect on GC PAL31 content (Figure 22A), despite an increase in cyclin D2 level (Figure 22B). To exclude the possibility that the vehicle alone might influence PAL31 expression, subsequent replicates included a corn oil control. Granulosa cell PAL31 content in the estrogen-treated group was increased compared to the corn oil control (Figure 22C; $P<0.05$).

Figure 21: Effect of estradiol on follicular growth *in vivo*

21 to 24-day animals were injected with estradiol benzoate (3 mg/day, s.c) and sacrificed 24 and 48 h thereafter. Ovaries were excised and weighed, and ovarian sections were stained with hematoxylin and eosin to facilitate the assessment of ovarian morphology. Bar = 500 μm.

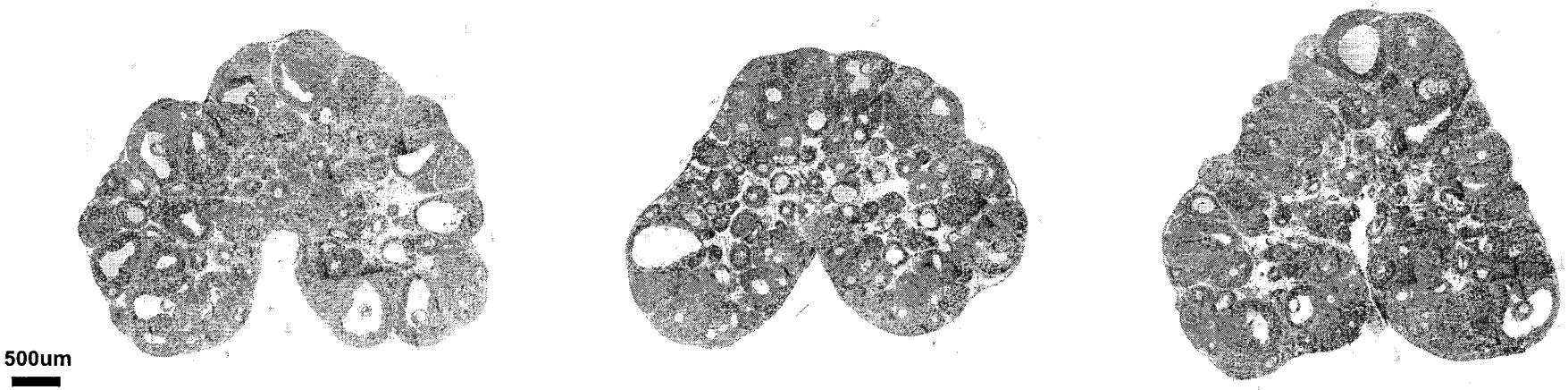
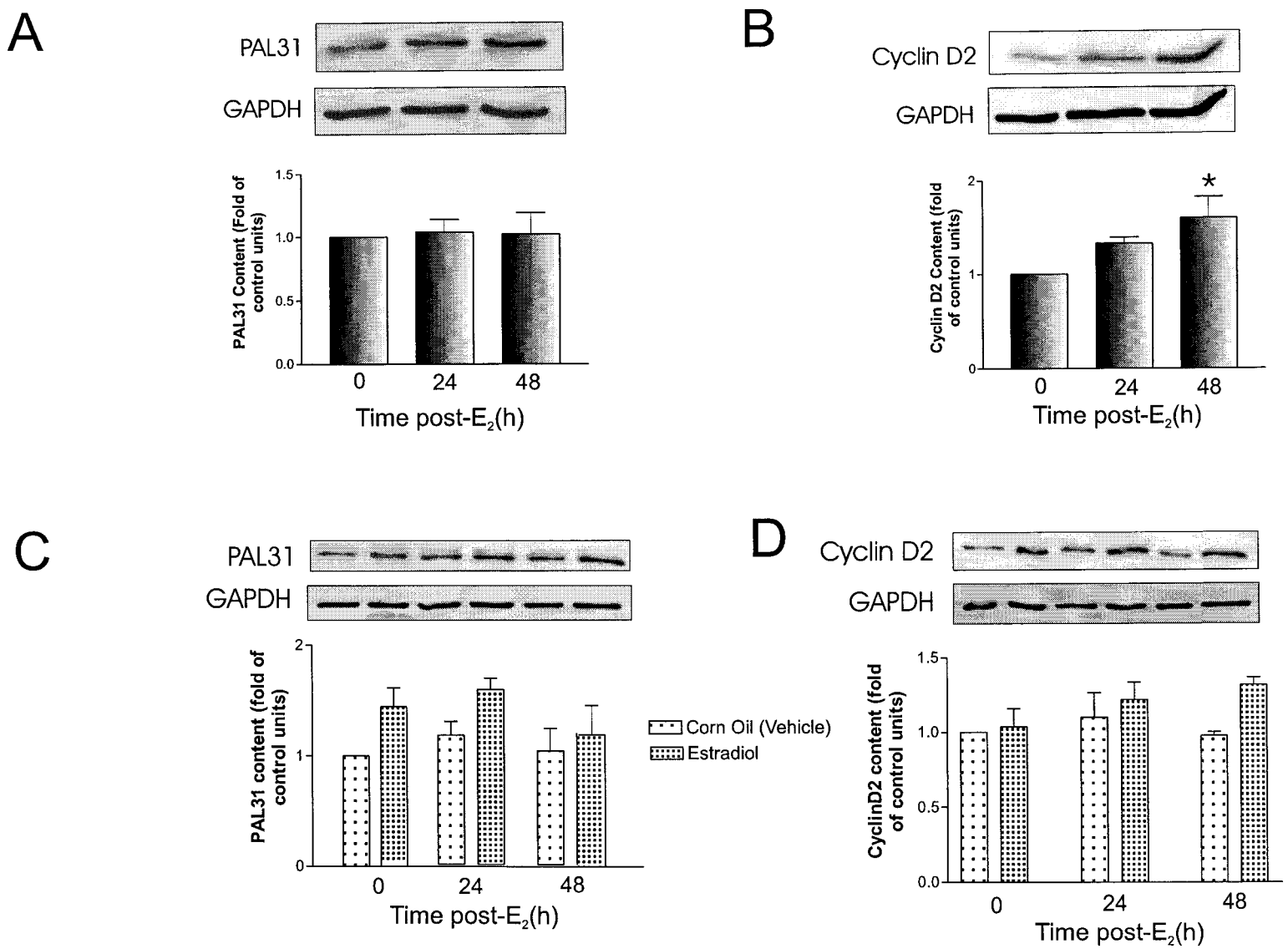

500um
0 h Estradiol
24 h Estradiol
48 h Estradiol

Figure 22: Effect of estradiol on GC PAL31 content *in vivo*

21 to 24-day animals were injected with 3 mg/day of estradiol benzoate or corn oil vehicle control, and sacrificed 24 and 48 h thereafter. Ovaries were excised and weighed, and GCs were harvested by follicle puncture with a 28 1/2-gauge needle. Large ovarian pieces and ovarian debris were allowed to settle by gravity, and supernatant was collected as the GC preparation. Cells were sonicated and centrifuged, and protein concentration was determined. Total protein between treatment groups were normalized, subjected to SDS-PAGE, electro-transferred onto nitrocellulose membrane and probed with the respective antibodies in the indicated dilution [rabbit polyclonal α-PAL31 (1:1000; provided by K. Shiota, University of Tokyo), mouse monoclonal α-GAPDH (1:30,000-40:000; Abcam)]. Separate gels were run for α-Cyclin D2 (1:400; Santa Cruz) to confirm estrogen responsiveness.

A & B: Data are the mean ± SEM of 5-7 independent experiments and were analyzed by one-way ANOVA and a Tukey's multiple comparison post-test. **A.** $P>0.05$. **B.** Time effect, $P<0.01$. Asterisk represents a significance difference compared to 0 h ($P<0.01$).

C & D: Data are the mean ± SEM of 5 independent experiments and were analyzed by two-way ANOVA and a Bonferonni post-test. Treatment effect, $P<0.05$.


A
PAL31
GAPDH
PAL31 Content (Fold of control units)
1.5
1.0
0.5
0.0
0
24
48
Time post-E2(h)
B
Cyclin D2
GAPDH
Cyclin D2 Content (fold of control units)
2
1
0
*
0
24
48
Time post-E2(h)
C
PAL31
GAPDH
PAL31 content (fold of control units)
2
1
0
0
24
48
Time post-E2(h)
Corn Oil (Vehicle)
Estradiol
D
Cyclin D2
GAPDH
CyclinD2 content (fold of control units)
1.5
1.0
0.5
0.0
0
24
48
Time post-E2(h)

CHAPTER 4: DISCUSSION

With the increase in maternal age, a growing number of couples are forced to conceive through the use of reproductive technologies. A thorough understanding of the events leading up to ovulation and subsequent impact on embryonic development is therefore necessitated. In addition, certain subsets of infertile patients are afflicted by conditions of ovarian dysfunction such as polycystic ovarian syndrome. The pathophysiology of this disease is multi-factorial and is often manifested in the form of endocrine disruption and anovulation. Aberration in the expression and/or regulation of cell survival or cell cycle-related proteins may contribute to abnormal GC proliferation and follicular arrest. An increased understanding of the gonadotropic and intra-ovarian regulation of GC factors such as PAL31 is therefore required. The aim of this thesis was to study the hormonal regulation of granulosa cell PAL31 expression during rat ovarian follicular development and atresia.

Original studies on PAL31 have shown that it is highly expressed in the embryonic brain and adult tissues containing proliferating cells (e.g. spleen, liver, testis, ovary) (Mutai et al., 2000). In rat brain and in mitotic PC12 cells, PAL31 is co-expressed with PCNA, suggesting the involvement of PAL31 during cell cycle progression (Mutai et al., 2000). In proliferating Nb2 cells, PAL31 mRNA and protein expression is highest during the G_1-S transition, and the expression patterns parallel that of cyclin E (Sun et al., 2001). Down-regulation of PAL31 markedly attenuates prolactin-induced Nb2 proliferation and induces G1 arrest, suggesting that PAL31 is required for entry into S phase. In the trophoblast giant cells of the placenta, PAL31 mRNA and protein are

down-regulated after cytodifferentiation (Oda et al., 2001). Interestingly, it has been suggested that PAL31 is anti-apoptotic in its intact form, and upon cleavage by caspase-3, induces apoptosis (Sun et al., 2005).

4.1. PAL31 is ubiquitously expressed in the rat ovarian follicle

During early follicular growth, the granulosa cells of preantral follicles are highly proliferative, while those of late antral follicles undergo a gradual process of differentiation, and express markers of follicle maturation such as the aromatase enzyme. In the pre-ovulatory follicle, the granulosa cell layer acquires functional specializations and becomes two populations of cells, the cumulus GCs and the mural GCs (Peters et al., 1980). The cumulus cells maintain close proximity to the oocyte and remain proliferatively active, while the mural granulosa cells eventually withdraw from cell cycle and become highly differentiated (Peters et al., 1980). In this thesis, we have demonstrated for the first time, that PAL31 is expressed in the immature rat ovary. Immunohistochemical analysis indicates the presence of PAL31 protein in all of the ovarian cell types during follicular development. Uniform PAL31 staining was observed in the oocyte and the theca cells, while the granulosa cells exhibited heterogeneous PAL31 expression. The latter observation is consistent with the notion that only a subset of GCs in the follicle may be at the appropriate stage of the cell cycle (Rao et al., 1991b). Despite the presence of PAL31 in the other cell types, oocyte contamination likely did not account for the differences in granulosa cell PAL31 protein contents after eCG treatment, since PAL31 protein levels in the GC preparations were not significantly

different from those in which the oocytes have been specifically removed, and the theca contamination are believed to be negligible.

In the growing follicle, intercellular communication between the follicular support cells (GCs, TCs) and the oocyte facilitates processes such as oocyte maturation and cumulus expansion (Eppig, 2001). In preantral follicles, the oocyte enters an extensive growth phase, undergoes rapid RNA and protein synthesis, and acquires a zona pellucida (van den et al., 2005). During this period, oocyte-derived growth factors (e.g. GDF-9, BMP-15) are the main regulators of GC function, and this aspect will be discussed in Section 4.5. At the preantral / early antral transition, the oocyte acquires the ability to resume meiosis and after the LH surge, undergoes nuclear and cytoplasmic maturation to become a mature oocyte (Matzuk et al., 2002). The theca layer provides structural integrity for the growing follicle and supplies androgen precursors for GC production of steroid hormones. In the rat, a distinct theca layer is observed as early as the preantral stage and proliferates to a limited extent as the follicle grows (Hirshfield, 1991). The TCs of late antral follicles become differentiated into two layers, the theca interna and the theca externa, and are closely associated with the mural GCs (Peters et al., 1980). The presence of PAL31 in the theca layer and in the oocyte was surprising since these cell types have relatively low or no proliferative capacity compared to the granulosa cells. Although the oocyte is susceptible to non-specific staining during immunohistochemistry, this possibility was excluded by the observation that a blocking peptide was able to eliminate the immuno-signal in the oocyte, thus confirming the specificity of the antibody used. The observed pattern of PAL31 expression in the follicle is consistent with the

expression of PCNA, which is uniformly expressed in the oocytes and GCs of preantral and antral follicles (Oktay et al., 1995). In rat brain and proliferating PC12 neuronal cells, PAL31 was co-expressed with PCNA, suggesting that these two molecules have similar expression patterns during mitosis (Mutai et al., 2000).

To examine the basal levels of PAL31 at different stages of follicle development, PAL31 mRNA abundance and protein content were assessed in granulosa cells at 14-days (preantral follicles) and 22-days of age (early antral/antral follicles). As the rats matured, granulosa cell PAL31 mRNA abundance slightly increased, but failed to reach statistical significance. Similarly, PAL31 protein content increased at 22-days of age and was significantly higher than that of 14-day granulosa cells. Although granulosa cell PAL31 mRNA abundance is not developmentally regulated as the animals mature, the increase in PAL31 protein content at 22-days suggests that the stability of the PAL31 protein increases with follicular developmental stage. During rat trophoblast giant cell differentiation, PAL31 mRNA decreased within a day of cytodifferentiation and was almost undetectable on Day 5 (Oda et al., 2001). In contrast, a decrease in PAL31 protein content was not observed until Day 4 and remained detectable on Day 6, suggesting that PAL31 protein levels are stabilized to a greater extent than the mRNA (Oda et al., 2001). Currently, the precise regulatory mechanisms of PAL31 expression are not well understood, however, these data suggest that PAL31 mRNA abundance and protein content are regulated in a concurrent fashion.

4.2. Possible role for PAL31 in the rat follicle

The role of PAL31 in the ovarian follicle is not known, although, in other systems, it is highly expressed during proliferation and is important for the G_1-S transition of cell cycle (Mutai et al., 2000; Sun et al., 2001). Additional studies suggest that PAL31 may function as an anti-apoptotic protein in its intact form, and may be pro-apoptotic after its cleavage by caspase-3 (Sun et al., 2005). Immunohistochemical analysis suggests that PAL31 is expressed throughout follicular development. During early follicular growth, intra-ovarian regulators and FSH play important roles in the control of GC proliferation and cell survival, and the incidence of GC apoptosis is relatively low compared to the penultimate stage (Peters et al., 1980). To facilitate follicle growth to this critical period, the GCs of preantral and early antral follicles may express high PAL31 to sustain cell survival and promote proliferation. As the follicle grows, there is a close correlation between maturation of GCs and exit from cell cycle. During this process, the GCs gradually acquire FSHR, increase E_2 production and induce LHR expression. It is possible that as the cells eventually exhaust their proliferative potential during differentiation and the mitogenic role of PAL31 ceases, PAL31 expression is attenuated. Precisely how PAL31 expression is down-regulated is not known and awaits further investigation.

Gonadotropin withdrawal at the penultimate stage (by anti-eCG neutralizing antibody) prevented an increase in ovarian weight, but failed to activate caspase-3 and modulate PAL31 contents. The absence of caspase-3 activation after anti-eCG treatment is contradictory to previous work, in which GC apoptosis and follicular atresia were

markedly increased after antibody treatment of eCG-primed rats (Boone et al., 1997a). Although the reason for this is not clear, it is possible that the observed effects on ovarian weight were not associated with GC apoptosis. However, whether this indeed the case, remains to be confirmed. Currently, the putative proliferative and anti-apoptotic actions of PAL31 in the GCs are not known. Moreover, whether PAL31 is more important at the preantral than at the antral (penultimate) stage of development in terms of cell survival, requires further investigation. During the later stages of follicle growth, other mitogens and cell survival factors (e.g. XIAP and phospho-Akt) may influence GC fate/function to a greater extent than does PAL31. Future studies using gene-knockdown strategies such as adenoviral anti-sense PAL31 cDNA *or* siRNA in cultured GCs *in vitro*, would determine whether PAL31 plays definitive roles in GC proliferation or cell survival.

Prior to meiotic arrest, the germ cell is mitotically active and synthesizes DNA during S phase. In the post-natal period, however, the oocyte undergoes meiotic divisions and is a non-proliferative follicular cell type (Peters et al., 1980). In the present studies, we observed PAL31 expression in the oocyte at all stages of follicular development. Although the function of PAL31 in the oocyte is not known, we speculate that high PAL31 in the oocyte confers resistance to apoptotic stimuli, and ensures survival of the follicle during development. Across species, more than 2/3 of the potential female germ cell population is lost through apoptosis by the time of birth (Morita et al., 1999). During the post-natal period, the incidence of oocyte-initiated follicle demise is low, and the majority of atresia occurs at the penultimate stage (rat: 300-400 μm follicle diameter) (Hirshfield, 1991; Morita et al., 1999). This form of

atresia is initiated by the granulosa cells, but spreads to the theca cells and finally to the oocyte (Tilly, 1996). In the dominant follicle, the GCs closest to the oocyte (cumulus cells) remain proliferative active, while the GCs beside the basement membrane (mural GCs) undergo further differentiation. We speculate that the presence of high PAL31 in the oocyte may facilitate cumulus cell proliferation and cell survival, thereby allowing the dominant follicle to reach ovulation. Despite this interesting possibility, the role of PAL31 in the oocyte remains unknown. Down-regulation of PAL31 expression in the oocyte by microinjection of adenoviral anti-sense cDNA *or* siRNA would confirm the role of oocyte PAL31 during follicular growth *in vitro*.

Although the proliferative index of theca cells is less than that of the granulosa cells, TC survival plays an important role in the maintenance of structural integrity and steroidogenesis. The presence of high PAL31 protein in TCs was not expected, since this ovarian cell type undergoes limited proliferation during follicular growth. In the present study, we observed that PAL31 was uniformly expressed in the theca layer, and increased during folliculogenesis. Although the importance of TC PAL31 expression is not known, it is possible that high PAL31 levels may promote theca cell survival. During follicular atresia, the GCs are the first cell type to undergo apoptosis, while theca cell death occurs at a slower rate and may facilitate resorption of the non-viable follicle (Foghi et al., 1998; Palumbo et al., 1994). Based on these findings, we speculate that high PAL31 in the theca layer may facilitate TC survival, thereby maintaining communication with the GCs and ensuring their viability. At the penultimate stage, the GCs of subordinate follicles succumb to the apoptotic insult, and undergo programmed cell death. Meanwhile, the

presence of high PAL31 expression in the theca layer may delay apoptosis of the cells during the initial stages of atresia. Although the role of PAL31 in theca cell survival is not known, future studies involving the down-regulation of PAL31 in theca-interstitial cell cultures or in TCs co-cultured with GCs, may provide some insight into the role of PAL31 in the theca cells and its relevance to follicular growth and steroidogenesis.

The immunohistochemical results in this thesis were obtained through semi-qualitative evaluation of PAL31 immunoreactivity. It should be noted that the epitope for the PAL31 antibody is located within the N-terminal region of the protein (1-171 aa) and hence, recognizes both the intact and cleaved forms of PAL31. Currently, the relative proportion of intact and cleaved PAL31 protein contents in the follicular cell types is not known. Western analyses of GC lysates demonstrated that the intact PAL31 (31 kDa) band was more immunoreactive than the 19 kDa cleavage product, suggesting that the PAL31 antibody strongly detects the 31 kDa species in granulosa cells. At the present time, it is not possible to immunodetect the 31 kDa, 19 kDa or the 12 kDa protein products individually due to the constraints of the antibody.

Quantitation of immuno-signal strength has previously been reported from our laboratory and by others to examine the relative changes of proteins during follicular development and atresia (Bayrak et al., 2003; Li et al., 1998; Thompson et al., 2004). Due to the small sample size, a multivariate analysis of variance (MANOVA) was not possible and data were subsequently analyzed by regular ANOVA using the mean intensities from all follicle stage-cell type combinations. The lack of interaction between

cell type and follicle stage with respect to PAL31 immunoreactivity, may be due to the low number of observations in the present study. It is worth noting that the oocyte sample size was much lower than the number of GC and TC observations. Within a given ovarian section, there are few "ideal" follicle cross-sections and hence, many of the follicles observed do not contain a visible oocyte. Therefore, to examine potential interactions between cell type, follicle stage and treatment, future studies would incorporate a larger sample size and data could be analyzed by three-way ANOVA. In addition, this approach would provide a more accurate mean relative intensity for the oocyte. Due to the difficulty associated with quantitation of immuno-signal, a growing number of laboratories have been expressing data as: 1) % of immunopositive cells relative to total cell number, 2) % of immunopositive cells relative to a positive control standard, or 3) fold change in densitometric units. In future studies, these methods may be more appropriate for evaluation of PAL31 protein content. The results obtained from immunohistochemistry were subsequently extended to include Western analysis for the assessment of the effect of eCG on GC PAL31 contents *in vivo*.

4.3. Granulosa cell PAL31 is down-regulated during cytodifferentiation

During folliculogenesis, FSH induces aromatase in GCs, which in turn, increases estrogen synthesis. However, as the GCs gradually become more differentiated, they lose their proliferative capacity. A number of studies suggest that timing of follicle maturation overlaps that of cell cycle exit, and that GCs exhaust their replicative potential by the 10^{th} generation of cell division (near the penultimate stage) (Hirshfield, 1991). Although the growth capabilities of antral follicles are greater than those of smaller

follicles, it is well established that the overall rate of GC proliferation decreases as the time of ovulation approaches (Hirshfield, 1991). During late stage follicle growth, FSH acts as a permissive signal that facilitates emergence of the dominant follicle. From this point forward, the follicle acquires LHR and upon exposure to LH, the GCs become terminally differentiated; this is accompanied by marked increase in progesterone production and end-stage follicle maturation. Thus, granulosa cell differentiation is a continual process beginning with induction of FSHR and terminating with the luteinization of granulosa cells.

In the present studies, we utilized an established eCG-primed immature rat model to examine the regulation of PAL31 contents during follicle maturation. Equine chorionic gonadotropin significantly decreased GC PAL31 content in 21- to 24-day immature rats 48 h after treatment, a response associated with increased aromatase level *in vivo*. In the infantile rats, however, there was a slight but non-significant decrease in GC PAL31 content after 48 h eCG treatment. During this period, the GCs possess functional FSH receptors and are responsive to eCG (Dunkel et al., 1994), however, whether they have reached the terminal stage of differentiation, is not known. Nonetheless, these observations are consistent with previous studies in the placenta, where PAL31 expression is high in proliferating Rcho-1 stem cells and down-regulated after differentiation into trophoblast giant cells (Oda et al., 2001). In the follicle, FSH is a key regulator of GC differentiation and stimulates induction of LHR in granulosa cells (Richards et al., 1995), thereby facilitating LH-induced terminal differentiation. Indeed, rats pre-treated with a low dose of LH/hCG required lower FSH to induce late stage

follicular development, suggesting that exposure to LH/hCG is an important aspect of follicle maturation (Uilenbroek et al., 1997).

Equine chorionic gonadotropin is a single hormone molecule that possesses FSH and LH-like activities across species (Moore, Jr. et al., 1980; Papkoff et al., 1978a). Although the potency of eCG has been compared to other gonadotropin preparations, the precise biological ratio of FSH to LH in the immature rat ovary, is not known. In the present studies, the observed decrease in PAL31 contents may have been caused by FSH-induced GC differentiation. However, another explanation is that the LH component of eCG caused premature terminal differentiation of granulosa cells. It has been shown that concentrations of eCG similar to those used in the present studies (10-15 IU) stimulated LHR expression and increased LH receptor concentration to a greater extent than that of FSHR (Camp et al., 1991b; Vidyashankar et al., 1984). Previous studies from our laboratory have shown that GCs from eCG-primed rats are more responsive to FSH *in vitro* than the less differentiated ones from DES-primed rats (Karakji et al., 1995a; Karakji et al., 1995b), and that only the former group responds in increased cAMP production to LH *in vitro* (Carnegie et al., 1984). Thus, the ability of FSH to stimulate GC differentiation *in vitro* likely depends on the status of the GCs and the stage of follicle development in which they were isolated. Currently, the precise regulation of granulosa cell PAL31 is not known. However, we speculate that LH causes the granulosa cells to enter terminal differentiation, and inhibits proliferation and PAL31 expression. The relative importance of the FSH and LH components in the eCG preparation in eliciting the observed changes in PAL31 content, has not been investigated. Future

studies involving recombinant FSH treatment of immature rats *in vivo* or treatment of GCs with LH *in vitro* would shed new light on this important question.

4.4. Possible regulation of GC PAL31 expression by estrogens

In the present studies, estradiol induced granulosa cell cyclin D2 expression, and increased PAL31 content *in vivo*. The proliferative effects of estrogen in GCs are partially mediated through cyclin D2, which associates with cyclin-dependent kinases and activates a number of genes involved in the G_1-S transition (Robker et al., 1998b). Although the relationship between estradiol and PAL31 is not clear, a number of studies suggest that FSH, E_2 and intra-ovarian growth factors act synergistically to regulate E_2-mediated GC proliferation at this stage. The lack of classical estrogen response element in the PAL31 promoter suggests an ERE-independent mode of regulation. However, whether this indeed the case, requires further investigation. Interestingly, the PAL31 promoter contains AP2 and Sp1 transcription factor binding sites and thus, PAL31 gene expression may be regulated to a greater extent by protein-protein interactions and phosphorylation rather than direct ERE-DNA binding.

During follicular growth *in vivo*, estrogen is a potent mitogen of rat granulosa cells, however, this response is lost when cells are cultured with estradiol *in vitro* (Dorrington et al., 1988; Drummond et al., 1999), suggesting that other follicular cell type(s) and local growth factors may be involved in E_2-stimulated GC proliferation. Theca-derived transforming growth factor α (TGFα) is an inhibitor of FSH-induced E_2 synthesis and LHR expression in cultured rat GCs (Adashi et al., 1986; Adashi et al.,

1987). Previous studies in our laboratory suggest that TGFα regulates the "switch" from the proliferative to differentiated state in granulosa cells, and potentiates FSH-induced differentiation of GCs in antral follicles (Karakji et al., 1995a). More recently, FSH increased E_2-dependent TGFα secretion from TCs of cultured rat follicles, which in turn, stimulated GC XIAP expression and follicular growth (Wang et al., 2002). Whether theca-derived TGFα is a mediator of estradiol-induced PAL31 content *in vivo* is not known and requires further investigation. Alternatively, a recent report suggests that oocytes play a key role in estrogen amplification of FSH signaling in GCs (Otsuka et al., 2005). Therefore, it is possible that modulation of ER and FSHR pathways by the oocyte indirectly regulates GC PAL31 expression and proliferation. Future experiments using Luciferase-based assays at the PAL31 promoter or immunoprecipitation to examine protein-protein interactions, may provide further insight into the precise mechanism of estrogen-induced PAL31 expression in granulosa cells.

4.5. Possible regulation of GC PAL31 by oocyte-derived growth factors

The present studies demonstrate that eCG treatment decreases granulosa cell PAL31 contents during late stage follicle development. However, early follicular growth can occur independent of gonadotropins, as evidenced by normal preantral and early antral follicle development in FSHR knockout mice (Dierich et al., 1998). Recently, oocyte-derived growth factors such as GDF-9 and BMP-15 have been shown to regulate GC proliferation and cell survival during folliculogenesis. Recombinant GDF-9 stimulates GC proliferation in small antral and preovulatory follicles, and suppresses FSH-induced GC differentiation (Vitt et al., 2000). Studies in our laboratory and others

have shown that GDF-9 also stimulates preantral follicle growth, and this effect is enhanced by FSH (Hayashi et al., 1999; Orisaka et al., 2005). In addition, GDF-9 is an anti-apoptotic factor and attenuates ceramide-induced apoptosis in GCs from early antral follicles (Orisaka et al., 2005). Like GDF-9, BMP-15 stimulates mitosis of GCs from small antral follicles (Otsuka et al., 2000), inhibits FSHR expression (Moore et al., 2003) and is part of a negative feedback loop with KL to negatively regulate KL-stimulated proliferation (Otsuka et al., 2002). Given the reported roles for PAL31 in cell cycle progression and cell survival, it is possible that GDF-9 or BMP-15 positively regulate PAL31 expression during early follicle development, whereas gonadotropins negatively regulate PAL31 during late stage follicle development. Thus, coordinated regulation of PAL31 by oocyte-derived growth factors and gonadotropins may determine the steady-state levels of PAL31 in GCs during follicular growth. However, whether this is indeed the case, requires further investigation.

4.6. Possible involvement of PAL31 during granulosa cell apoptosis and follicular atresia

In Rat1 fibroblast cells, PAL31 acts as anti-apoptotic protein in its intact form and after proteolytic cleavage by caspase-3, becomes pro-apoptotic (Sun et al., 2005). In the present studies, gonadotropin withdrawal had no effect on granulosa cell caspase-3 activation and PAL31 contents. Although the reason for this is not clear, we propose that the anti-apoptotic action of PAL31 may be exerted to a greater extent during early follicle development, whereas at the penultimate stage, cell survival proteins such as XIAP and phospho-Akt may be more important for GC survival. Studies in our laboratory have shown that XIAP protein is higher in antral follicles relative to preantral and early antral

follicles (Li et al., 1998). In addition, XIAP has been shown to regulate the phosphatidylinositol 3-kinase (PI3K)-Akt cell survival pathway, which in turn, stimulates FSH-induced cell survival and follicular growth *in vitro* (Asselin et al., 2001; Wang et al., 2003). As previously mentioned, we speculate that PAL31 promotes the proliferation and continual survival of granulosa cells during preantral follicle development.

The current results indicate that anti-eCG failed to activate caspase-3 and cause PAL31 cleavage in granulosa cells *in vivo*. Although the basis of this phenomenon remains unknown, it is worth noting that the effect of the anti-eCG antibody preparation on ovarian weight was marginal although significant. This is only partially consistent with previous studies, whereby anti-eCG treatment prevented the gain in ovarian weight observed between 24 and 48 h after eCG injection, and induced GC apoptosis (Boone et al., 1997a). The subdued biological effect of the anti-eCG suggests that the antibody may have lost some of its activity over time. However, whether this is indeed the case, remains to be investigated.

Regulation of caspase-3 substrate recognition is multi-factorial, and is influenced by the three-dimensional conformation of the protein. In addition, the accessibility of the cleavage site to caspase-3 and/or presence of phosphorylation near the P1 and P4 positions of the recognition motif, may influence caspase-3 substrate recognition (Fischer et al., 2003; Tozser et al., 2003). Currently, a number of questions pertaining to the role of PAL31 in GCs remain unanswered. Whether granulosa cell PAL31 is indeed a physiological substrate for caspase-3 and whether caspase-3 and PAL31 co-localize in

the cellular milieu, is not known. Although the 19 kDa band detected in the present studies is consistent with that reported previously (Sun et al., 2005), whether this is indeed a cleavage fragment of the intact PAL31 protein requires confirmation. Future studies involving the incubation of recombinant PAL31 protein with active caspase-3 *in vitro* in the presence or absence of DEVD-CHO (a specific caspase-3 inhibitor) would confirm this possibility. Other experiments of interest would include: 1) confirmation of apoptosis in the GCs of eCG-primed rats treated with the anti-eCG antibody [DNA ladder, Hoechst staining or terminal deoxynucleotidyl transferase-mediated dUTP nick end labeling (TUNEL)], 2) examination of PAL31 and active caspase-3 localization during GC apoptosis by immunocytochemistry, and 3) over-expression of N-terminal (19 kDa) and C-terminal (12 kDa) PAL31 cleavage fragments in cultured GCs to examine the role(s) of the PAL31 cleavage products during apoptosis.

4.7. Conclusion and future research directions

In this thesis, the expression and regulation of ovarian PAL31 were investigated using established immature rat models and a granulosa cell culture system. We have demonstrated for the first time that PAL31 is expressed in all of the follicular cell types, and is dramatically down-regulated as the granulosa cells undergo late stage differentiation / terminal differentiation. These findings are in agreement with previous reports in the placenta, where PAL31 protein content was decreased during rat trophoblast giant cell differentiation (Oda et al., 2001). Unfortunately, the same phenomenon was not observed in granulosa cells treated with purified FSH *in vitro*, suggesting that the LH component of eCG may have induced terminal differentiation or

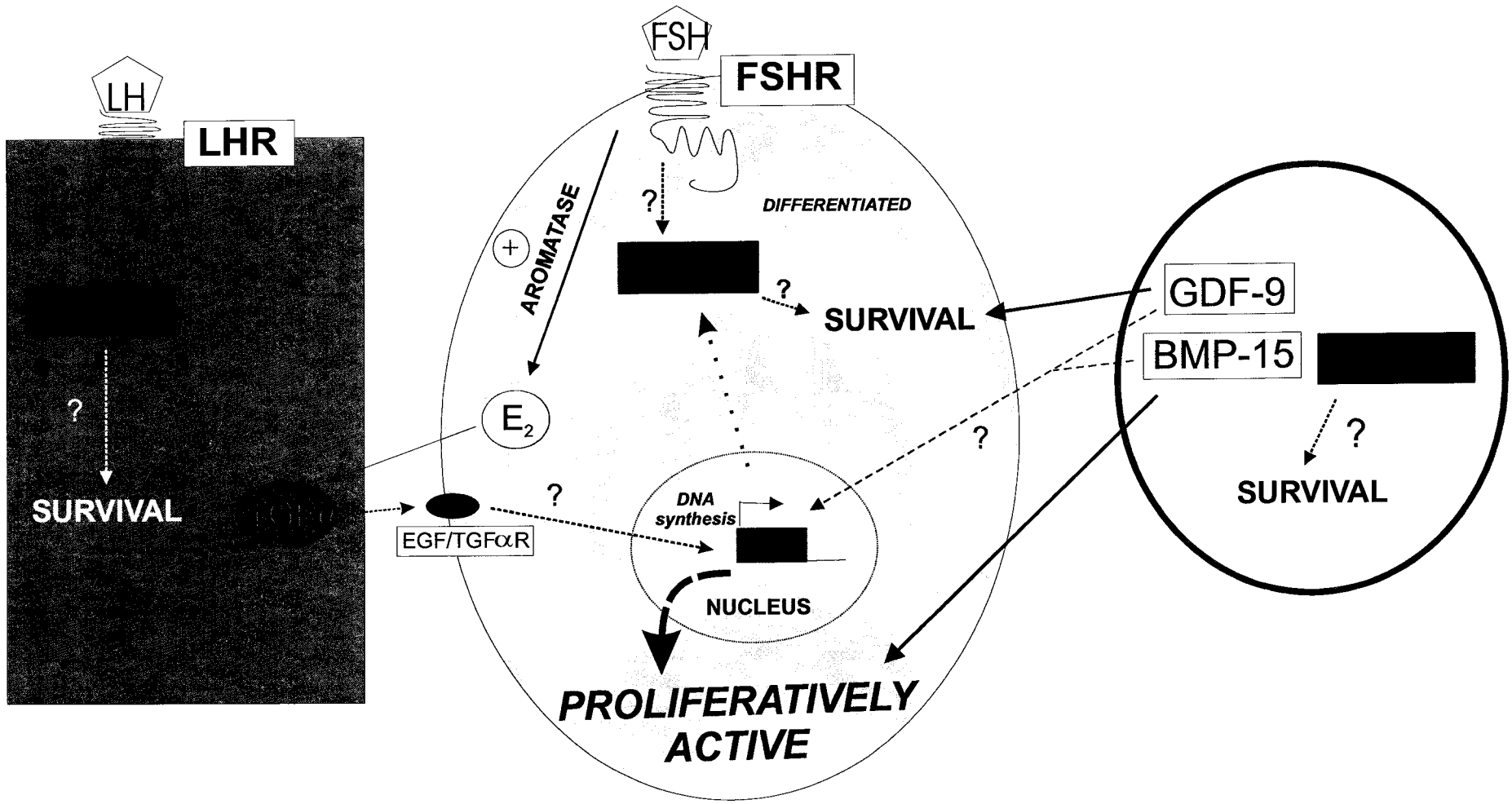
luteinization of the granulosa cells. As hypothesized, granulosa cell PAL31 content was up-regulated by estradiol treatment *in vivo*, and this was associated with induction of cyclin D2, a marker of estrogen responsiveness and granulosa cell proliferation. Gonadotropin withdrawal in eCG-primed immature rats had no effect on caspase-3 activation, suggesting that anti-eCG antibody is only partially effective with respect to the induction of follicular atresia. Whether granulosa cell PAL31 is a physiological substrate for caspase-3 at the penultimate stage of follicle development remains to be determined. The data presented in this thesis are quite novel and demonstrate for the first time that PAL31 is regulated by key reproductive hormones (gonadotropins, estrogens) during different stages of follicular development (Figures 23, 24).

These results presented herein represent correlative changes in PAL31 expression during different stages of follicle development. In addition to the future experiments suggested in this discussion, it is necessary to demonstrate the role of PAL31 in the follicle with a number of functional experiments. We are fortunate to have adenoviral preparations containing anti-sense and sense-PAL31 cDNAs, intact PAL31 cDNA and the two cleavage fragments. This technology will permit PAL31 gene manipulation in cultured rat granulosa cells and intact follicles at high infection efficiency. We are particularly interested in studying the putative regulation of PAL31 by oocyte-derived GDF-9, and in determining whether PAL31 has a role GDF-9-stimulated GC proliferation *or* GDF-9-induced GC survival during ceramide-induced apoptosis. To study the relationship between GDF-9 and PAL31, GDF-9 can be down-regulated by intra-follicular microinjection of anti-sense oligos, and follicular PAL31 expression can

Figure 23: Proposed model depicting the regulation of PAL31 in the rat ovarian follicle at the preantral and early antral stages

Early follicular development is regulated by circulating gonadotropins and intra-ovarian regulators. The granulosa cells of preantral and early antral follicles are proliferatively active and exhibit minimal characteristics of differentiation. Oocyte-derived growth factors have been shown to promote rat granulosa cell survival and proliferation, however, whether this phenomenon is mediated through up-regulation of PAL31 is not known. The stimulatory effect of estradiol on PAL31 expression at the preantral / early antral stage may occur through theca-derived TGFα, which in turn, stimulates GC survival and proliferation. Currently, the role of PAL31 in the follicular cell types during early stage folliculogenesis remains unclear.

Preantral / Early Antral Follicle

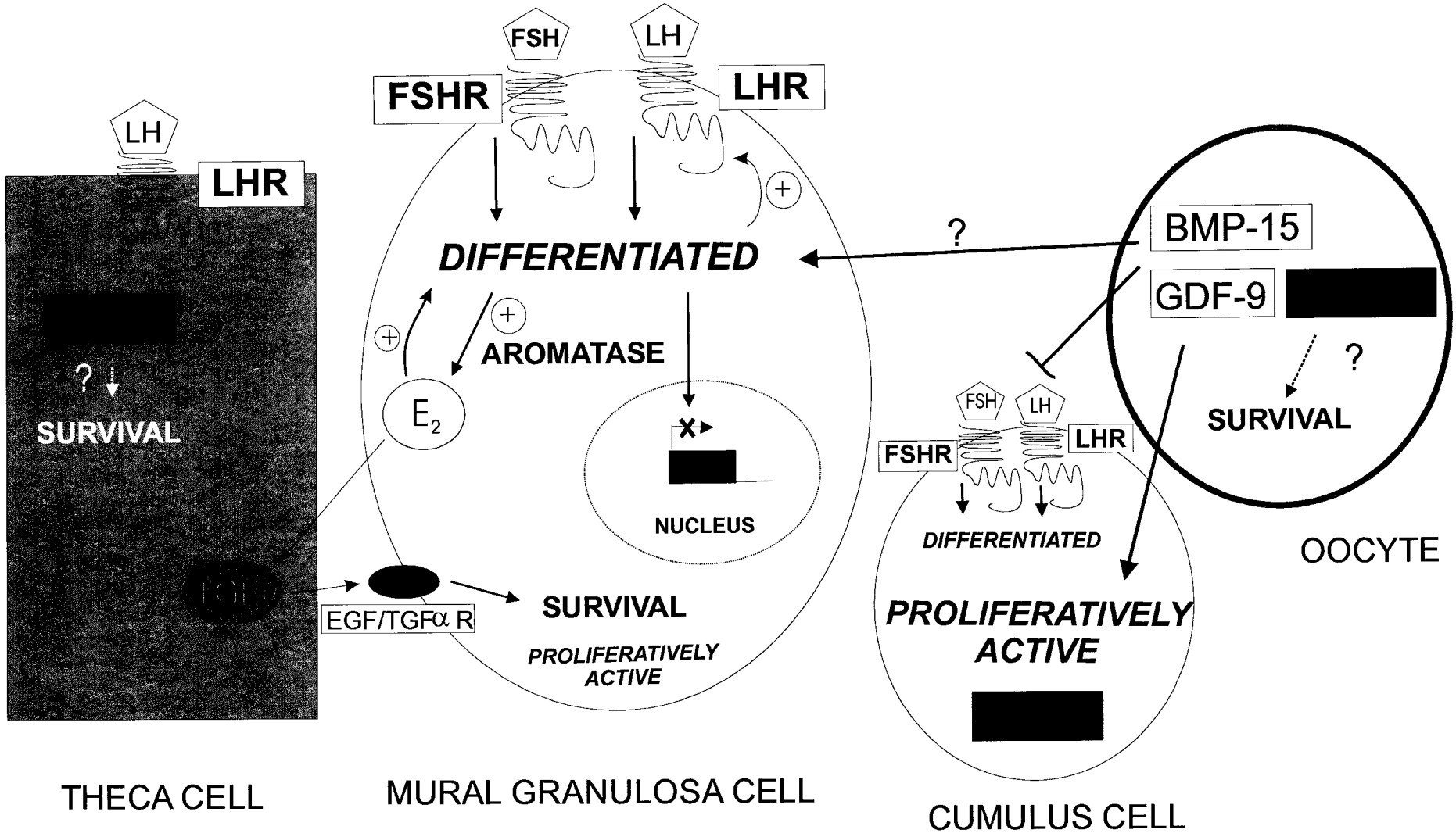


THECA CELL GRANULOSA CELL OOCYTE

Figure 24: Proposed model depicting the regulation of PAL31 in the rat ovarian follicle at the antral and pre-ovulatory stages.

At the antral and pre-ovulatory stages of development, the mural granulosa cells (GCs closest to the TCs) gradually acquire characteristics of differentiation (e.g. LHR induction, increased aromatase enzyme and estrogen synthesis), while the cumulus cells (GCs closest to the oocyte) remain proliferatively active. Estradiol stimulates theca cell TGFα production, which in turn, promotes granulosa cell survival and follicular growth. Meanwhile, granulosa cell differentiation is associated with down-regulation of PAL31 expression. Oocyte-secreted factors (e.g. GDF-9, BMP-15) maintain the cumulus phenotype by stimulation of proliferation and the inhibition of FSH-induced differentiation. The role of these factors in mural GCs of late antral and pre-ovulatory follicles is poorly understood.

Antral / Pre-ovulatory Follicle



then be examined by immunohistochemistry. Down-regulation of PAL31 in cultured rat GCs by anti-sense cDNA *or* siRNA ± GDF-9, would lend support for a role of PAL31 during proliferation, cell cycle progression and/or cell survival.

Ovarian dysfunction is often manifested in the form of polycystic ovarian syndrome, a condition strongly associated with infertility. The polycystic ovary contains follicles that are arrested in the early stages of development and unable to grow to reach ovulation. Currently, there is much to be learned with respect to the pathways and candidate proteins involved in GC proliferation during FSH-induced follicular growth. To gain a more thorough understanding of the cellular mechanisms involved in follicular development and atresia, the regulation of PAL31 was examined in the present research. Insight into the possible role of PAL31 in GC function and follicular growth would provide a better understanding of the ovarian events occurring during the normal menstrual cycle and how these events might be altered in pathological conditions. This could potentially lead to the use of PAL31 levels as a marker of normal or pathological status in infertile women.

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CURRICULUM VITAE

Carmen K.M. Cheung

EDUCATION

Sept. 2002 – July 2005	M.Sc. Cellular and Molecular Medicine (Physiology), University of Ottawa, Canada
Sept. 1998 – June 2002	B.Sc. (Hon.) Bio-medical Science, University of Guelph, Canada

AWARDS AND ACCOMPLISHMENTS

Apr. 2003 – Apr. 2005	Strategic Training Initiative in Research in Reproductive Health Sciences (STIRRHS) Scholarship (Master's level) Canadian Institutes of Health Research – Association of Professors of Obstetrics and Gynaecology
May 2004	Canadian Workshop in Human Reproduction and Reproductive Biology – 2nd Prize Poster Presentation (M.Sc. Category)
Apr. 2003	The Gerry Taichman Award – Best Achievement by a Graduate Student in a Master's Program.
May 2003	Ottawa Reproductive Biology Workshop – 2nd Prize Research Paper (Trainee Category).
Jan. 2003	Ottawa Health Research Institute Research Day – 2nd Prize Poster Presentation (Trainee Category).
Jan. 1999, Jan. 2002	University of Guelph, Dean's Honor List, in recognition of academic achievement.
Sept. 1998	University of Guelph Registrar's Entrance Scholarship.
Sept. 1998	Campbellford District High School Pinkerton University Entrance Scholarship.

RESEARCH AND PROFESSIONAL EXPERIENCE

Sept. 2002 – June 2005 **Graduate Student**
Ottawa Health Research Institute
Investigated the gonadotropic regulation of a cell cycle-related protein, PAL31 during ovarian follicular development and atresia.

May 2001 – Sept. 2001 **Summer Research Student**
University of Guelph, Department of Animal and Poultry Science
Investigated factors that influence sperm physiology as it relates to artificial insemination. Employed laboratory techniques to determine sperm motility and viability before and after cryopreservation, and tested the effects of different extenders on these end-points.

May 2000 – Sept. 2000 **Student Alumni Ambassador**
University of Guelph Alumni Affairs and Development
Facilitated a strong relationship between Guelph alumni and the University through continued communications and customer service. Collaborated with colleagues and different departments of the University during the organization of Alumni Weekend and other internal events.

June 1999 – Sept. 1999 **Research Assistant**
University of Guelph Department of Human Biology and Nutritional Sciences
Care of laboratory rats being used in a nutritional-based study. Duties included the administration of specialized diets to the animals and determination of body weight throughout the study.

LABORATORY SKILLS

Extensive experience working with laboratory rats including handling, restraint, injections and euthanasia.

Technical skills include: animal tissue extraction, Western analysis, RNA isolation and RT-PCR, real-time PCR, plasmid cDNA purification and amplification using *E. Coli*, Immunohistochemistry, mammalian cell culture, adenoviral infection of mammalian cells, microsomal protein extraction.

2002 – present	Completion of WHMIS training and Radiation training (Ottawa Health Research Institute).
June 2004	Completion of the Institutional Animal User Training Program from the Canadian Council on Animal Care (Ottawa Health Research Institute).

Post-secondary courses in human physiology, reproductive biology, cellular biology, biochemistry and anatomy.

COMPUTER SKILLS

Regular use of Microsoft Windows, Microsoft Office, Prism3 statistical analysis software, Scion Imaging, Adobe Acrobat, Adobe Photoshop, CorelDraw, Empix Northern Eclipse imaging software, Internet (including NCBI Pubmed search engine).

PUBLICATIONS

Jiang, J.-Y., **Cheung, C.K.M.**, Wang, Y., and B.K. Tsang. 2003. Regulation of Cell Death and Cell Survival Gene Expression during Ovarian Follicular Development and Atresia. Front. Biosci. 8:d222-237.

RESEARCH PRESENTATIONS

Nov. 2004	**Cheung, C.K.M.,** Jiang, J.-Y., Hattori, N., Shiota, K. and B.K. Tsang. Gonadotropic Regulation of Proliferation-Associated Leucine-Rich Protein 31 during Rat Ovarian Follicular Development and Atresia. STIRRHS Satellite Meeting, Association of Professors of Obstetrics and Gynaecology Annual General Meeting, Toronto, Ontario, Canada
May 2004	**Cheung, C.K.M.,** Jiang, J.-Y., Hattori, N., Shiota, K. and B.K. Tsang. Gonadotropic Regulation of Proliferation-Associated Leucine-Rich Protein 31 Content and Processing during Ovarian Follicular Development and Atresia. Canadian Workshop on Human Reproduction and Reproductive Biology: From Physiology to Genes to Therapy, Ottawa, Ontario, Canada
July 2003	**Cheung, C.K.M.,** Hattori, N., Shiota, K. and B.K. Tsang Gonadotropic Regulation of Proliferation-Associated Leucine-Rich Protein 31 Content and Processing during Ovarian Follicular

Development and Atresia. Abstract 284. Society for the Study of Reproduction 36th Annual Meeting, Cincinnati, Ohio, United States of America

May 2003 **Cheung, C.K.M.,** Hattori, N., Shiota, K. and B.K. Tsang. Gonadotropic Regulation of Proliferation-Associated Leucine-Rich Protein 31 Content and Processing during Ovarian Follicular Development and Atresia. Ottawa Reproductive Biology Workshop, Ottawa, Ontario, Canada

April 2003 **Cheung, C.K.M.,** Hattori, N., Shiota, K. and B.K. Tsang. Gonadotropic Regulation of Proliferation-Associated Leucine-Rich Protein 31 Content and Processing during Ovarian Follicular Development and Atresia. Southern Ontario Reproductive Biology Meeting, Kingston, Ontario, Canada

January 2003 **Cheung, C.K.M.,** Hattori, N., Shiota, K. and B.K. Tsang. Gonadotropic Regulation of Proliferation-Associated Leucine-Rich Protein 31 Content and Processing during Ovarian Follicular Development and Atresia. Ottawa Health Research Institute Research Day, Ottawa, Ontario, Canada