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EXPRESSION, HORMONAL REGULATION AND FUNCTION OF KIT LIGAND, THE LIGAND FOR THE C-*KIT* RECEPTOR, IN THE RAT REPRODUCTIVE SYSTEM

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Submitted to the School of Graduate Studies and Research
in partial fulfillment of the requirements for the degree of
Doctor of Philosophy

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December, 1997

ABSTRACT

Mice having mutations at the *Kit* and *Mast Cell Growth Factor* loci suffer from severe development abnormalities in gametogenesis suggesting that the protein products produced at these loci, Kit receptor and kit ligand (KL), serve an essential role in the reproductive system. The purposes of this study were: (1) to identify the expression Kit and KL in the ovary which is the organ in which gametogenesis occurs in the female animal, (2) to determine the expression of Kit and KL in the female reproductive tract, (3) to determine the influence of hormones and growth factors on the expression of Kit and KL, and (3) to identify the functional significance of KL/Kit interactions in the reproductive system.

Kit receptor expression was identified in the oocytes of antral follicles and KL expression was seen in granulosa cells in growing and antral follicles. In antral follicles, KL expression was greatest in cumulus granulosa cells and only those mural granulosa cells closest to the oocyte and antral cavity. Low KL expression was seen in the mural granulosa cells closest to the basement membrane. This pattern of KL expression in antral follicles suggested a role for KL in oocyte function.

There was an inhibition of oocyte meiotic maturation when oocytes from antral follicles were cultured in the presence of KL compared to untreated oocytes. In addition, under conditions where 50 % of oocytes were maintained in meiotic arrest for a 24 hr period of time, ablation of oocytic Kit receptor expression resulted in oocyte maturation in a greater proportion of oocytes compared to oocytes in which Kit receptor expression was not disturbed. Thus spontaneous meiotic maturation was inhibited by KL/Kit receptor interactions and KL stimulation of Kit receptors may be one mechanism by which oocytes are maintained in meiotic arrest within the follicle.

Luteinizing hormone (LH) is the key stimulator of oocyte maturation within the follicle. *In vivo* stimulation with LH resulted in a loss of KL expression in cumulus granulosa cells and a shift in the production of membrane-bound KL to more soluble KL in mural granulosa cells. Thus, LH-stimulated oocyte maturation may be partly mediated by the removal of KL in the cumulus granulosa cells and the production of a potentially less active form of KL by mural granulosa cells. These experiments support the hypothesis that *in vivo* oocyte maturation occurs as a result of the withdrawal of meiosis-inhibiting substances in the follicle.

The expression of KL in granulosa cells was upregulated at the transcript level by treatment with FSH, LH, or both *in vivo* and *in vitro*, as well as with membrane-permeable analogues of cyclic adenosine monophosphate (cAMP), whereas KL mRNA expression decreased with steroid hormone stimulation.

KL but not Kit mRNA expression was seen in the ovarian surface epithelium. KL expression in ovarian surface epithelium derived-cells was influenced by both dibutyryl cAMP and transforming growth factor-β stimulation. These studies would indicate that the ovarian surface epithelium is a site of KL production and that this expression can be regulated by ovarian factors.

Preimplantation embryos have been previously shown to express Kit receptors. In this study KL expression was demonstrated in oviducts and uteri and gonadotropin-stimulated increases in oviductal KL mRNA expression were seen. Furthermore, preimplantation embryo survival and development were enhanced with KL suggesting that KL has stimulatory effects on the mitotic cell cycle of the embryo.

Dedicated

to my parents, Azra and Shahid

ACKNOWLEDGEMENTS

I would like to thank my supervisor, Dr. Barbara Vanderhyden, for giving me the opportunity to work with her and to learn from her. The excellent training has not only developed my research but has also made me a better scientist. She has been an ideal mentor and good friend.

I would like to thank the members of Barbara's laboratory, Angela Tonary, Elizabeth MacDonald, Eniko Keszthelyi and Manon Dubé for their valuable suggestions at lab meeting and keeping lab meetings a social/science event. I am grateful to Elizabeth for her assistance with the animal work and her extensive involvement with OSE cell steroidogenesis and proliferation assays. I would like to thank Angela Tonary for her willingness to proof read my written material. I will definitely miss the long chats and the laughter I've shared with Elizabeth and Angela.

I would like to express my gratitude to Michaela Cada for her assistance in the cell attachment studies and Megan Hersh for assistance with the PCR work. It has been entertaining working with both these summer students.

I would like to thank Dr. Paul Morley for his interest in my career development.

I would like to express my gratitude to the individuals at the Cancer Research Group. I have had the good fortune of working with a group of people who have all contributed in making this an excellent learning experience while still maintaining a friendly environment. There are some individuals who I would like to thank in particular: Dr. Michael McBurney, for his valuable suggestions and criticisms of my research, Karen Jardine who taught me

northern blot analysis. Josée Coulombe who answered my many questions with patience, and Dave Stojdl for dealing with my computers woes as I was writing this manuscript.

I would like to thank Dr. Yuji Okawara for the teaching me *in situ* hybridization.

I would like to thank the members of my advisory committee Dr.Douglas Gray, Dr. John Bell and Dr. Jim Fryer who have offered valuable suggestions that have improved this project.

I would like to thank Donna Mulder and Denyse Blais in the Department of Physiology who have always been most helpful in dealing with the administrative side of being a graduate student.

I would like to express my gratitude to Beth Mason who has gone above and beyond the call of duty in her assistance with this document. A special thanks for her friendship..

I would like to thank my parents, Azra and Shahid, and Zarina, Razeena, Majid and Zuhaib. Without their encouragement and support I would not have been able to accomplished my doctorate. I would like to thank my grandmother for her blessings.

I would like to express my gratitude to my husband, Riyaz, for his friendship and support, and especially for his encouragement and cooperation in helping me finish my thesis. A special thanks to my daughter Shireen who has been most tolerant of the long hours that I have spent at work. She will always be my inspiration.

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α-MEM	α-minimum essential medium
3β-HSD	3β-hydroxysteroid dehydrogenase
BCIP	5-bromo-4-chloro-3-indolyl-phosphate
bp	base pair
Ca^{2+}_i	intracellular calcium
cAMP	cyclic adenosine monophospate
cDNA	complementary DNA
c-*fms*	CSF-1 receptor
CL	corpus luteum
cm	centimeter
cp-cAMP	8-(chloroylphenylthio)-adenosine 3':5'-cyclic monophosphate
cpm	counts per minute
CSF-1	colony stimulating factor-1
Cys	cysteine
dbcAMP	dibutyryl cyclic AMP
DAB	diaminobenzidene
DES	diethylstilbestrol
DMAP	6-dimethylaminopurine
dNTPs	deoxynucleotides
E	embryonic day
E_2	17β-estradiol
EC	embryonal carcinoma
EGF	epidermal growth factor
F1	first generation
FBS	fetal bovine serum
FSH	follicle-stimulating hormone
g	force of gravity
G	gauge
G_2 to M	meiotic maturation to metaphase II
GVBD	germinal vesicle breakdown
GAP Ras	$p21^{ras}$GTPase-activating protein
hCG	human chorionic gonadotropin
HPLC	high purification liquid chromatography
hr	hour

IBMX	3-isobutylmethylxanthine
Ig	Immunoglobulin
IU	international units
J	Joules
kb	kilobases
Kit	c-*kit* receptor
KL	kit ligand
KL-1	KL mRNA isoform which includes exon 6
KL-2	KL mRNA isoform which excludes exon 6
KL^{248}	product of KL-1
KL^{220}	product of KL-2
L	liters
LH	luteinizing hormone
LiCl	lithium chloride
µg	microgram
µM	micromolar
µm	micrometer
µl	microliter
mm	millimeter
mM	millimolar
M	molar
mgc	mural granulosa cells
min	minutes
MIS	Müllerian inhibiting substance
MPF	maturation promoting factor or M-phase promoting factor
mRNA	messenger RNA
ηg	nanogram
NBT	4-nitroblue tetrozolium chloride
OCC	oocyte-cumulus cell complexes
OSE	ovarian surface epithelial cells
p	probability
^{32}P	^{32} phosphorus
P_4	progesterone
PAGE	polyacrylamide gel electrophoresis
Pb formation	formation of the first polar body
PBS	phosphate-buffered saline

pc	postcoitum
PGC	Primordial germ cells
PI3K	phosphatidylinositol 3'-kinase
PKA	cAMP-dependent kinase, protein kinase A
PKC	protein kinase C
pl	picoliter
pmoles	picomoles
PLCγ	phospholipase Cγ
PMSG	pregnant mares' serum gonadotropin
POD	horseradish peroxidase
PTP	protein tyrosine phosphatases
Ras	$p21^{ras}$
RIAs	radioimmunoassays
sec	seconds
SCF	stem cell factor
S.E.M.	standard error of the mean
SSC	standard saline citrate
SDS	sodium dodecyl sulphate
SH2	src homology 2
T	testosterone
TBS/BSA	tris-buffered saline supplemented with 5% BSA
TE	Tris-EDTA, pH 7.5
TGF-α	transforming growth factor-α
TGF-β	transforming growth factor-β
TPA	active phorbol ester 12-O-etra-decanoyl phorbol-13-acetate
U	units
v/v	volume per volume
WAY	Waymouth MB 752/1 medium
ZF-oocytes	zona-free oocytes

CHAPTER 1- INTRODUCTION AND LITERATURE REVIEW

Mutations at the *Kit* and *Mgf* loci (formerly known as *White-spotting* and *Steel*, respectively) profoundly affect the development of three migratory embryonic stem cell populations: neural crest-derived melanocytes, hematopoeitic precursors and primordial germ cells (Russell, 1979). Numerous mutant alleles have been identified at these loci, and these mutations confer a wide range of phenotypic defects in the heterozygous and homozygous states. For instance, many mutations associated with *Kit* and *Mgf* alleles are embryonic lethal, while viable homozygotes carrying somewhat milder mutations are white-spotted, deficient in both erythrocytes and mast cells, and sterile. Using transplantation experiments, geneticists have determined that the products of the *Kit* locus are cell autonomous and act within the stem cell population, while the defects due to the *Mgf* mutant locus can be rescued by a normal environment and thus are associated with the microenvironment of the targeted cell (Bernstein, 1970; Wolf, 1978). Furthermore, when cells isolated from $Kit^{W/W}$ mice are manipulated to express Kit, normal activity is restored in these cells (Ray et al., 1991).

The c-*kit* receptor (Kit), a 120-145 kDa protein, is encoded by the *Kit* locus on mouse chromosome 5 (Gokkel et al., 1992) and has been mapped to human chromosome 4 (Gospodarowicz et al., 1977). The mouse *Kit* locus is within a region of the mouse genome that has been found to be frequently involved in germline mutations and rearrangements (Qiu et al., 1988; Majumder et al., 1988; Chabot et al., 1988; Geissler et al., 1988; Gokkel et al., 1992). The *Kit* gene encodes a 5.5 kb mRNA transcript, with the exception of unique 3.5

and 2.3 kb transcripts identified in mouse male germ cells (Sorrentino et al., 1991), and the coding region is distributed over 21 exons. Kit is a transmembrane receptor expressed on the surface of many cells and belongs to the tyrosine kinase receptor family (reviewed in Gokkel et al., 1992; Yarden and Ullrich, 1988). Similar to all family members, Kit has an amino-terminal signal peptide that targets Kit expression to the plasma membrane of the cell in which it is expressed (Fig. 1.1A). The extracellular ligand binding domain resides in the amino-terminal half of the molecule and is extensively post-translationally modified with cysteine bridges and glycosylations. Membrane anchorage is achieved by a single stretch of highly hydrophobic amino acids which traverse the plasma membrane. The cytoplasmic domain, contained in the carboxy-terminal portion of the receptor, is highly conserved among receptor tyrosine kinases and represents sequences that specify tyrosine-specific protein kinase activity. Kit receptor belongs to the same subclass of receptor tyrosine kinases as platelet-derived growth factor receptor and the colony stimulating factor-1 (CSF-1) receptor. The unique features of this subclass are: an extracellular ligand binding domain that contains five immunoglobulin-like repeats, and a hydrophilic amino acid sequence found within their intracellular tyrosine kinase domain which divides the kinase domain into an ATP-binding region and a phosphotransferase region (Qiu et al., 1988). Ligand binding of Kit receptor results in the phosphorylation of Kit on a number of tyrosine phosphorylation sites (Fig 1.1A).

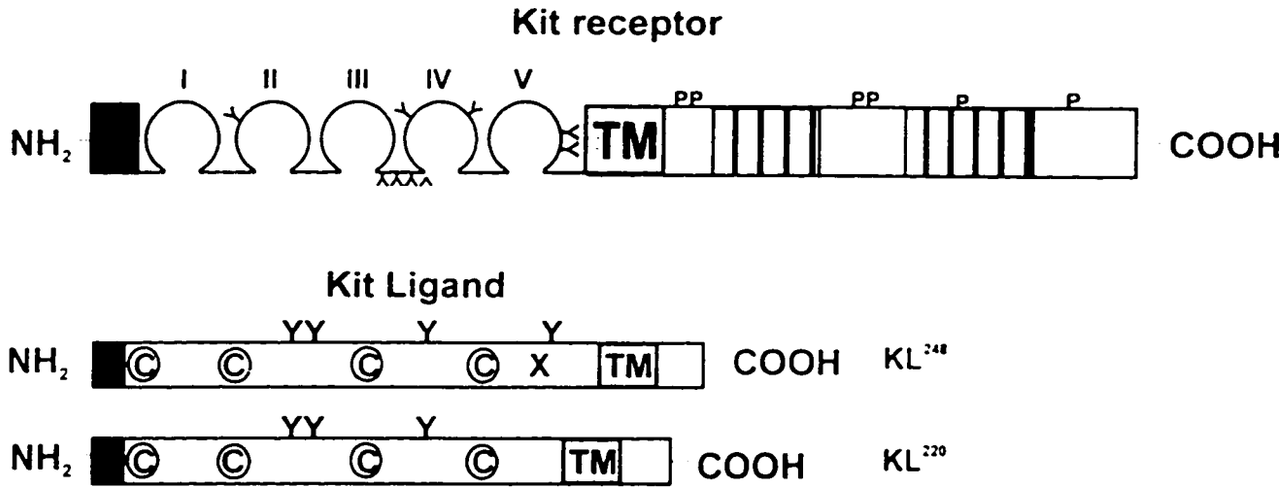
A Kit isoform has been identified in some Kit-bearing cell types which arises through the use of an alternative splice donor site identified in exon 9 (Reith et al., 1991; Gokkel et al., 1992). The alternatively spliced mRNA contains 12 additional bases and results in a four

Figure 1.1

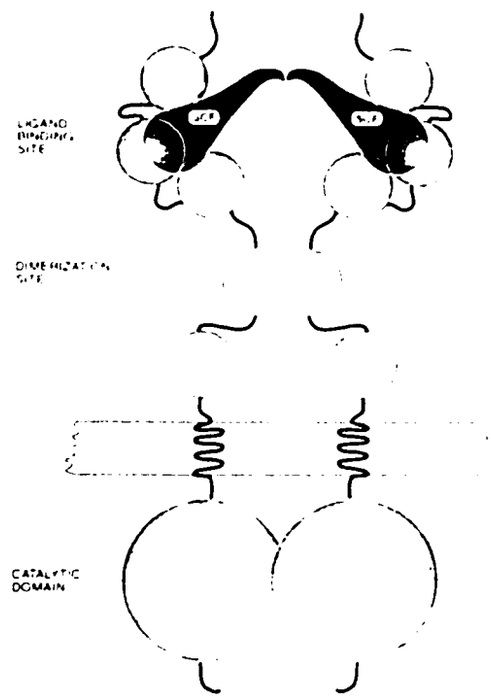
Model of the structure of Kit and KL and postulated interaction between receptor and ligand.

The structures of Kit receptor and kit ligand (KL) are illustrated in (A). The extracellular domain of the Kit receptor, located in the amino-terminal portion has extensive secondary structures containing both nitrogen-linked glycosylation sites (Y) and intramolecular disulphide bonds which form five immunoglobulin-like repeats. The two major isoforms of KL shown, differ in that KL^{220} lacks the major proteolytic cleavage site (X) present in KL^{248}. Like Kit, KL is extensively glycosylated in its extracellular domain, having both nitrogen- and oxygen-linked glycosylation sites (Y). A postulated model of interactions of Kit receptor and KL, also called stem cell factor (SCF), is shown in B. Noncovalently-associated KL interact with domains I, II, III of Kit and domain IV of Kit is responsible for receptor dimerization. Models are based on findings of Majumdar et al. (1988); Majumdar et al. (1994); Huang et al. (1992); Besmer (1995) and Blechman and Yarden, (1995).

A.


Kit receptor
I
II
III
IV
V
PP
PP
P
P
NH2
TM
COOH
Kit Ligand
YY
Y
Y
NH2
C
C
C
C
X
TM
COOH
KL248
YY
Y
NH2
C
C
C
C
TM
COOH
KL220
signal peptide
transmembrane domain
kinase domain
amino terminal
carboxy terminal
Y
glycosylation sites
C
cysteine residue
P
phosphorylation sites
X
major proteolytic cleavage site
immunoglubulin-like repeats

B.


CATALYTIC DOMAIN

amino acid insert within the extracellular domain which encodes a proteolytic cleavage site (Hayashi et al., 1991; Gokkel et al., 1992). Proteolysis results in the release of the extracellular domain of Kit (Turner et al., 1995; Kawakita et al., 1995; Wypych et al., 1995) and soluble Kit was first identified as a 95 kDa protein in the supernatants of hematopoeitic cells (Turner et al., 1995). Soluble Kit circulates in normal human plasma at a concentration of 325 ± 105 ng/ml (Yee et al., 1994; Wypych et al., 1995). Proteolytic processing to generate soluble Kit is enhanced with the activation of protein kinase C (PKC) and/or increased intracellular calcium [Ca^{2+}] (Yee et al., 1993).

The ligand for Kit, called kit ligand (KL) and variously known in the literature as stem cell factor, c-kit ligand and mast cell growth factor, is encoded by the *Mgf* locus which maps to mouse chromosome 10 (Flanagan and Leder, 1990; Williams et al., 1990; Witte, 1990; Anderson et al., 1990; Copeland et al., 1990; Zsebo et al., 1990; Huang et al., 1990) and human chromosome 12 (Anderson et al., 1991; Geissler et al, 1991). The *Mgf* locus encodes a 6.5 kb mRNA transcript with less dominant RNA transcripts of 5.5 and 3.0 kb (Matsui et al., 1990; Copeland et al., 1990). The major KL transcript is comprised of 9 exons and is alternatively spliced to include or exclude exon 6 (84 bp in length) giving rise to two proteins containing either 248 amino acids or 220 amino acids (Anderson et al., 1990; Flanagan et al., 1991) (Fig. 1.1A). Both proteins are membrane-associated (Flanagan et al., 1991; Huang et al., 1992); however, the translated product of the exon 6-containing KL mRNA (KL-1) yields a protein, KL^{248} which is efficiently proteolytically cleaved in the extracellular domain to release a soluble product. The translation of the shorter (lacking exon 6) mRNA, KL-2, results in KL^{220} that is more stably associated with the membrane

(Huang et al., 1992). Cleavage of KL from the cell surface can be induced by PKC or by agents that increase cytosolic calcium levels (Huang et al., 1992) and can be prevented by serine protease inhibitors (Pandiella et al, 1992). The mature soluble protein has a total of 165 amino acids, but when separated by SDS-PAGE it migrates at 28-40 kDa indicating extensive glycosylation with up to 30% of its weight being contributed by carbohydrates (Huang et al., 1990; Arakawa et al., 1991). KL has considerable secondary structure including regions of α-helices and β-sheets (Arakawa et al., 1991). The 4 Cys residues of KL are involved in intramolecular disulfide bonds and the presence of these bonds is critical to maintain the full bioactivity of soluble KL (Langley et al., 1992; Matous et al., 1996). Soluble KL has been found in plasma at a average concentration of 3.3 ng/ml (Shiohara et al., 1993; Wodnar-Filipowicz et al., 1993) and shown to circulate as noncovalently-associated dimers (Arakawa et al., 1991). Whether the transmembrane form of KL also exists as a dimer is not known.

1.1 Signal transduction of the Kit receptor

The signal transduction cascade initiated upon KL binding to Kit has been extensively studied in a variety of cell types including mast cells (Rottapel et al., 1991; Reith et al., 1991), hematopoeitic progenitor cells (Okuda et al., 1992), myeloid cells (Miyazawa et al., 1991) and transfected fibroblasts (Lev et al., 1991; Herbst et al., 1991 and 1992). Dimers of KL bind to Kit and cause receptor dimerization and catalytic activation of the Kit kinase domain via tyrosine phosphorylations (Lev et al., 1992b). Three amino-terminal Ig-like motifs are involved in ligand binding and the dimerization site resides in the fourth Ig-

like motif which symmetrically interacts with its dimer mate (Blechman and Yarden, 1995) (Fig. 1.1B). Several src homology 2 (SH2)-containing proteins have been shown to associate with Kit upon ligand binding. Activated Kit kinase strongly associates with the p85 subunit of phosphatidylinositol 3'-kinase (PI3K) through interactions with a specific phosphorylated tyrosine (Serve et al., 1994), resulting in PI3K phosphorylation (Rottapel et al., 1991; Lev et al., 1991; Lev et al., 1992a; Herbst et al., 1992). KL activation of Kit results in the phosphorylation of Janus Kinase 2 (JAK2) and its association with activated Kit receptors (Weiler et al., 1996). Kit also associates (directly or indirectly) with and activates $p21^{ras}$ (Ras) (Duronio et al., 1992), $p21^{ras}$GTPase-activating protein (GAP Ras) (Herbst et al., 1991 and 1992), Raf-1protein kinase (Rottapel et al., 1991; Lev et al., 1991; Herbst et al., 1991), phospholipase Cγ (PLCγ) (Herbst et al., 1991 and 1992) and members of the MAP kinase family (Okuda et al., 1992; Tsai et al., 1993; Paulson et al., 1996). In addition, Kit activation results in the recruitment and subsequent phosphorylation of two src-related tyrosine kinases, MATK (Jhun et al., 1995) and Tec (Tang et al., 1994) which are found only in hematopoeitic cells.

The phosphorylation of growth factor receptors on specific tyrosine residues is critical for regulation of their actions. The activation of receptor tyrosine kinases, resulting in the phosphorylation of downstream effector molecules, is an important event in the signal transduction cascades which ultimately result in growth or differentiation. The regulation of these pathways requires a delicate balance between the signals that activate the receptor tyrosine kinases and the mechanisms which inactivate and thereby attenuate their signals. Dephosphorylation of proteins at specific tyrosine residues by protein tyrosine phosphatases (PTP) has been shown to be essential for the normal regulation of growth factor activity.

Two phosphatases, PTP1C and PTP2C, each containing two SH2 domains (Feng and Pawson, 1994), have been shown to regulate Kit receptor activity by interacting via their SH2 domains with tyrosine phosphorylated residues of Kit (Feng and Pawson, 1994). Whether this is a direct binding to Kit and which of the six phosphotyrosine residue(s) of activated Kit are involved in the tyrosine phosphatase association have not been determined. However, both PTP1C and PTP2C dephosphorylate, and therefore, inactivate Kit. Interestingly, association of both PTP1C and PTP2C to Kit results in the recruitment of Grb2, an SH2-containing adapter molecule, to the activated Kit receptor phosphatase complex (Tauchi et al., 1994). In addition, PTP1C and PTP2C binding results in the activation of the Ras signalling pathway, (Tauchi et al., 1994; Kon-Kozlowski et al., 1996) probably as a consequence of the Grb2 adapter molecules. Therefore, not only does PTP1C or PTP2C negatively regulate Kit phosphorylation and hence deactivate the kinase but they also recruit the Ras signalling pathway for transmission of the Kit signals.

1.2 Mutant alleles of Kit and Mgf

The pleiotropic effects of mutant alleles at the *Kit* and *Mgf* loci in mice have been studied extensively and a great deal has been elucidated about the function of Kit and KL in hematopoeisis, melanogenesis and gametogenesis. Mice homozygous for severe mutations at either locus die during gestation or within days of birth (reviewed in Geissler et al., 1981). The heterozygous mice carrying severe mutations, as well as mice carrying somewhat milder homozygous mutations, are all viable but exhibit varying degrees of coat colour dilution, anemia and fertility. Mutations at the *Kit* and *Mgf* loci include deletions, rearrangements

and base substitutions, and have been documented in humans, mice and rats. To exemplify the variety of phenotypes that can arise due to mutations at or around these loci, some of the mutant alleles associated with *Kit* and *Mgf* are summarized in Tables 1.1 and 1.2, respectively. Null mutations in which there is a reduced or no appropriate expression of Kit protein, although severely affected in the homozygous state, give rise to relatively mildly-affected mice when the allele is present in the heterozygous state. All point mutations described to date are within the kinase domain of Kit resulting in the expression of a protein with defective kinase activity, and such mutations give rise to a more dominantly-affected animal in the heterozygous state. The dominant negative phenotype suggests that the coexpression of wild-type and mutant Kit proteins in the same cell inhibits proper signalling of the wild-type protein, most probably due to the partnering of a wild-type receptor with a mutant receptor resulting in an inactive Kit receptor dimer (Blechman and Yarden, 1995).

1.3 Cellular Responses of Kit and KL interactions

Identification of mutant alleles in the *Kit* and *Mgf* loci was very exciting for developmental biologists, owing to their pleiotropic effects on several cell lineages during embryo development (see Table 1.3). In agreement with the cell autonomous nature of the *Kit* mutants, Kit is normally expressed on those cells targeted by the mutations, during both embryo and postnatal development. However, the expression of KL is complementary to the expression pattern for Kit such that KL-producing cells are found in the microenvironment of the Kit-bearing cells. During embryo development, KL expression is associated with the migratory pathways and the sites of destination of the Kit-bearing cells and function to aid

Table 1.1

Mutant alleles associated with the *Kit* locus: Alterations in Kit protein and their effects on gametogenesis

Some mutant alleles are listed to exemplify the wide range of phenotypic deficiencies associated with mutations at the *Kit* locus. The type of mutation, the effect on protein expression and the phenotype of the mutant mice are described with particular interest in the gametogenesis in the affected mammals.

	Mutation	Effect on Kit Protein	Phenotype		References
			Gametogenesis	Other	
W^{v}	substitution at the 5' splice donor site (null)	1) deletion of transmembrane and part of catalytic domain 2) no cell surface localization 3) no catalytic activity	severely affected gametogenesis	lethal severely affected hematopoiesis and pigmentation	Hayashi et al., 1991
W^{37}	substitution in kinase domain (dominant)	1) no kinase activity 2) rapid ligand-independent turnover	ND	lethal severe anemia	Nocka et al., 1990
W^{42}	substitution in kinase domain (dominant)	1) no kinase activity	ND	lethal severe anemia	Rottapel et al., 1991; Geissler et al., 1981
W^{m}	substitution in kinase domain (null)	1) no posttranslational processing 2) no cell surface localization 3) expression of truncated receptor in specific cell types	ND	lethal severe anemia	Koshimizu et al., 1994; Tsujimura et al., 1993
W^{v}	substitution in kinase domain (dominant)	1) reduced kinase activity	sterile	viable severe anemia, black-eyed white,	Little and Cloudman, 1937; Nocka et al., 1990
W^{44}	4 Kb insert in intron (null)	1) reduced receptor expression	sterile males and fertile females	viable black-eyed white	Reith et al., 1990; Geissler et al., 1981
W^{41}	substitution in kinase domain (dominant)	1) reduced kinase activity	unaffected	viable severe anemia, black-eyed white,	Nocka et al., 1990; Geissler et al., 1981
W^{l}	substitution in kinase domain (dominant)	1) reduced kinase activity 2) spontaneous transformation of melanocytes *in vitro*	unaffected	viable severe anemia, patchy coat colour	Tarue et al., 1992; Tsujimura et al., 1993

Table 1.2

Mutant alleles associated with the *Mgf* locus: Alterations in KL protein and their effects on gametogenesis

Some mutant alleles are listed to exemplify the wide range of phenotypic deficiencies associated with mutations at the *Mgf* locus. The type of mutation, the effect on protein expression and the phenotype of the mutant mice are described with particular interest in the gametogenesis in the affected mammals.

	Mutation	Effect on protein	Phenotype		References
			Gametogenesis	Other	
Sl	complete deletion of region coding for Kl.	no protein produced	ND	lethal severe anemia	Williams et al 1990; Bennett, 1956
Sl^{12h}	deletion?	ND	ND	lethal, loss of preimplantation embryos	Cattanach, Raspberry and Beechey, 1988
Sl^{d}	intragenic deletion	no membrane-bound Kl. due to protein lacking intracellular and transmembrane domains	sterile	viable severe anemia, black-eyed white,	Bernstein, 1960; Nakayama et al., 1988; Brannan et al., 1991; Flanagan et al., 1991
Sl^{pan} =*Sl*^{11h}	inversion 115 Kb 5' of coding regions	reduced expression of Kl. mRNA in some tissues	sterile females fertile males	viable mildly anemic black-eyed white	Beachey and Searle, 1985; Huang et al., 1993; Bedell et al., 1995
Sl^{con}	rearrangements 195 Kb 5' of coding regions	differential effects on expression of Kl. mRNA	sterile females fertile males	viable mild anemia diluted coat colours	Bedell et al., 1995; Beechey and Searle, 1983
Sl^{17h}	point mutation in affecting splicing of carboxy-terminal exon	encodes Kl. lacking cytoplasmic tail	sterile males fertile females	viable black-eyed white with patches of black,	Brannan et al., 1992
Sl^{t}	ND	ND	female sterile, male fertile	viable no anemia seen, black-eyed off-white	Kohrogi et al., 1983; Kuroda et al., 1988

Table 1.3

Cellular responses of Kit and KL interactions in Kit expressing cells

Many cellular responses of Kit and KL interactions have been identified in cell types typically affected by mutations at the *Kit* and *Mgf* loci. The cells affected by mutations (targeted cells), the phenotype seen in mutant mice and the function of Kit stimulation are listed.

Targeted cells	Phenotype	Kit/KL function	References
primordial germ cells	sterility	proliferation, survival and migration	Dolci et al., 1991a; Matsui et al., 1991; Homa et al., 1991; Pesce et al., 1993; Packer et al., 1995
neural crest derived-melanocytes	lack of coat colour	proliferation, survival and migration	Klüppel et al., 1997; Ray et al., 1991
hematopoeitic precursors a. erythroid progenitors	anaemia	proliferation, survival and migration, adhesion	Horie and Broxmeyer, 1995; Brandt et al., 1992; Yonemura et al., 1997 and reviewed in Broudy, 1997
b. myeloid progenitors	mast cell deficiency	proliferation, migration and survival of precursors, as well as, mature cells; maturation, and secretion of mediators	Mekori et al., 1997; Wang et al., 1997 and reviewed in Morrison-Graham and Takahashi, 1993
Cajal cells	impaired autonomic gut motility	survival of intestinal pacemaker cells (Cajal cells)	Ward et al., 1995; Yamataka et al., 1996; Klimpel et al., 1995

in the appropriate migration of these cells. During postnatal development, its expression is found in the cells surrounding the Kit-bearing cells and may play a role in continued maintenance of these cells. Complementary expression of Kit and KL has been demonstrated in all tissues associated with the mutant phenotype and, utilizing extensive molecular biological techniques, the role of Kit and KL interactions in the normal development of primordial germ cells, hematopoeitic progenitor cells, mast cells, neural crest-derived melanocytes, and Cajal cells of the gut have been well characterized. Interactions of Kit and KL have been shown to influence a number of activities in the physiological systems in which Kit and KL are expressed, and are summarized in Table 1.3. Without the expression of Kit and KL embryo lethality occurs, implying the importance of these proteins in the development of a viable embryo. However, the continued expression of KL in the stromal tissues that surround the Kit-bearing cells indicate the importance of KL/Kit interactions in the normal functioning of the mature Kit-bearing cells such as mast cells, melanocytes and spermatogonia in the developing animal after birth (Table 1.3). In addition Kit and KL expression has been observed in the central nervous system and may be involved in neuronal organization, hippocampal learning and astrocyte survival (Morrison-Graham and Takahashi, 1993; Motro et al., 1996).

Kit and KL are expressed in a number of tissues that have no obvious deficiencies in either mutant embryos or adult mice. Complementary expression has been observed in the olfactory epithelium, lungs, heart and kidney (Matsui et al., 1990; Morrison-Graham and Takahashi, 1993); however, the role of Kit/KL in these tissues is not known, and may be redundant in their normal development and function.

1.3.1 Kit and KL and the cell cycle

Kit and KL interactions have been shown to result in a wide variety of cellular responses: cell proliferation, survival, migration, adhesion, and in some cases, differentiation and maturation (see Table 1.3). The best characterized of these cellular responses is cell proliferation. Many of the cell types examined respond to activation of the Kit receptor by stimulating the mitotic cell cycle. When progenitor hematopoeitic cells and mast cells are cultured in the presence of KL, there is a dose-dependent increase in cell number and in [^{3}H]thymidine incorporation (Metcalf and Nicola, 1991; Tsai et al., 1991). Furthermore, KL stimulates colony formation of primitive hematopoeitic precursors from bone marrow (Metcalf and Nicola, 1991). Like the Kit-bearing somatic cells, primordial germ cells respond to KL in culture with increased proliferation (Matsui et al., 1991) and in male mice KL selectively stimulates DNA synthesis in type A spermatogonia (Rossi et al., 1993).

In contrast to its proliferative effects described above, there is evidence to support an inhibition of the cell cycle by Kit in some cells. Introduction of Kit expression into breast cancer cells and melanoma cells results in a suppression of cell growth when transfected cells were stimulated with KL (Nishida et al., 1996; Huang et al., 1996). In addition, Kit-transfected melanoma cells were highly susceptible to KL-induced apoptosis (Huang et al., 1996). Furthermore, a dramatic loss of Kit expression is seen in breast (Natali et al., 1992a), testicular (Strohmeyer et al., 1991), and melanoma (Natali et al., 1992b) tumour specimens compared to normal cells suggesting that Kit is involved in the inhibition of growth and this function is lost with the transformation of the cells. Thus Kit activation can also result in the inhibition of the cell cycle.

1.4 Role of Kit and KL in gametogenesis in the mouse embryo

Based on the phenotypes of the various homozygous mutant mice, it is clear that the defect in melanogenesis, hematopoeisis and gametogenesis are not dependent on each other for their appearance (Geissler et al., 1981). In addition, the defect in gametogenesis can specifically effect one sex and not the other as is the case with Kit^{W-44} mice (Table 1.1) and Mgf^{Sl-1}, Mgf^{Sl-pan}, Mgf^{Sl-con} and Mgf^{Sl-17h} (Table 1.2), therefore indicating that functional Kit and KL are imperative for normal reproductive development in both male and female mice. However, because of the different effects in male and female reproductive capacity, there must be different requirements either in terms of the dosage or quality of Kit and KL between the male and female gonads.

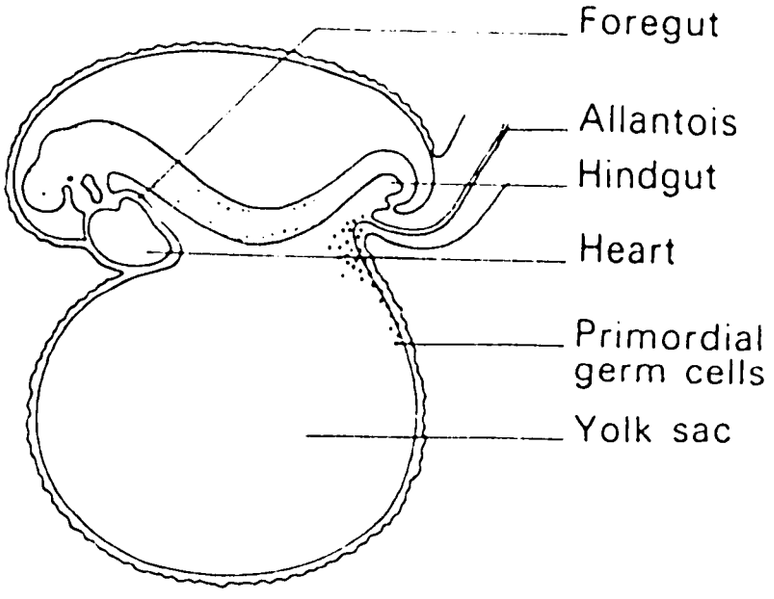
Primordial germ cells (PGC) are the embryonic precursors cells that give rise to oocytes and are integral to the normal development of the ovary and the maintenance of ovarian structures in the adult animal. In the mouse embryo, PGC develop in the epithelium of the yolk sac near the allantois (Fig. 1.2a). Between embryonic day (E) 9.5 and E 11.5, these cells actively proliferate as they migrate along a pathway leading from the endoderm and mesentery of the hindgut to the genital ridge; the area of the somatic mesenchymal tissue which forms the matrix of the developing gonad (Bennett, 1956) (Fig.1.2b). The PGC move by amoeboid movements in response to chemotactic factors produced by the genital ridge (Fig 1.2). After the PGC reach the genital ridge, they continue to proliferate and this colonization is complete by day E 13 (Fig. 1.2).

In mice homozygous for *Kit* and *Mgf* mutations, PGC are generated normally at E 8, but fail to increase in number and few reach the gonad (Buehr et al., 1993; Bennett, 1956;

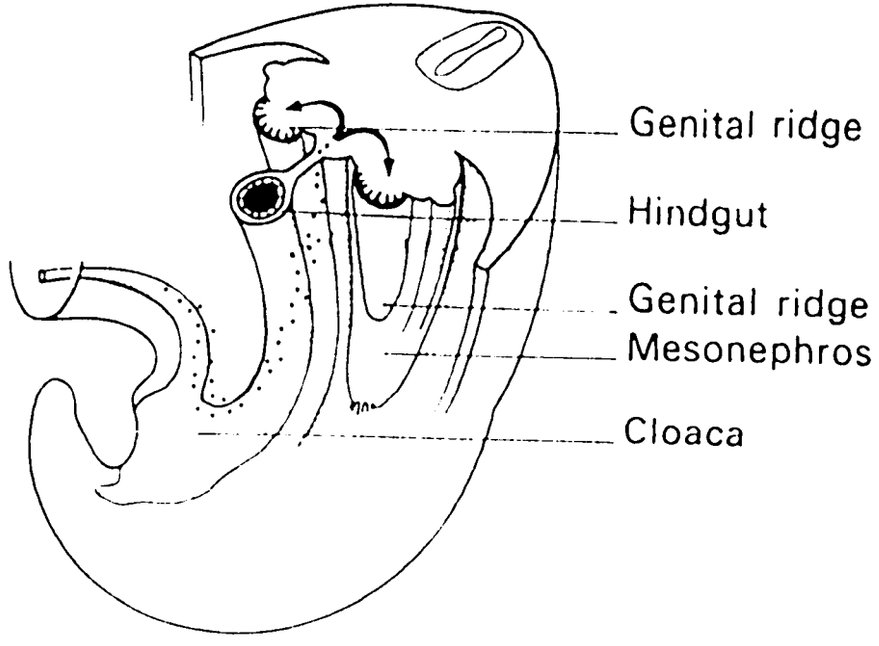
Figure 1.2

PGC formation and migration in the developing embryo.

Diagram of a sagittal cross-section of a mouse embryo at E 9 showing the origin of PGC in extragonadal tissues (a). The route of PGC migration from the allantois through the hindgut to the indifferent genital ridge between E 9.5 and E 11.5 is shown using a transverse cross-section of a mouse embryo (b). Taken from Johnson and Everitt (1980).


Foregut
Allantois
Hindgut
Heart
Primordial germ cells
Yolk sac

(a)


Genital ridge
Hindgut
Genital ridge
Mesonephros
Cloaca

(b)

Mintz and Russell, 1957). In normal embryos, the PGC strongly express Kit mRNA (Orr-Urtreger et al., 1990; Keshet et al., 1991; Manova and Bachvarova, 1991). Interestingly, when Keshet et al., (1991) performed simultaneous *in situ* hybridization analysis of both Kit and KL expression in sections of developing mouse embryos, their studies revealed that there is highest KL mRNA expression in the genital ridge with a gradient of KL expression along the PGC migratory path to the genital ridge. Once colonization of the genital ridge had occurred, the gradient of KL mRNA disappeared. This data suggests that KL serves as a chemotactic factor acting as a homing device to guide the (Kit-positive) germ cells to the developing gonad (Matsui et al., 1990; Keshet et al., 1991). Indeed, using a bicameral culture system, it has been shown that mast cells, also a Kit-bearing cell type, migrate in the direction of high amounts of KL, reinforcing the hypothesis that KL acts as a beacon for PGC migration (Meininger et al., 1992). In addition to its function in PGC migration, KL stimulation of cultured PGC results in increased survival (Matsui et al., 1991; Godin et al., 1991; Dolci et al., 1991a and1991b; Pesce et al., 1993) by preventing apoptosis (Pesce et al., 1993 and 1996). KL was also able to cause the proliferation of PGC but only when cultured in direct contact with KL-bearing feeder cells, probably due to a requirement for the transmembrane form of KL for this activity (Matsui et al., 1991). Based on these data, a strong case can be made that KL is a growth factor that has multiple functions in normal embryonic development of the ovary.

Once colonization of the ovary has occurred, PGC continue to proliferate until mesenchymal cells originating from the cortex of the ovary split into cell clusters and surround the germ cells to initiate the formation of *primordial follicles* (Fig. 1.3). Only

those PGC surrounded by the cortex-derived cells survive and all other PGC are eliminated by apoptotic death. These primitive follicles form the functional unit of the mature ovary so that the germ cells will give rise to *oocytes* and the mesenchymal cells will differentiate to form *granulosa cells*.

By days E 13.5 to 15, as the PGC become enclosed by the primitive granulosa cells, PGC mitotic activity ceases and meiosis is initiated, and at this time there is a decrease in PGC Kit expression (Manova and Bachvarova, 1991). PGC begin to enter meiotic prophase I and remain arrested in this extended prophase I until after birth. As a consequence of this initiation of meiosis, the female mammal has her complete supply of oocytes before birth which are slowly depleted during her reproductive lifespan.

As investigators interested in ovarian development and function, the sterility seen in the viable mutants was curious to us. The normal development of germ cells in both male and female embryos is dependent on the expression and function of Kit and KL. Because PGC develop in extragonadal tissues and must migrate to the developing genital ridge, lack of gametogenesis in the mutants occurs as a result of the incomplete migration of PGC during embryo development. In general, sections through meagrely developed ovaries of mutant mice indicate that there are few, if any, oocytes or normal ovarian structures in mutant animals (Kuroda et al., 1988; Huang et al., 1993). This phenotype would indicate that KL and Kit interactions are necessary for the embryonic development of the ovary.

1.5 Follicular development in the rodent ovary

In the neonatal rodent ovary, the follicles are predominantly *primordial follicles*, each containing a small non-growing, *meiotically-arrested oocyte* surrounded by a single layer of flattened *granulosa cells* and a *basement membrane* which compartmentalizes the follicle from the rest of the ovary (Fig 1.3). During the first two weeks after birth, several oocytes along with their surrounding granulosa cells start to grow. It is not known whether the initiation of oocyte growth is in response to signals within the follicle or by an oocyte-autonomous programme. The transition from primordial to *primary follicle* is indicative of the start of the growth phase and is marked by a change in morphology of granulosa cells from flattened to cuboidal (Fig. 1.3). Oocyte growth is completed in 2-3 weeks at which time the oocyte has increased in diameter from 10 μm to 80 μm and has acquired a non-cellular glycoprotein layer called the *zona pellucida*. Coincident with oocyte growth, the granulosa cells proliferate and enclose the growing oocyte with multiple layers of cells. After oocyte growth is complete the follicle continues to develop in response to gonadotropins and two changes occur: a fluid-filled cavity called the *antrum* arises among the multi-layered granulosa cells, and the stromal cells outside of the basement membrane differentiate into *theca cells* (Fig. 1.3). This mature follicle is called an *antral or Graafian follicle*. The granulosa cells within antral follicles differentiate to *mural granulosa cells* which are attached to the basement membrane and *cumulus granulosa cells* immediately surrounding the oocyte (Fig 1.4). After a surge in luteinizing hormone (LH), the follicle ruptures and the oocyte is released into the oviduct where fertilization occurs. The follicular

Figure 1.3

Diagram of a mammalian ovary.

Sequential development of the follicle, formation of the corpus luteum and follicular atresia are shown. A section of a wall of a mature antral follicle is enlarged in the upper right corner. Taken from Ganong (1981).

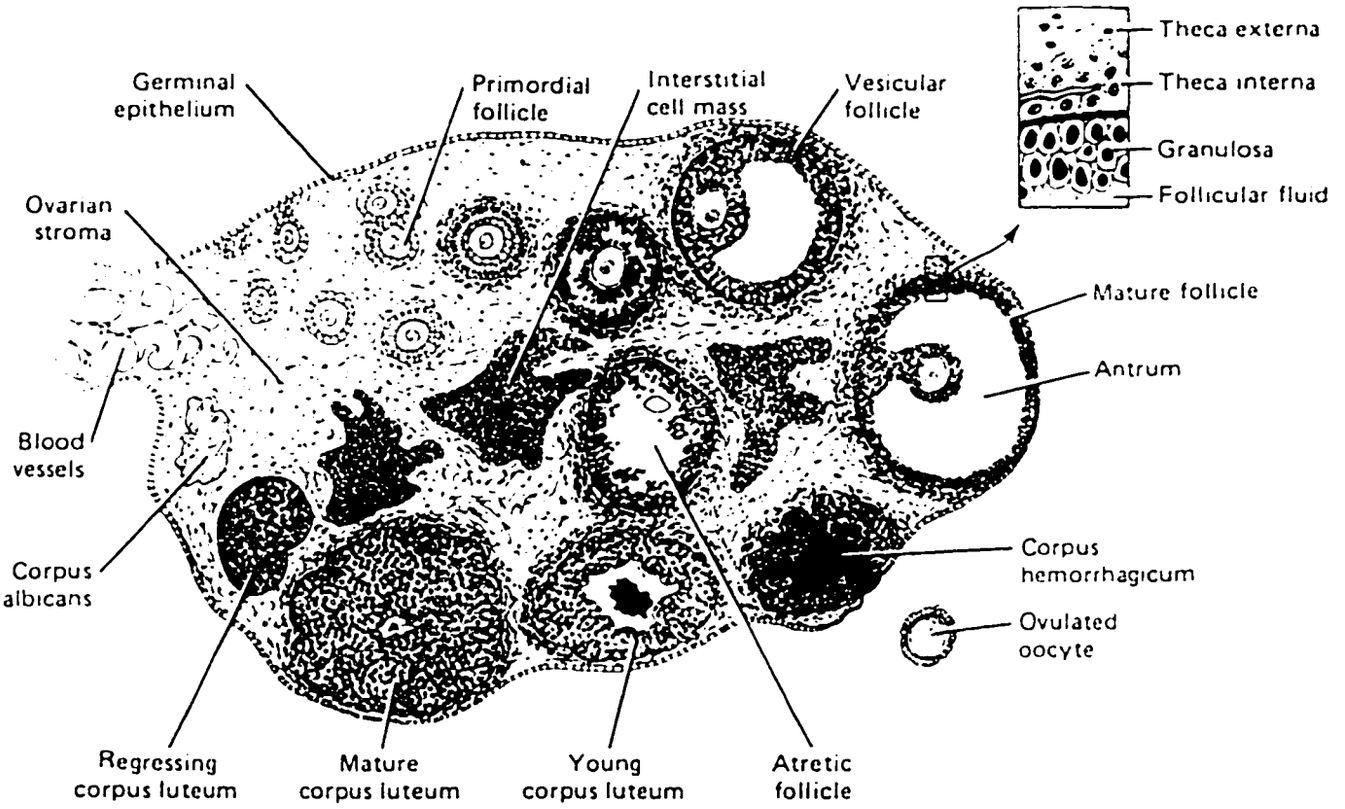

Theca externa
Theca interna
Granulosa
Follicular fluid
Germinal epithelium
Primordial follicle
Interstitial cell mass
Vesicular follicle
Ovarian stroma
Mature follicle
Antrum
Blood vessels
Corpus albicans
Corpus hemorrhagicum
Ovulated oocyte
Regressing corpus luteum
Mature corpus luteum
Young corpus luteum
Atretic follicle

Figure 1.4

Cross-section of an antral follicle.

Scanning electron micrograph of a rat preovulatory antral follicle. mgc= mural granulosa cells. cum=cumulus cells. ooc=oocyte. a=antral cavity. Taken from Richards (1979).


a

cell structures remaining in the ovary after ovulation differentiate to form the *corpus luteum (CL)* (Fig. 1.3). The main purpose of the CL is to make large quantities of steroid hormones to enable the uterus to prepare for and subsequently support the growth of the embryo during pregnancy.

Membrane specializations called *gap junctions* located between the oocyte and cumulus granulosa cells enable the transfer of low molecular weight products from one cell to another. Granulosa cells communicate with each other via homologous gap junctions (Albertini and Anderson, 1974). Cumulus granulosa cells have cytoplasmic bridges which extend through the porous zona pellucida and synapse at the oolemma to form heterologous gap junctions (Fig. 1.5) (Anderson and Albertini, 1976). Since gap junctions metabolically couple all granulosa cells, mural and cumulus, and the oocyte, these cells constitute a functional syncitium mediated by gap junctions. The oocytes and granulosa cells are dependent on each other for a number of cell functions (Eppig, 1977; Herlands and Schultz, 1984; Buccione et al., 1990) which are mediated through the gap junctions, as well as paracrine factors.

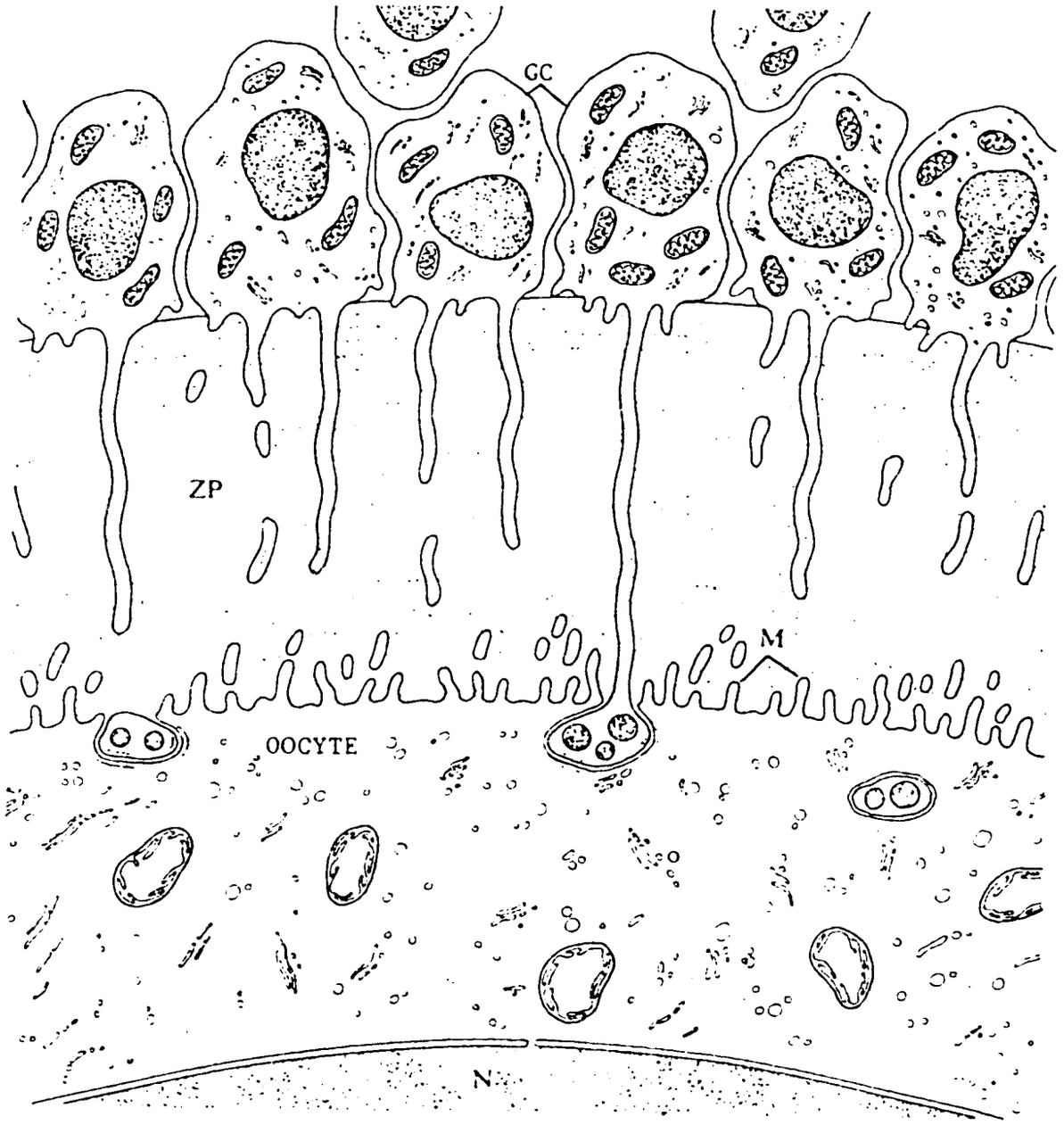
1.6 KL and Kit expression in the postnatal mouse ovary

In the postnatal mouse ovary, Kit mRNA and protein expression have been demonstrated in oocytes at all stages of follicular development, from primordial oocytes to fully-grown, ovulated oocytes and in preimplantation embryos (Manova et al., 1990; Horie et al., 1991), as well as in theca-interstitial cells of antral follicles of adult mice

Figure 1.5

Structure of the zona pellucida of an antral follicle.

The zona pellucida is penetrated by microvilli (M) arising from the oocyte on one side and cytoplasmic bridges from the granulosa cells (GC) on the other side. Heterologous gap junctions between the cytoplasmic bridges and the oocyte plasma membrane enable the transfer of small molecules between the oocyte and granulosa cells. N=oocyte nucleus. ZP=zona pellucida. Taken from Baker (1982).


GC
ZP
M
OOCYTE
N

(Manova et al., 1993; Motro and Bernstein, 1993). In addition, KL mRNA has been demonstrated in granulosa cells of preantral, multi-layered follicles (Keshet et al., 1991). In agreement with findings reported in mouse ovaries, Kit mRNA and protein have been detected on the surface of oocytes at all stages of follicular development in human and monkey ovaries (Smikle, et al 1996a).

In mouse ovaries, KL protein and mRNA expression were observed in the granulosa cells of primary and multi-layered preantral follicles and antral follicles, with highest KL expression seen in the antral follicles (Motro and Bernstein, 1993; Keshet et al., 1991; Manova et al., 1993). Interestingly, after granulosa cell differentiation to mural and cumulus subpopulations, KL staining was restricted to the mural granulosa cells in antral follicles, presumably directed towards the thecal Kit receptors (Motro and Bernstein, 1993; Manova et al., 1993). KL was not present in the flattened granulosa cells of primordial follicles located in the cortex region of the ovary (Manova et al., 1993). In sheep ovaries, KL mRNA has been localized to granulosa cells of all stages of follicles (Tisdall et al., 1997) and both KL mRNA and protein have been localized to ovine CL (Gentry et al., 1996). KL mRNA has been demonstrated in granulosa-luteal cells isolated from late human antral follicles (Laitinen et al., 1995). Thus, Kit is present in all oocytes of mouse, primate and human ovaries. KL expression, on the other hand, is restricted to the granulosa cells surrounding developing follicles with some species-specific differences in distribution in follicles.

1.7 Role of Kit/KL interactions in oocyte growth

Kit and KL interactions are clearly imperative for normal embryonic ovary development by enabling the proper migration, survival and proliferation of PGC. However, once the ovary has differentiated, *Mgf* and *Kit* remain transcriptionally active during the rest of embryonic and postnatal ovary development, suggesting that there is another function(s) in the growth or maintenance of the normal ovary. Indeed, the milder *Mgf*-mutants, $Mgf^{Sl\text{-}d}$, $Mgf^{Sl\text{-}pam}$, and $Mgf^{Sl\text{-}com}$ result in viable homozygous animals in which male reproduction appears to be unaffected but the female mice are sterile most likely due to an impairment of ovarian follicular development.

In $Mgf^{Sl\text{-}d}$ mice, primary follicular development occurs but no advanced growing follicles are evident in sections of ovaries indicating that follicular progression is arrested after primary follicle development (Kuroda et al., 1988). Using chimera ($Mgf^{Sl\text{-}d}$/wild-type) mice, Kuroda and coinvestigators (1993) showed that the defect is not intrinsic to the oocyte itself, but may be associated with other ovarian cell types. Since no data is available describing the molecular nature of the defect, one can speculate that a different amount, form or site of KL expression is required for follicular development to progress past the primary follicle stage in these mutant mice.

In $Mgf^{Sl\text{-}pam}$ and $Mgf^{Sl\text{-}com}$ mutants, DNA rearrangements located 115 and 195 kb, respectively, upstream of the KL-coding sequences caused a decrease in the abundance of KL mRNA expression, although there were no alterations in the protein produced (Bedell et al., 1995). Whether this decrease in KL mRNA amount was due to alterations in the rate of transcription initiation or stability of the mRNA species has not been determined.

Although with both mutations there is a decrease in KL expression, $Mgf^{Sl\text{-}pan}$ mutation results in mice that are phenotypically more affected than $Mgf^{Sl\text{-}con}$ mutant mice. The ovaries of $Mgf^{Sl\text{-}pan}$ mice had only 20 % of oocytes compared to the wild-type (Bedell et al., 1995). Like in the $Mgf^{Sl\text{-}l}$ mice, follicular growth was arrested in the primary follicle stage of $Mgf^{Sl\text{-}pan}$ mice. At 4 days after birth, the authors observed a slight increase in size of the oocytes of the $Mgf^{Sl\text{-}pan}$ mice consistent with the size of oocytes seen in primary follicles; however, the oocytes did not deposit a zona pellucida. Furthermore, by 19 days of age, much of the ovary was comprised of disorganized follicle-like clusters which lacked oocytes. Given that at the earlier age, the follicles were all oocyte-containing, these data suggests that the diminished KL expression resulted in the elimination (possibly apoptotically) of the oocytes. This elimination of the oocyte probably contributes to the mutant ovary phenotype in the older animals. Thus, diminished KL expression seen in the ovaries of $Mgf^{Sl\text{-}pan}$ mice resulted in the arrest of follicular development at the primary follicle stage, and furthermore, may be associated with an inability of the follicle to support the survival of the oocyte.

In $Mgf^{Sl\text{-}con}$ mice, there were fewer oocytes and half as many maturing follicles compared to wild-type mice. Although the onset of follicle development was delayed, some follicles were able to develop to the preantral stage and occasionally to the antral stage. However, after 5 weeks of age, all remaining follicles started to degenerate. Although there was generally less KL expression in the ovaries of $Mgf^{Sl\text{-}con}$ mice, KL levels were comparable to wild-type within the antral follicles of mutant mice. This is consistent with earlier findings that in some cases, $Mgf^{Sl\text{-}con}$ homozygous mice may give birth to only a single litter (Beechey and Searle, 1983), suggesting that a reduced number of follicles can develop

normally. In summary, a critical level of KL expression is required for complete follicular development to occur.

The decrease in the number of oocytes in the ovaries of the above-described mutants is most likely due to a reduction in the number of germ cells that colonize the ovary during prenatal ovary development. Based on the phenotype of the $Mgf^{sl\text{-}t}$ and $Mgf^{sl\text{-}tm1}$ ovaries, the initiation of oocyte and follicular growth seen from primordial to primary follicle transition is KL-independent but KL is required for the primary to multi-layered preantral follicular development and onwards.

Packer et al (1994) demonstrated that when early growing oocytes isolated from 8-day old mice were cultured under specific conditions *in vitro*, stimulation of Kit by KL resulted in enhanced oocyte growth. In addition, when oocytes were cultured on granulosa cell monolayers in the presence of a Kit blocking antibody, a mild but significant inhibition of oocyte growth was seen (Packer et al., 1994). Furthermore, a marked inhibition of oocyte growth was seen when oocytes from primordial follicles were isolated and cultured in the presence of ACK2, a Kit blocking antibody shown previously to block Kit function *in vivo*. (Yoshinaga et al., 1991; Ogawa et al., 1991; Nishikawa et al., 1991; Maeda et al., 1992; Yoshida et al., 1993 and 1996). These results indicated that KL/Kit interactions may play a role in the initiation of early oocyte growth associated with the change from primordial to primary follicles (Packer et al., 1994). These data are further supported by the findings that KL expression is lost in the cumulus granulosa cells after mouse antral follicle development (Motro and Bernstein, 1993; Manova et al., 1993), as at this stage of follicular development

oocyte growth is complete and KL would not be required. However, the observations that KL stimulation enhances oocyte growth could not be repeated with oocytes isolated from 12-day old mice (Cecconi et al. 1996) and somewhat contradict what is seen in Mgf^{Sl-t} and Mgf^{Sl-pan} mice where clearly the initiation of growth is not impaired but the progression of oocyte and follicular growth does not occur. Perhaps in these mutant mice there may be just enough KL for this primordial to primary follicle transition to occur. Nonetheless, KL/Kit may regulate oocyte growth *in vitro* under suboptimal conditions.

Yoshida and coinvestigators (1997) have recently demonstrated a requirement for Kit-KL interactions for primordial follicle transition, primary follicle growth and follicular fluid accumulation. To do this, prepubertal mice were given an intraperitoneal injection with ACK2 and postnatal ovarian development was investigated . When Kit function was blocked during the first 5 days after birth, both granulosa cell proliferation and the transition of granulosa cells from a squamous to cuboidal cell morphology were inhibited, indicating an impairment in the transition from primordial to primary follicles. When Kit function was blocked 10-14 days after birth (at a time when oocyte growth is complete and antral follicles are starting to develop) there was a suppression of granulosa cell DNA synthesis and a lack of accumulation of follicular fluid in multi-layered preantral follicles. Hence, blocking Kit function had no effect on primordial follicle formation, or primordial and preantral follicle survival, but prevented antral follicle development and survival.

Immunoneutralization of Kit receptors on oocytes resulted in the suppression of granulosa cell proliferation (Yoshida et al., 1997). Because there are no Kit receptors on granulosa cells this data suggests that stimulation of Kit receptors on oocytes results in the

production of signals from the oocytes that control granulosa cell proliferation. This data is in agreement with previous reports demonstrating the role of the oocyte in granulosa cell proliferation and antral follicle development (Vanderhyden et al., 1992; Vanderhyden et al., submitted).

Although, KL may be involved in the regulation of oocyte growth, highest KL expression in mouse ovaries is observed in the antral follicle, a stage at which oocyte growth is complete and, therefore, a signal to enhance oocyte growth would not be required. Since Kit and KL continue to be expressed in antral follicles, the outcome of functional interactions of Kit and KL in antral follicles is unknown and remains to be elucidated.

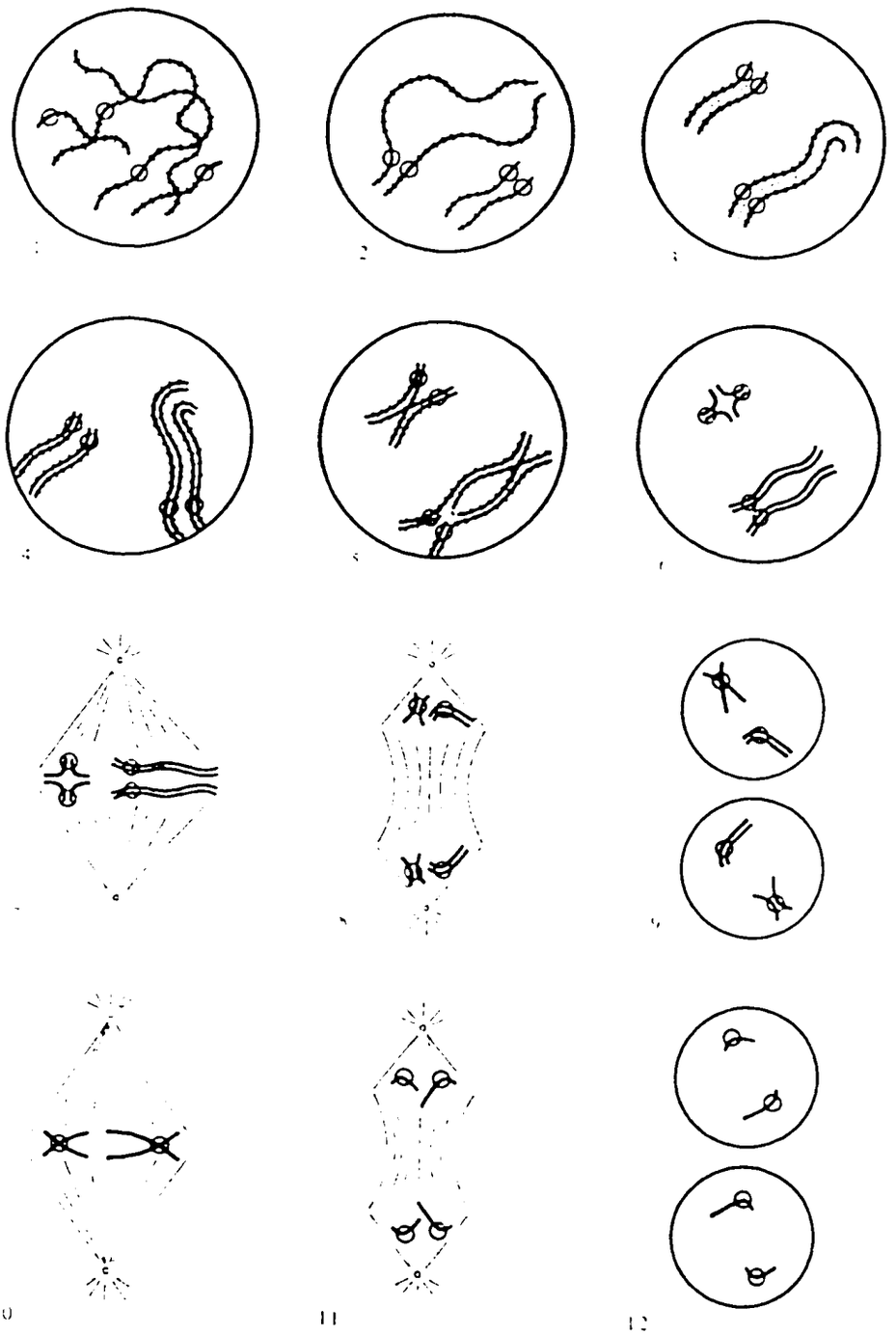
1.8 Meiosis and meiotic maturation in oocytes

Meiosis is a process that is essential for the production of genetically unique organisms and ensures the genetic diversity of the species. It is a type of nuclear division that is unique to germ cells and is responsible for the generation of haploid cells (oocytes) from diploid cells (PGC). This reduction division is quite similar to mitosis in terms of the cell machinery utilized and is illustrated in Figure 1.6. During interphase of both processes, the chromosomes duplicate so that each chromosome is comprised of two identical chromatids joined at the centromere. The nuclear envelope break down occurs during mitotic prophase. In contrast to mitosis, meiotic prophase I is extended and further subdivided into four stages. In leptotene, the chromosomes remain long and thin; in zygotene, the homologous pairs of chromosomes lie side by side; during pachytene, they associate along their length to allow for recombination to occur; and, in diplotene/diakinesis, recombination is completed and the cell now enters metaphase I. In metaphase I, the nuclear

Figure 1.6

Diagram showing the behaviour of chromosomes during meiosis.

Chromosome behaviour at the different stages of the meiotic cell cycle are illustrated using two pairs of chromosomes as an example. For simplicity only the cell nuclei are shown. 1. leptotene; 2. zygotene; 3. pachytene; 4. late pachytene; 5. diplotene; 6. diakinesis; 7. metaphase I; 8. late anaphase I; 9. telophase I; 10. metaphase II; 11. anaphase II; 12. telophase II. Taken from Baker (1982).

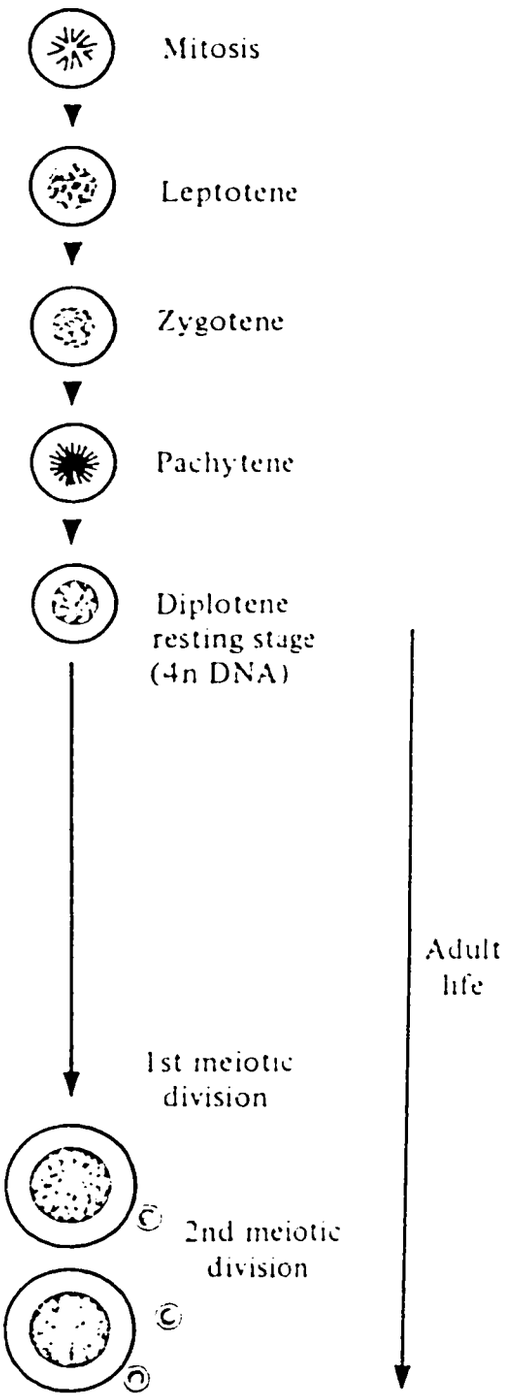


envelope breaks down and the pairs of chromosomes lie on the metaphase plate which is the region at the equator of the spindle. Homologous chromosomes separate in anaphase I and unlike mitosis where the nuclear envelope reforms in telophase, in meiotic telophase I, the cells immediately enters a short interphase between meiosis I and II, and no DNA replication occurs. (DNA replication occurs at each mitotic interphase.) The already replicated chromosomes go through the stages corresponding to a mitotic division in which individual chromosomes segregate to opposite poles to produce two haploid nuclei. Oocyte meiosis in mammalian females is a protracted process involving a series of meiotic arrests and resumptions (see Fig 1.7). Although oocytes enter meiosis during fetal life, the process is arrested at the time of birth in the diplotene stage of meiotic prophase I. This extended diplotene stage corresponds to the G_2 phase of the cell cycle (Dekel, 1995). Although Kit expression is transiently lost in oocytes entering meiosis, Kit mRNA expression reappears as the oocytes are entering the diplotene stage of meiosis during fetal life and persists throughout all stages of follicular development (Manova et al., 1990). Beginning at sexual maturity, which corresponds to 4 weeks after birth in rodents, cohorts of oocytes develop along with their surrounding granulosa cells. In response to a surge in gonadotropins, the oocyte completes the first meiotic division (telophase I) (Lindner et al., 1974; Tsafriri et al., 1982; Schultz et al., 1983; Eppig et al., 1983) and an asymmetric cytokinesis occurs forming a secondary oocyte and a polar body, both of which contain a haploid chromosome complex. The polar body, being devoid of most cytoplasmic components, subsequently degenerates. The second meiotic division is initiated immediately in the secondary oocyte, but the oocyte becomes arrested at metaphase II until the penetration by the spermatozoan at fertilization.

Figure 1.7

Life cycle of female germ cells.

PGC divide mitotically until gonadal sex differentiation. During fetal development, all female germ cells enter meiosis and remain arrested in prophase I of the meiotic cell cycle until after birth. Beginning at sexual maturity, small cohorts of oocytes develop and resume meiosis. Taken from Byskov (1982).


Mitosis
Leptotene
Zygotene
Pachytene
Diplotene
resting stage
(4n DNA)
Adult
life
1st meiotic
division
2nd meiotic
division

Meiosis II continues in the secondary oocyte, and again, an unequal cytoplasmic division occurs resulting in the formation of a polar body. Following the completion of meiosis II, the oocyte nucleus (haploid) and sperm nucleus (haploid) fuse resulting in a diploid cell, the *zygote*. All subsequent cell divisions in this newly formed preimplantation embryo occur via the mitotic cell cycle. Thus, in the female, each PGC gives rise to only one (haploid) oocyte.

Oocyte maturation is defined as the reinitiation and completion of the first meiotic division and the subsequent progression to metaphase II (Eppig, 1993). The nucleus of the oocyte is called the *germinal vesicle*. The first morphological indicator of oocyte maturation is the disappearance of the oocyte's germinal vesicle, a process which is termed germinal vesicle breakdown (GVBD). The second morphological indicator of oocyte maturation is the formation of the first polar body (Pb). Both GVBD and Pb formation morphologically indicate progression of the oocytic cell cycle to metaphase II phase which is commonly referred to as G_2 to M transition.

1.9 Role of Granulosa Cells in the inhibition of oocyte meiosis in antral follicles

One very important function of granulosa cells in antral follicles is to regulate oocyte meiotic maturation by preventing the progression of meiosis in fully grown oocytes until such time as the follicle is fully developed. The ability of oocytes to resume meiosis is acquired during the growth and development of the follicle. Only oocytes of antral follicles are able to undergo meiotic maturation and are considered *meiotically competent*, whereas oocytes from preantral follicles are *meiotically incompetent*. This acquisition of meiotic competence is correlated not only with follicular morphology but also with oocyte size

(Eppig, 1993). Some morphological differences have been identified in competent and incompetent oocytes which indicate that the acquisition of meiotic competence may be considered as the reinitiation of meiotic prophase I (reviewed Eppig, 1993).

Although meiotic maturation of oocytes is initiated by an *in vivo* gonadotropin surge, oocytes removed from antral follicles will spontaneously resume meiosis (Pincus and Enzmann, 1935), leading to the hypothesis that the granulosa cells of the follicle produce meiosis-inhibiting factors to maintain the oocytes in a meiotically-arrested state until GVBD is induced by the preovulatory gonadotropin surge. Thus, atretic degeneration of the follicle would cause a disruption of this meiosis-arresting system and result in oocyte maturation (Eppig, 1993). Normal oocyte growth and the acquisition of developmental capabilities require this maintenance of meiotic arrest. Indeed, disruption of meiotic inhibition within the follicle can, under certain circumstances, result in parthenogenetic activation of the oocyte and the formation of intrafollicular teratomas (Eppig, 1978; Furuta et al., 1995).

In the last 20 years, a number of researchers have investigated the role of cumulus cells in mediating the signals that maintain the oocyte in meiotic arrest. A number of molecules found within follicles have been shown to delay or inhibit spontaneous oocyte maturation including cyclic adenosine monophosphate (cAMP) (Cho et al., 1974; Dekel and Beers, 1978), follicle-stimulating hormone (FSH) (Eppig et al., 1983; Salustri et al., 1985; Vanderhyden and Armstrong, 1990), purines (Eppig et al., 1985) and müllerian inhibiting substance (MIS) (Takahashi et al., 1986).

1.9.1 Role of cAMP

Oocytes removed from antral follicles experience a sudden drop in intracellular cAMP levels (Aberdam et al., 1987) and GVBD occurs in these oocytes. When oocytes are cultured in the presence of membrane-permeable analogues of cAMP, oocyte maturation is inhibited in a dose-dependent and reversible manner (Cho et al., 1974; Magnusson and Hillensjo, 1977; Dekel and Beers, 1978). Stimulation of isolated oocytes with forskolin, a reversible activator of the catalytic subunit of adenylate cyclase (Seamon et al., 1981), resulted in a maintenance of meiotic arrest for short periods of time (Dekel et al., 1984). GVBD was inhibited by preventing the normal decrease in oocytic cAMP levels by treatment with pharmacological agents that inhibit phosphodiesterase activity and prevent the degradation of cAMP, such as 3-isobutylmethylxanthine (IBMX) and theophylline (Cho et al., 1974; Magnusson and Hillensjo, 1977). Inhibition of cAMP-dependent kinase, protein kinase A (PKA), induced GVBD in IBMX-arrested oocytes, suggesting that phosphorylation via the PKA pathway mediates the actions of cAMP on the G_2 to M transition (Bornslaeger et al., 1986a).

These experiments convincingly demonstrate the importance of cAMP in the *in vitro* maintenance of G_2-arrested oocytes, however, they do not demonstrate the importance of cAMP in the maintenance of intrafollicular meiotic arrest. This was shown using a culture system in which oocytes were developed within preantral follicles so that they become GVBD competent during the incubation period but remain G_2-arrested while within the follicle. GVBD was induced in follicles cultured in the presence of Rp-adenosine-3'-5'-cyclic phosphorothioate, a membrane-permeable antagonist of cAMP that competes with

endogenous cAMP for the regulatory subunit of PKA (Eppig, 1991). Thus, endogenous intrafollicular maintenance of meiotic maturation *in vivo* requires the participation of cAMP and its dependent pathways.

1.9.2 Role of FSH

Follicular development is dependent on FSH stimulation. Along with its many effects in follicular development, FSH may participate in the maintenance of meiotic arrest within the follicle. FSH transiently inhibits meiotic maturation in oocytes cultured in the presence of cumulus cells (Schultz et al., 1983; Eppig et al., 1983; Salustri et al., 1985; Vanderhyden and Armstrong, 1990). This inhibition is due to an accumulation of intraoocyte cAMP which is generated in the cumulus cells and passed to the oocyte by the metabolic coupling that exists between the two cell types. FSH-stimulated inhibition of G_2 to M transition is potentiated by the presence of testosterone (Schultz et al., 1983).

1.9.3 Role of purines

Hypoxanthine and adenosine have been isolated in millimolar quantities in rodent follicular fluid (Eppig et al., 1985). These two substances synergistically and reversibly maintain meiotic arrest for up to 24 hr in oocytes cultured in the presence or absence of the cumulus cells *in vitro* (Eppig et al., 1985; Downs et al., 1985). Adenosine has been shown to elevate cAMP levels in granulosa cells (Polan et al., 1983) and hypoxanthine has been shown to be a phosphodiesterase inhibitor (Chasin and Harris, 1976). Furthermore, a combination treatment of oocytes with hypoxanthine and adenosine resulted in a greater

accumulation of cAMP than with either treatment on its own (Downs et al., 1989). These purines may act synergistically by a stimulation of cAMP generation by adenosine and an inhibition of cAMP degradation by hypoxanthine. The concentration of purines required to inhibit oocyte maturation *in vitro* is well within the quantities isolated from follicular fluid.

1.9.4 Role of müllerian-inhibiting substance

Müllerian-inhibiting substance (MIS), a protein similar in structure to transforming growth factor-β (TGF-β), has been detected in the cumulus granulosa cells of antral follicles of the rat ovary (Ueno et al., 1989; Hirobe et al., 1992). High MIS activity was detected in those rat ovaries with the highest number of antral follicles (Ueno et al., 1989). Addition of MIS to either cumulus-enclosed or cumulus-free oocytes resulted in dose-dependent inhibition of spontaneous GVBD (Takahashi et al., 1986). There was no synergistic effect of MIS in combination with IBMX or cAMP indicating that MIS-stimulated inhibition of GVBD occurs in a cAMP-independent manner (Takahashi et al., 1986).

1.9.5 Role of protein synthesis

In mice and rats, GVBD occurs in 2 to 3 and 3 to 4 hours, respectively. GVBD is not blocked by incubation with inhibitors of protein synthesis such as cyclohexamide (Schultz and Wassarman, 1977), indicating that the necessary proteins are already present. When oocytes are cultured in the presence of 6-dimethylaminopurine (DMAP), an inhibitor of protein phosphorylations, GVBD is inhibited demonstrating the requirement for protein phosphorylations for GVBD (Eppig, 1993). Moreover, 6-DMAP treatment after GVBD

induces chromosome decondensation and the reformation of the germinal vesicle, morphologically reverting the cell nucleus to a prophase-like stage (Szollosi et al., 1991).

1.9.6 Role of maturation promoting factor

Maturation promoting factor (MPF), which has come to be known as M-phase promoting factor, was first identified in oocytes as a cytosolic factor which would promote the G_2 to M transition (Balakier and Czolowska, 1977; Masui and Clarke, 1979). It was later shown that this molecule is a key player in entry to the mitotic cell cycle of somatic cells (Sunkara et al., 1979). MPF is a complex of two components: $p34^{cdc2}$, a 34kD protein that is homologous to cdc2 of the fission yeast (Nurse and Thuriaux, 1980) and a 45kD cyclin protein similar to other B-type cyclins (Fig. 1.8). The B-type cyclin levels vary as they are subjected to periodic synthesis and degradation, whereas $p34^{cdc2}$ levels remain stable throughout the cell cycle. Well before G_2 to M transition, a pool of inactive MPF is present in the cell. Activation of MPF requires the removal of phosphate from tyrosine and threonine of the $p34^{cdc2}$ and this activity is performed by cdc25 which itself must be activated by serine/threonine phosphorylations (Kumagai and Dunphy, 1991; Gautier et al., 1991; Strausfeld et al., 1991). Thus, while oocytes are G_2-arrested, $p34^{cdc2}$ is present as a phosphorylated protein (Goren and Dekel, 1994), and its phosphorylation renders the complex inactive (Morla et al., 1989). Phosphorylated forms of $p34^{cdc2}$ are detected in G_2-arrested oocytes and disappear after the onset of meiosis (Choi et al., 1991; Goren and Dekel, 1994). The substrate for active $p34^{cdc2}$ is H1 histone. The obligatory dephosphorylations

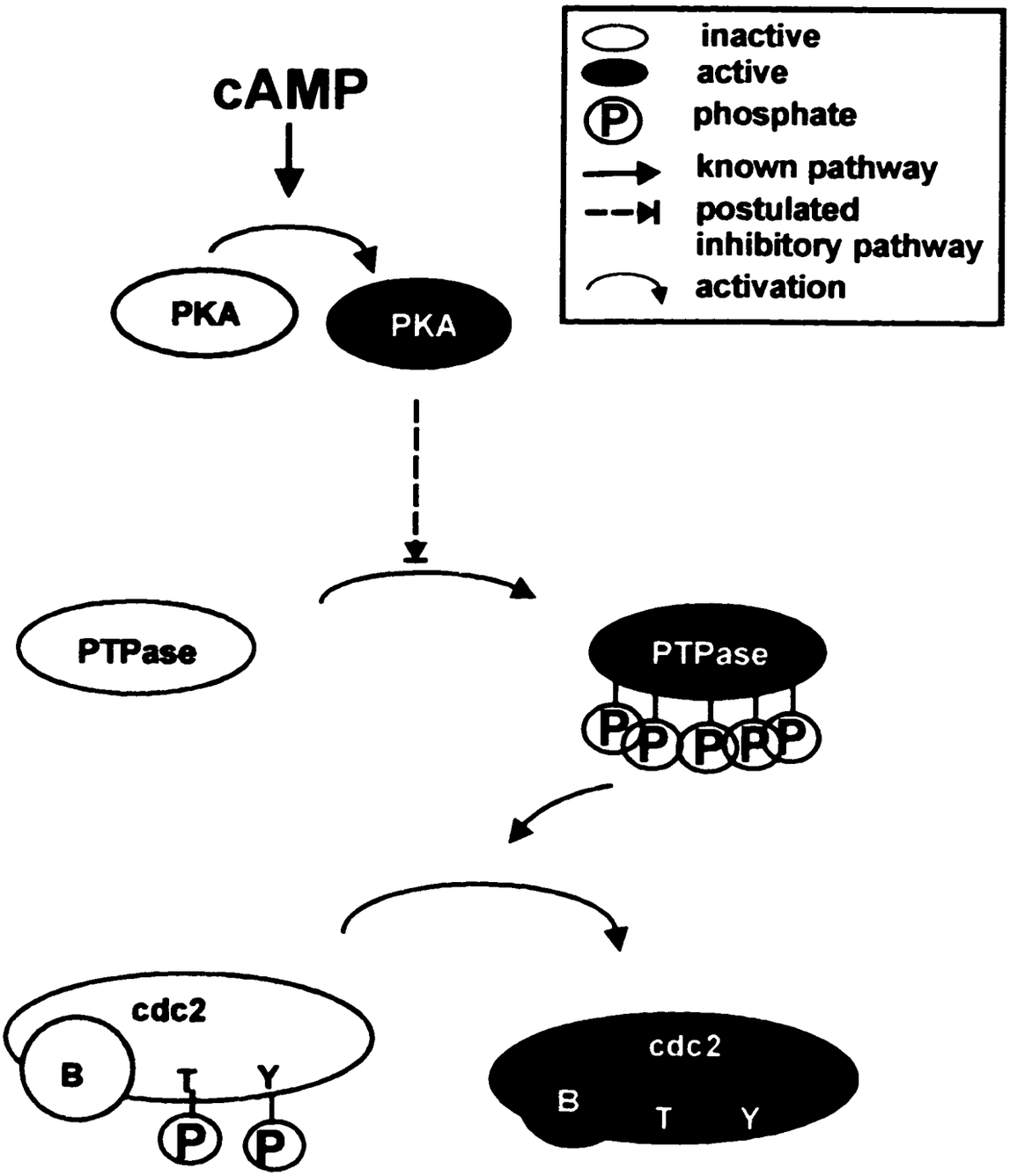
which render $p34^{cdc2}$ active, result in an increase in H1 histone kinase activity and correlates with a transition to metaphase I (Choi et al., 1991).

Cyclic AMP may be an important physiological regulator of G_2 to M transition; however, its role in relation to $p34^{cdc2}$ is not yet clear. Although approximately 2 to 4 hr is required from the time at which the oocytes are removed from the antral follicle to the time they undergo observable germinal vesicle breakdown, rat oocytes experience an irreversible commitment to undergo meiotic maturation within 1 hr of isolation from follicles (Dekel and Beers, 1980). Within 1 hr of oocyte retrieval, a drop in intraoocytic cAMP levels (Aberdam et al., 1987) is observed but $p34^{cdc2}$ remains phosphorylated and, therefore, inactive (Goren and Dekel, 1994). The dephosphorylation of $p34^{cdc2}$ which is associated with the reinitiation of meiosis in both rat and mouse oocytes can be prevented in the presence of IBMX, indicating that elevated cAMP levels may be involved in maintaining $p34^{cdc2}$ in a phosphorylated state (Goren and Dekel, 1994; Choi et al., 1991). In addition, treatment of rat oocytes with vanadate, a general tyrosine phosphatase inhibitor, resulted in a continued phosphorylation of $p34^{cdc2}$ and these oocytes remained G_2-arrested despite a drop in oocytic cAMP levels (Goren and Dekel, 1994; Goren et al., 1994). Taken together these data suggest that i) cAMP acts upstream of $p34^{cdc2}$ activity in the maintenance of meiotic arrest, and that ii) elevated cAMP levels may maintain $p34^{cdc2}$ in a phosphorylated state by indirectly preventing a PTPase activity which is associated with G_2 to M transition (Fig 1.8).

Figure 1.8

Postulated role of cAMP in the inhibition of spontaneous meiotic maturation.

Elevated levels of cAMP activate cAMP-dependent kinase A (PKA) which inhibit the activation of a vanadate-sensitive phosphatase (PTPase) by preventing its phosphorylations. In the presence of inactive PTPase, cdc2-cyclin B complex is maintained in a phosphorylated, and thus inactive, state and the cell remains arrested in G_2 of the cell cycle. Without cAMP stimulation, the inhibitory actions of PKA on PTPase activity are relieved and PTPase is activated by phosphorylations. Phosphorylated PTPase activates the cdc2-cyclin B complex by dephosphorylating on threonine (T) and tyrosine (Y) residues and this action allows the meiotic cell cycle to progress to M phase.



G_2-arrested

G_2 to M transition

1.10 Rationale

It is clear that expression of KL and Kit are essential for the normal embryonic development of mammals. Furthermore, the phenotypes of the various viable mutants suggest that normal expression of these genes is necessary for fully-functional reproduction in both males and females. The ovary is an organ that continues to develop in the animal after birth and, for this reason, serves as an excellent model to investigate developmentally important genes. Appropriate signals for both oocyte growth and follicular development must be coordinated in order to attain full reproductive function and perturbations in the system are easily identified.

Since many of the *Kit* and *Mgf* mutants suffer from reproductive aberrations my hypothesis is that these genes serve an essential function in the reproductive system. The aims of the work described in this thesis were to characterize the expression of Kit and KL in the rat reproductive system and to determine the function of KL/Kit interaction with a particular interest in the expression and function of KL in the ovary. My specific aims are summarized below:

1 To describe the site of Kit and KL production in rat ovarian follicles and to investigate the possible hormonal and developmental regulation of follicular KL production.

2 To determine if KL might be one of the granulosa cell products that inhibits meiotic maturation in oocytes. In addition, experiments were done to compare the meiosis-inhibiting actions of KL and cAMP, and to determine if there was a possible potentiation of KL-induced inhibition of meiosis with cAMP treatment.

3 To identify developmental regulation of the isoforms of KL mRNA. In particular, these experiments were designed to determine if the resumption of oocyte meiosis in response to LH is associated with a decrease in Kit receptor levels or a change in quality or quantity in granulosa cell KL expression.

4 To compare the relative effects of membrane-bound versus soluble KL in the inhibition of meiotic maturation and to determine factors which alter KL expression.

5 To investigate Kit and KL expression in the rat ovarian surface epithelium. Experiments were designed to determine the effect of hormone stimulation on Kit and KL expression in ovarian epithelial cells. The effects of hormone treatment on epithelial cell steroidogenesis and proliferation were also determined in order to correlate these parameters in relation to changes in gene expression.

6 To complete the investigation of KL expression in the female reproductive system, by determining KL mRNA expression in the uterus and oviduct and to determine the effects of KL on the preimplantation embryo development.

The rat has been particularly useful for the characterization of ovarian function in small mammals because of the relative ease with which large numbers of granulosa cells and oocytes can be collected. The rat model is particularly advantageous as a comprehensive knowledge base exists and enables us to interpret the findings described in this thesis with respect to known functional information, and it is for this reason that we chose to characterize the expression and function of Kit and KL in the rat ovary. Although the original mutations were first identified in mice, spontaneous *Kit* locus mutations have also been

reported in rats (Tsujimura et al., 1991). The work involving the effect of KL on embryo development was done using mouse embryos because, based on the literature, successful *in vitro* development of rat embryos is more difficult to achieve.

CHAPTER 2 - EXPRESSION OF KIT AND KL IN RAT OVARIAN FOLLICLES

2.1 Introduction

The patterns of expression and functional activity of the Kit receptor and its ligand in the developing embryo have been described in detail in Chapter 1. Although KL and Kit serve an essential function in the development of the ovary before the birth of the animal, Kit continues to be expressed in increasing amounts on the surface of primordial, growing and fully-grown mouse oocytes (Manova et al., 1990; Orr-Urtreger et al., 1990; Horie et al., 1991). In mouse preantral follicles, the source of KL appears to be the granulosa cells (Horie et al., 1991; Keshet et al., 1991). Although the presence of KL and its receptor may only suggest a possible function, strong evidence for the importance of ligand-receptor interactions is provided by the phenotypes of *Mgf*$^{Sl\text{-}d/Sl\text{-}d}$, *Mgf*$^{Sl\text{-}pan/Sl\text{-}pan}$ and *Mgf*$^{Sl\text{-}con/Sl\text{-}con}$ ovaries (see Table 1.2). Oocytes are present in these ovaries, albeit in reduced numbers, but follicular development is arrested, indicating the need for the presence of the ligand for continued ovarian function.

In postnatal testes, studies demonstrate that both Kit mRNA and protein are expressed at high levels in spermatogonia (Yoshinaga et al., 1991; Sorrentino et al., 1991) and experiments using homogeneous populations of mouse testicular cells reveal that Sertoli cells are the site of KL expression (Tajima et al., 1991; Rossi et al., 1991). Although it is clear that cAMP increased the mRNA levels for KL in Sertoli cells (Rossi et al., 1991), whether FSH is the biological stimulus for this response has not been resolved. Sertoli cells, like their female homolog the granulosa cells, mediate the actions of gonadotropins and

steroid hormones on germ cells, and it appears that KL may be one of the substances used by the somatic cells to transmit the hormonal signals.

The major focus of this chapter is to investigate the expression of KL mRNA and protein in the rat ovary. First, the expression of Kit on rat oocytes and the expression of KL mRNA in rat ovaries was determined. The second objective was to determine the site(s) of KL mRNA and protein expression in rat ovaries. Although in preantral mouse follicles it has already been demonstrated that granulosa cells produce KL, here the expression of KL in other stages of follicular development was investigated with a particular interest in the expression of KL in granulosa cells after their differentiation to mural and cumulus subpopulations. Furthermore, since hormones regulate many functions in the ovary, the expression of KL mRNA in response to gonadotropins or steroids was investigated to determine if KL serves as a mediator of hormone signals in the follicle.

2.2 Materials and Methods

2.2.1 Animals

Prepubertal female Sprague-Dawley rats were obtained from either the animal colony at the University of Ottawa or Charles River Canada Inc. (St. Constant, Quebec). Animals were allowed free access to food and water, and lighting was provided for 14 hr daily. Some animals were injected intraperitoneally at 26 days of age with 7.5 IU of pregnant mares' serum gonadotropin (PMSG; Equinex: Ayerst, Montreal). Animals were sacrificed at 40 hr after PMSG injection and ovaries were fixed for localization experiments or ovaries were

immediately used for isolation of total RNA. Other animals were injected subcutaneously with 1 mg diethylstilbestrol (DES; Sigma Chemical Co., St. Louis, MO) in 0.1 ml sesame oil at 19 days of age and were subsequently injected 24 hr and 48 hr after the initial injection with the same amount of DES. Animals were sacrificed 24 hr after the final DES injection and ovaries were used for RNA isolation. Age-matched rats that had not been hormone treated were sacrificed and used as control animals and ovaries from these animals were either fixed or used to isolate total RNA. In experiments to determine the KL expression in neonatal rats, 1- to 2-day old animals were obtained from the animal care unit of the University of Ottawa. Animals were sacrificed and ovaries dissected. Whole ovaries were either immediately homogenized in 8M urea/3M LiCl for RNA extraction, submerged in fixative for localization studies or transferred into Waymouth MB 752/1 medium (WAY; Sigma) supplemented with 75 mg/L penicillin (Sigma), 50 mg/L streptomycin (Sigma), 25 mg/L sodium pyruvate (Sigma) and 3 mg/ml bovine serum albumin (BSA; ICN Biomedical, Costa Mesa, CA) for granulosa cell or oocyte isolation. Ovaries of gonadotropin-treated rats were placed in medium and punctured with 25-gauge needles, to extrude the contents of antral follicles. The ovary remnants were removed from the media and granulosa cells were isolated by centrifugation (3000 g for 4 min) and/or oocyte-cumulus cell complexes (OCC) were collected using a mouth pipette.

2.2.2 Localization of Kit in rat oocytes

Immunofluorescence was done to determine the presence of Kit receptors in rat oocytes of antral. To do this, oocytes were isolated from antral follicles of PMSG-primed rats, stripped of their cumulus cells by repeated pipetting and briefly submerged in acid

Tyrodes (NaCl, 8.0 g/L; KCL, 0.2 g/L; $CaCl_2$, 0.24 g/L; glucose, 1.0 g/L, pH 2.5) to dissolve the zona pellucida, yielding zona-free oocytes (ZF oocytes). ZF oocytes were incubated for 45 min in WAY/BSA to allow for recovery and then stained for Kit receptors as follows: in microtest Terasaki flat bottom Falcon tissue-culture plates (Becton-Dickinson, Mississauga, ON). ZF oocytes were rinsed in wash solution (100 mM Tris-HCl, 150 mM NaCl, pH 7.5 supplemented with 5% BSA; TBS/BSA) for a total of 3 min with 3 changes of wash solution. ZF oocytes were then fixed in 4% paraformaldehyde in phosphate-buffered saline for 15 min at room temperature. Oocytes were briefly rinsed twice, and then washed 3x5 min each in TBS/BSA. Oocytes were incubated in 2 ng/µl of affinity purified polyclonal rabbit Kit antibody (Santa Cruz Biotechnology Inc., CA) [diluted in TBS/BSA with 0.02% Triton X-100] for 1 hr at room temperature (or overnight at 4°C) in a humidified light-protected chamber. Oocytes were again rinsed twice and washed 3x 5 min each in TBS/BSA, and then exposed to a CY3-conjugated affinity purified donkey anti-rabbit IgG (Jackson Immunoresearch, West Grove, PA) at 1:100 dilution in TBS/BSA for 45 min at room temperature in a humidified light-protected chamber. These oocytes were mounted in 15% glycerol on glass slides (with a constructed glass chamber to preserve the three dimensional structure of the stained oocytes), coverslipped and sealed with nail polish to prevent drying. Fluorescent staining was visualized under a fluorescent confocal microscope using a rhodamine filter. Control experiments in which one or both antibodies were omitted revealed no background fluorescence due to the antibodies, nor any autofluorescence inherent to the oocyte.

2.2.3 Localization of KL in rat ovaries

a) Collection of ovaries

Control and treated ovaries were fixed by immersion in 4% freshly prepared paraformaldehyde (0.1 M phosphate-buffered saline) for 48 hr and then washed/dehydrated in 70% ethanol (at 4°C) for 48 hr with occasional changes. Tissues were dehydrated through a graded ethanol (BDH, Toronto, ON) series (70, 95 and 100%), cleared in xylene (BDH) at room temperature and embedded in melted Paraplast (Fisher Scientific, Fair Lawn, NJ). Sections (6 μm) were mounted onto either 3-aminopropyltriethoxysilane-coated glass slides as described previously (Rentrop et al., 1986) or commercially available Superfrost coated slides (Fisher Scientific, Ottawa, ON). Sections were allowed to dry overnight at 37°C before *in situ* hybridization or immunohistochemistry.

b) In situ hybridization

Sections were deparaffinized using xylene, rehydrated using a graded ethanol series (100, 95 and 70%) and placed in distilled water. Sections were then treated with proteinase K (type XXVIII, 10 μg/ml in 100 mM Tris-HCl, pH 7.5; Sigma) at 37°C for 10 min. After washing in distilled water at room temperature for 10 min, sections were air dried and hybridized with 500 pg/μl digoxigenin-labelled probe to KL (see below) diluted in hybridization buffer consisting of 50% formamide (BDH); 6 X standard saline citrate (SSC; 0.15 M NaCl, 0.015 M sodium citrate, pH 7.0); 50 mM Tris-HCl, pH 7.0; 2 X Denhardt's solution; and 0.2% sodium dodecyl sulfate (SDS). After the probe was added to the sections, they were hybridized for 18 hr at 50°C in a chamber humidified with 50% (v/v)

formaldehyde (BDH). The slides were washed twice in 2 X SSC for 10 min at room temperature, once in 0.1 X SSC for 30 min at 65°C and once in 0.1 X SSC for 20 min at room temperature. For immunological detection of probe-mRNA hybrids, the slides were immersed briefly in buffer no. 1 (100 mM Tris-HCl pH 7.5 and 150 mM NaCl) and preincubated with 1% blocking agent (Boehringer Mannheim, Laval, Que) in buffer no. 2 [buffer no. 1 with 0.3% Triton X-100 (Fisher)]. The slides were then incubated with alkaline phosphatase-conjugated digoxigenin antibody (Boehringer Mannheim) in buffer no. 2 at a 1:500 dilution for 2 hr. The slides were washed twice in buffer no. 1 for 30 min, and subsequently incubated with buffer no. 3 (100 mM Tris-HCl, 100 mM NaCl and 50 mM $MgCl_2$, pH 9.5) for 2 min to adjust the pH to 9.5. Alkaline phosphatase was detected by colour reaction with 4-nitroblue tetrozolium chloride (NBT; Boehringer Mannheim) and 5-bromo-4-chloro-3-indolyl-phosphate (BCIP; Boehringer Mannheim) by incubating slides in 0.33 mg/ml NBT and 0.17 mg/ml BCIP in buffer no. 3 for 18 hr. Levamisole (Sigma) was added to a final concentration of 0.2 μM to the colour reaction during the 18 hr incubation to reduce the endogenous alkaline phosphatase activity. Colour development was stopped by rinsing the slides in water overnight. Sections were dehydrated in a graded ethanol series (70, 95 and 100%) and xylene, then embedded in Permount (Fisher) and coverslipped.

Oligonucleotide Probe

An oligonucleotide probe of 27 bases (5' CCA TTT ATG TTC CCC CTG TTG CAG CC 3') corresponding to nucleotides 727-753 of the rat KL gene (Martin et al., 1990) was synthesized using a DNA synthesizer 391 (Applied Biosystems, Foster City CA). This

sequence is complementary to the RNA encoding a portion of the extracellular region of KL (see Fig.2.1). The probe was 3' end-labelled with digoxigenin-11-dUTP (Boehringer Mannheim) using terminal deoxynucleotidal transferase (Boehringer Mannheim) according to a previously published procedure (Farquharson et al., 1990) and applied to sections without purification.

Controls

To evaluate the specificity of the probe, the following controls were performed: (1) omission of the labelled KL probe from the hybridization incubation; (2) incubation using the labelled KL probe with unlabelled probe in excess (100:1 unlabelled:labelled probe); (3) incubation with a heterologous probe, i.e. a 24-base oligonucleotide (5' ATG ATT GAA CAA GAT GGA TTG CAC 3') complementary to nucleotides 151-174 of bacterial neomycin transferase and which does not share any common nucleotide sequences with KL. For all post-hybridization steps, the control sections were developed according to the above protocol and together with sections which were hybridized with labelled probe. Endogenous alkaline phosphatase activity was assessed by omitting the digoxigenin antibody from the immunological detection procedure.

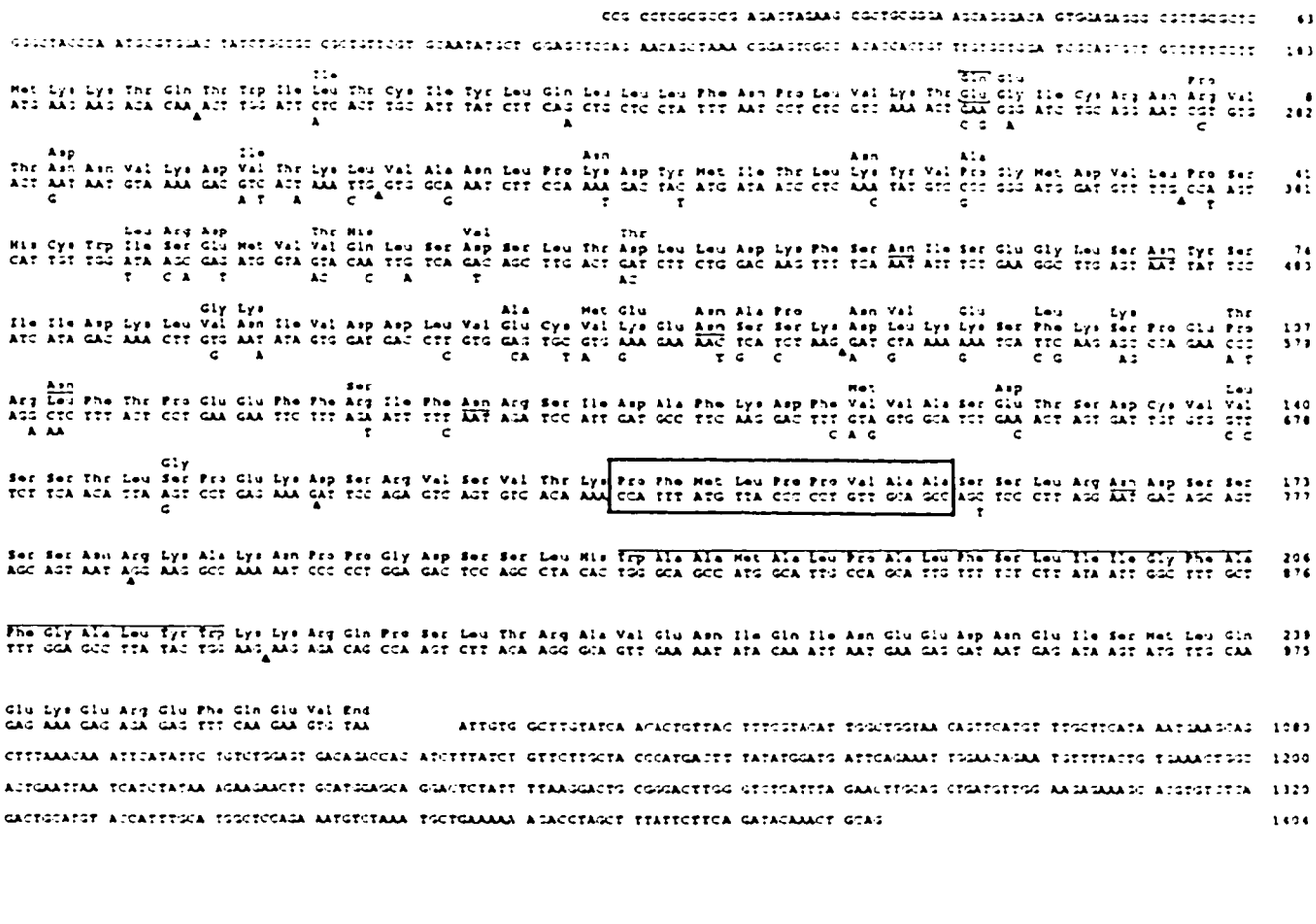
c) *Immunohistochemistry*

Sections of ovaries were deparaffinized using xylene, rehydrated using a graded ethanol series (100, 95 and 70%) and placed in distilled water. Endogenous peroxide activity

Figure 2.1

Sequence of KL cDNA as published by Martin and coworkers (1990).

The amino acid and nucleotide sequences of human KL are shown. The positions at which the rat amino acid and nucleotide sequences differ from those of human indicated above and below the human sequence, respectively. The nucleotides identified by the box have been used to generate oligonucleotide probes for *in situ* localization of KL mRNA. The hydrophobic transmembrane region is overlined. Triangles indicate the positions of intervening sequences. Taken from Martin et al., 1990.



was blocked using 1% H_2O_2 (BDH) diluted in phosphate-buffered saline (PBS). Slides were rinsed for 5 min with 3 changes in PBS, and the antigenic sites were exposed by treating slides with 0.1% trypsin in PBS for 15 min at room temperature. Slides were washed vigorously by rinsing three times and then washing for 5 min in PBS. Sections were incubated with 20 µg/ml of a polyclonal goat anti-mouse KL antibody (R & D Systems, Minneapolis, MN) diluted in 0.3% triton X-100 in PBS, for 1 hr at room temperature in a dark humidified chamber. After incubation, slides were rinsed twice and washed three times for 5 min each in PBS. Sections were then exposed to a biotinylated donkey anti-goat secondary antibody (Jackson Immunoresearch) diluted 1:200 in PBS for 30 min under the same conditions as the primary antibody and washed as above. Slides were incubated with a horseradish peroxidase (POD)-conjugated Streptavidin antibody (Jackson Immunoresearch) diluted 1:200 in PBS and again washed as described above. To determine reactive antigenic sites, sections were exposed to diaminobenzidene (DAB) substrate detection kit according to manufacturer's instructions (Boehringer Mannheim) for 10 min. After colour development, slides were dehydrated and mounted with permount. To ensure antibody specificity, primary antibody was excluded or competing peptide was included in some experiments.

2.2.4 Northern analysis of KL in ovaries and granulosa cells

To examine the developmental expression of KL in whole ovaries or the hormonal regulation of KL in ovaries and granulosa cells, hormone treated (PMSG and DES) and untreated (control) animals were sacrificed by cervical dislocation, ovaries were excised and

immediately used for RNA extraction or were placed in WAY/BSA for granulosa cell isolation and subsequent RNA extraction. Ovaries or freshly isolated granulosa cells were immediately immersed in 3M LiCl/8M urea. Tissues or cells were homogenized and RNA was precipitated overnight at 4°C. RNA was collected from the ovaries and granulosa cell homogenates using a LiCl precipitation protocol (Sambrook et al., 1989). RNA was also extracted from NIH 3T3 fibroblast cells, and P19 EC cells or Sl^4 fibroblast cells to use as positive (Huang et al., 1990) and negative (our findings and Williams et al., 1990) hybridization controls, respectively.

RNAs were fractionated on 0.9% agarose-formaldehyde gels and transferred overnight to HyBond nylon membranes (Amersham, Oakville, Ontario). To immobilize RNA the blots were either cross-linked at 125 J (nylon) in a cross linker, or exposed to ultraviolet light for 3 min. Blots were soaked in prehybridization solution (50% formamide, 1% SDS, 5 X SSPE, 2.5 X Denhardt's solution, 250 μg/ml carrier DNA) for 2-24 hr (Sambrook et al., 1989). Blots were then hybridized in prehybridization solution with [^{32}P] cDNA probes (specific activity at least 1 x 10^8 cpm/μg) labelled by random priming, using a Random Primed Labelling Kit (Boehringer Manneheim).

Probes

SalI fragment (2.06 kb) isolated from a plasmid containing a mouse KL cDNA (Williams et al., 1990) were used to probe the northern blots. After overnight hybridization, blots were washed in low stringency buffer (2 x SSC/0.1 % SDS) once for 30 min at room temperature, and high stringency buffer (0.2 x SSC/0.1 % SDS) twice for 15 min at 65°C with constant agitation. Signal was assessed by exposing blots to autoradiographic film (Life

Sciences Products; Guelph, ON) for 3-7 day exposure. In some cases, signal was assessed by exposing blots to phosphor screens which were scanned with a PSI phosphoimager (Molecular Dynamics; Sunnyvale, CA).

After hybridization to detect KL signal, blots were re-probed with [^{32}P] α-tubulin cDNA probes labelled by random priming in order to control for differences in loading. Intensity of the bands on the autoradiographic film were estimated by densitometry using an Ultrascan XL Enhance Laser Densitometer (LKB; Bromma, Sweden) and the ratios of KL/α-tubulin were calculated and standardized to untreated (control) values which were arbitrarily set to 1.0 for each northern blot. Histograms of mean ± standard error of the mean (S.E.M.) of at least 3 northern blots are presented. An unpaired t test was used to determine statistical significance between a single treatment compared to untreated values . Statistical comparisons involving multiple groups were made by analysis of variance (ANOVA) and when significant differences were seen, the Dunnett test was used for multiple comparisons. Significance was inferred at $p<0.05$ and is indicated with either letters or *.

2.3 Results

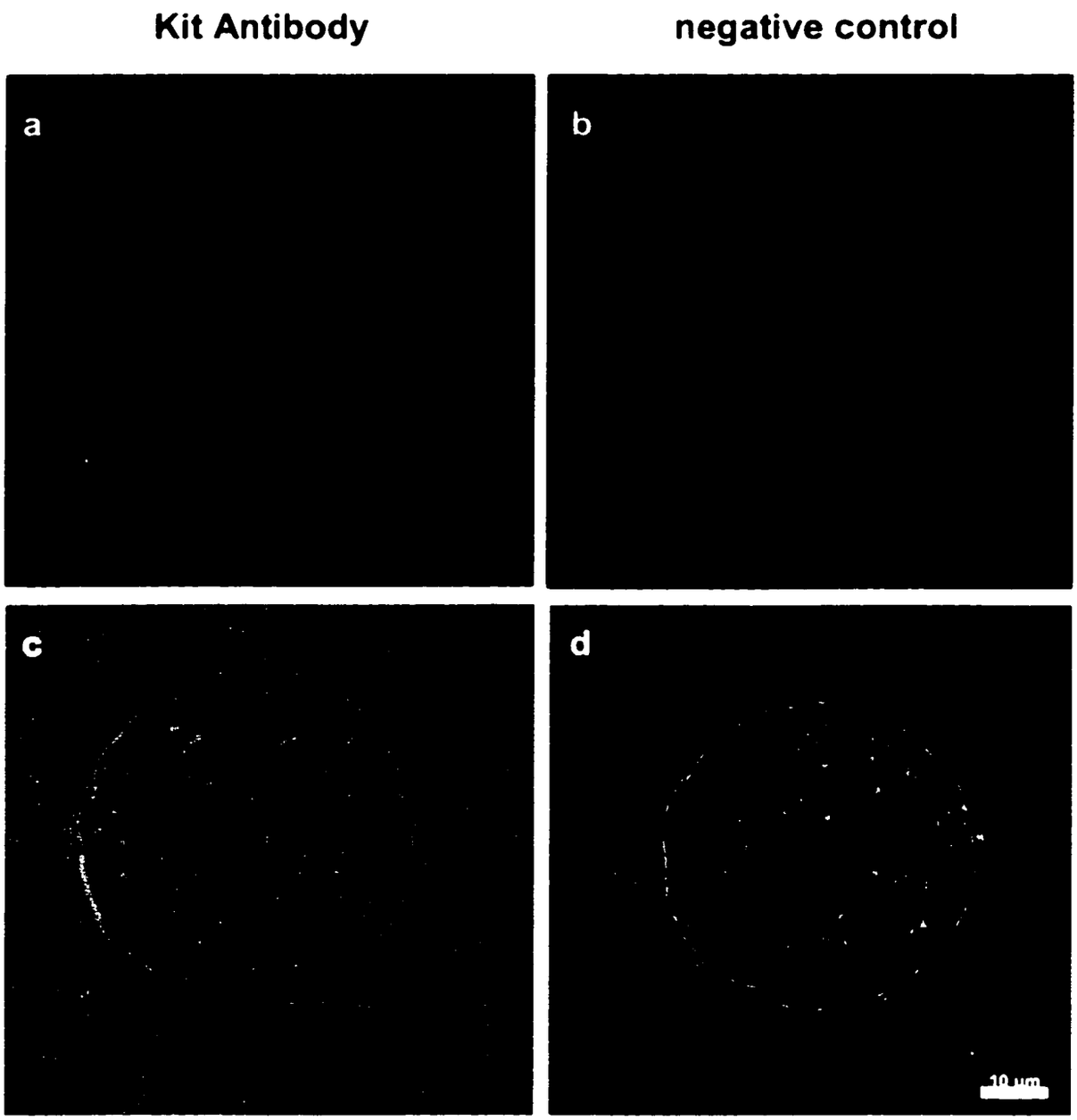
2.3.1 Localization of Kit receptors on rat oocytes

Immunofluorescence was performed to determine if Kit receptors were present on oocytes from PMSG-primed rats. Whole-mounted oocytes were exposed to Kit antibodies and subsequently visualized using fluorescent light on a confocal microscope. In Fig 2.2a, Kit receptors were detected on the surface of rat oocytes and were localized to the membrane of the oocyte. Fig. 2.2c is the transmitted light photomicrograph of the same

Figure 2.2

Detection of Kit receptors on rat oocytes using immunofluorescence

Kit was detected on the plasma membrane of oocytes when anti-Kit antibodies were used for detection (a), but not when these antibodies were not included (b). Transmitted light photomicrographs of (a) and (b) are shown in (c) and (d), respectively. No staining is evident in NIH 3T3 cells, a Kit negative cell line (data not shown).


Kit Antibody
negative control
a
b
c
d
10 μm

oocyte. Control experiments in which primary antibodies were omitted showed no fluorescence staining (Fig. 2.2b), indicating that there was no background staining due to the secondary antibody or autofluorescence of the oocyte. Furthermore MC/9 cells and LC-80 cells, two cell lines that express Kit receptors showed staining on the plasma membrane whereas NIH 3T3 cells, a line that does not express Kit receptors, showed no staining with this antibody (data not shown).

2.3.2 KL expression in the developing rat ovary

To determine the level of KL expression in the ovaries of neonatal and prepubertal rats, northern blot analysis was done using total RNA extracted from whole ovaries of 1-2 day old animals and 24 day old animals (Fig 2.3). To ensure the specificity of the probe, NIH 3T3 and Sl^4 fibroblast RNA preparations were run along side of the ovary RNA preparations to serve as positive and negative controls, respectively. In both neonatal and prepubertal rat ovary preparations, a single 6.5 kb band is evident and runs above the 28s ribosomal marker RNA (Fig. 2.3). When band intensities were normalized for loading differences, it was determined that there was a 3.5 ± 0.3 fold higher level of KL mRNA in the total RNA preparations isolated from the ovaries of neonatal rats compared to levels of KL mRNA in 24 day old rats.

2.3.3 Localization of ovarian KL

In situ hybridization was performed to localize KL mRNA transcripts in sections of 28 day old rat ovaries. A section of ovary from a rat primed with PMSG is shown in Fig. 2.4a,c,d and e. A positive hybridization signal for KL mRNA, as seen by a blue-purple

Figure 2.3

Northern blot analysis of KL mRNA in neonatal and prepubertal rat ovaries

Total RNA of ovaries from 1-2 day old and 24 day old rats were probed with a ^{32}P-labelled 2.06 kb KL cDNA probe (A). Gel electrophoresis was done with 15 μg of total RNA in each lane and total RNA samples from NIH 3T3 and Sl^{4} fibroblasts were included in the northern blot analysis to serve as positive and negative KL mRNA controls, respectively. The blot was then stripped and reprobed with ^{32}P-labelled α-tubulin (B). Intensity of the bands on phosphor screens was estimated using ImageQuaNT software provided with the PSI phophoimager, and the ratio of KL/α-tubulin values were standardized to levels seen in ovary RNA of 24 day old rats which were arbitrarily set to 1.0 (C). Each bar indicated in (C) represents mean KL/α-tubulin from 2 northern blots and error bars represent maximum and minimum data points. * indicates significant difference in KL/α-tubulin mRNA expression using unpaired two-tail t test where significant differences were inferred at $p>0.01$

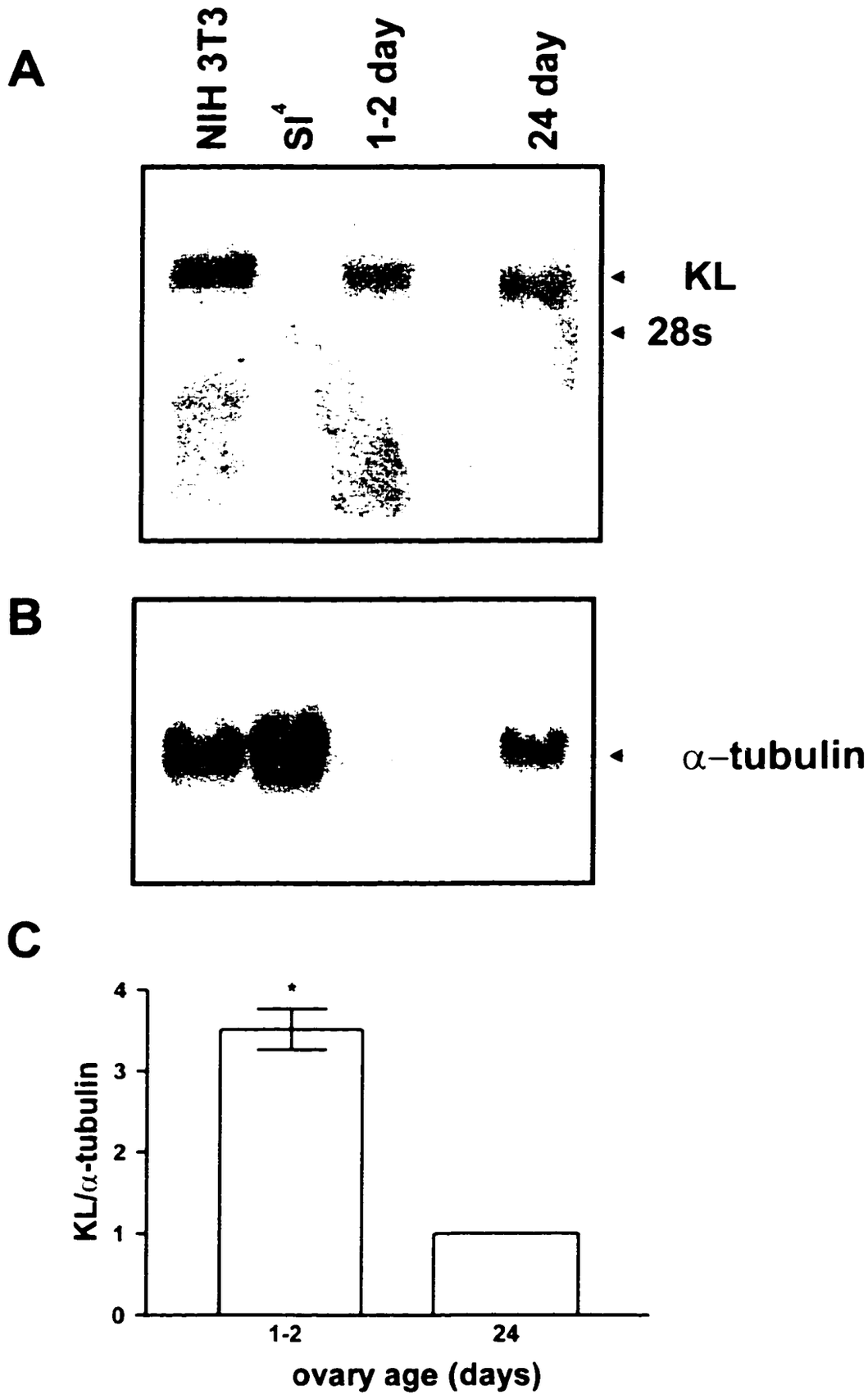
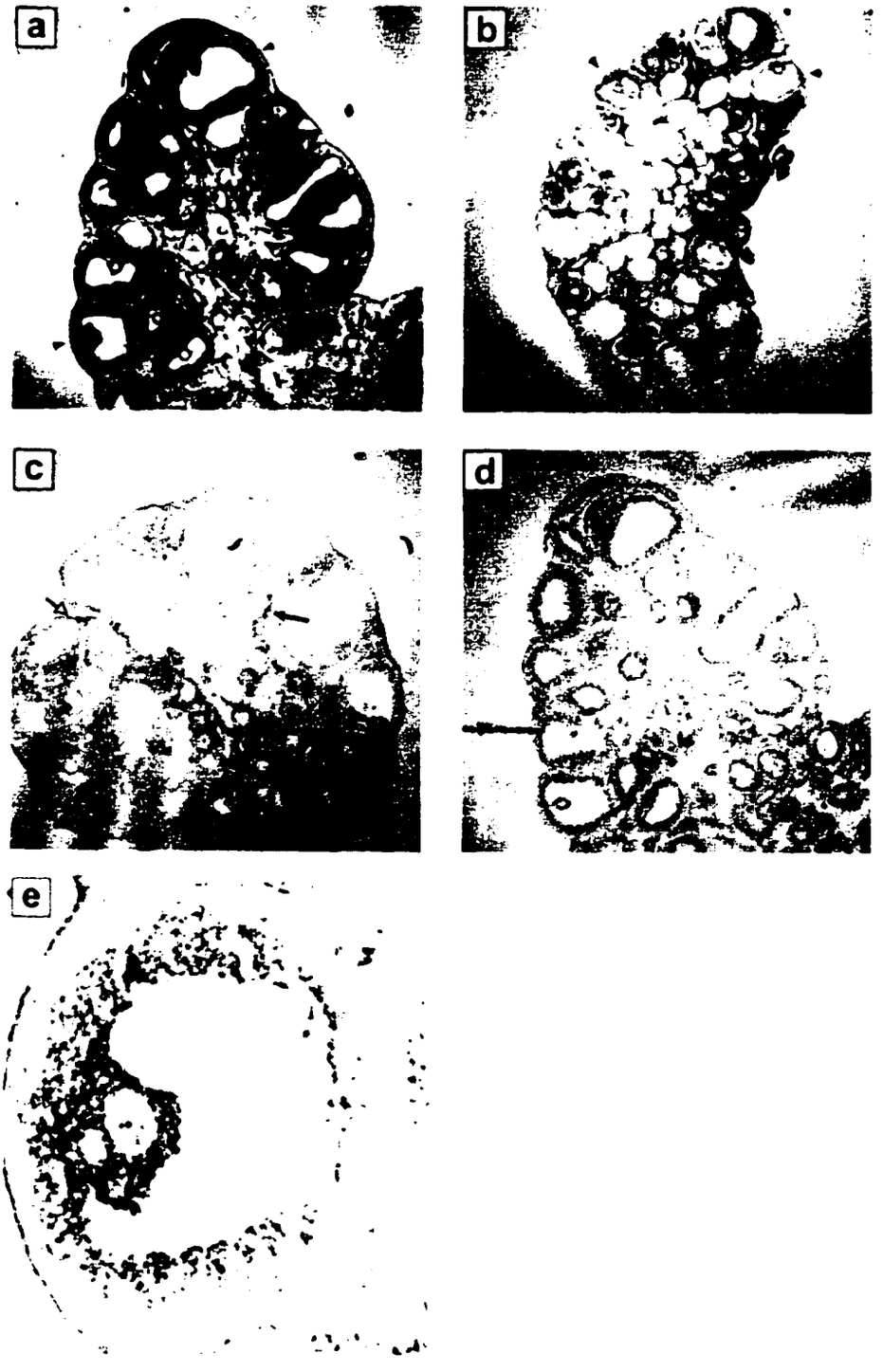

A
NIH 3T3
Sl4
1-2 day
24 day
KL
28s
B
α–tubulin
C
KL/α-tubulin
0
1
2
3
4
*
1-2
24
ovary age (days)

Figure 2.4

Localization of KL mRNA in the ovary by *in situ* hybridization.

Photographs are of bright-field images of 6 μm sections through whole ovaries from 28 day old animals. Sections are from PMSG-primed (a,c,d and e) or untreated (b) ovaries hybridized with a KL probe (a,b,e), 100 fold excess unlabelled KL probe (c), or neomycin transferase probe (d). Antral follicles are indicated by arrow heads. Non-specific binding of labelled probe in the presence of excess unlabelled probe (c) is found in some regions of the epithelium (open arrow) and the interstitium. (a-d: ×26; e: ×130).


a
b
c
d
e

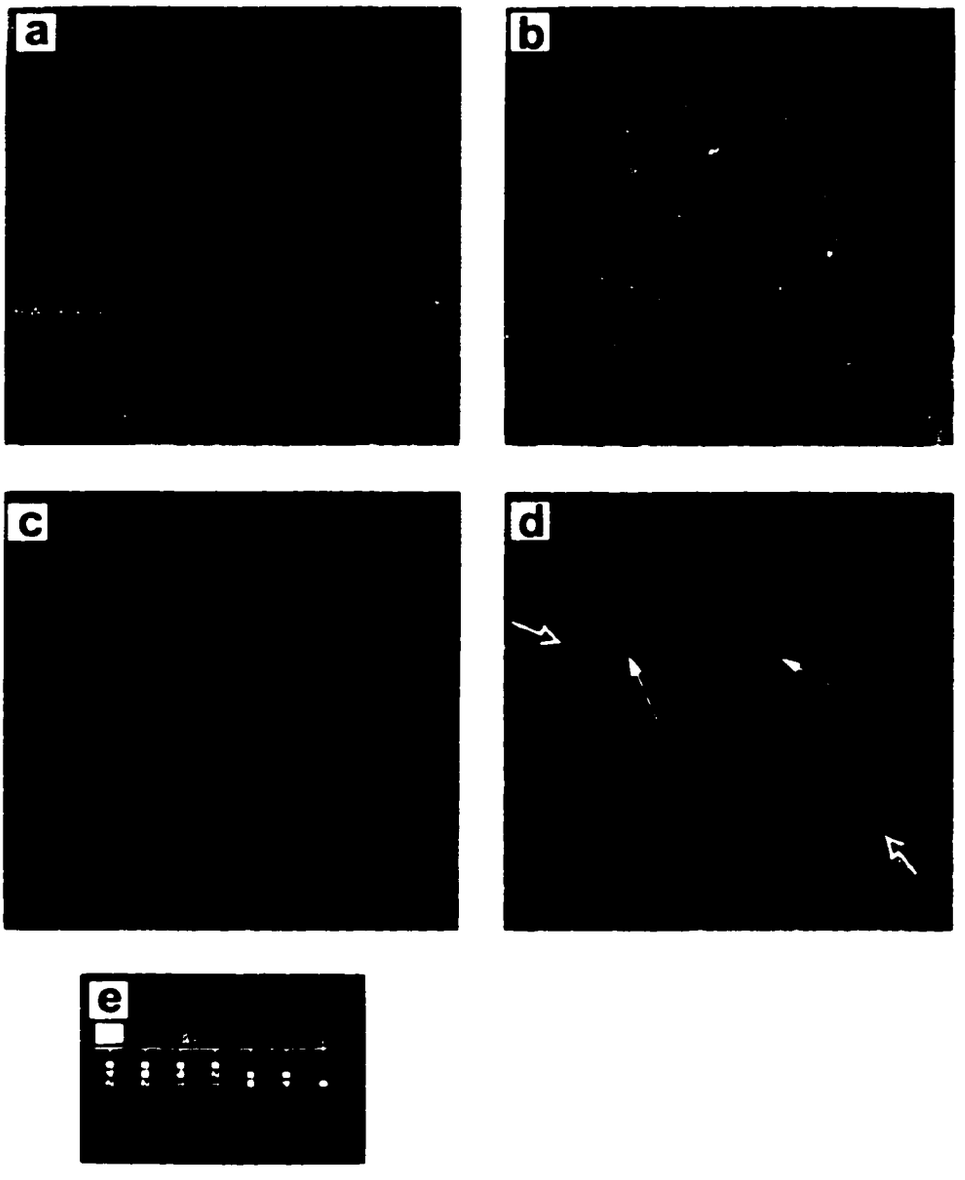
reaction product, was detected in the granulosa cells of the follicles in Fig 2.4a. In antral follicles, the number of KL transcripts was greatest in the cumulus granulosa cells that enclose the oocyte (Fig 2.4a). Furthermore, there was a gradient in the intensity of the staining reaction, with the cell layers closest to the oocyte and the antral surface having a greater intensity of colour than the cell layers closer to the basement membrane (Fig 2.4e). For the mural granulosa cells, the oocyte-containing side of the follicle generally displayed a darker staining reaction than on the opposite side of the follicle (Fig 2.4a). A section of an ovary from an untreated animal in shown in Fig 2.4b. A positive hybridization signal was evident in the granulosa cells of the follicles; however, there was less intense reaction products and the gradient in the intensity of the staining reaction away from the oocyte and antral surface was less pronounced in untreated follicles compared to PMSG-primed follicles (Fig 2.4a,b). There was no hybridization signal observed in the oocyte, antrum or theca layers of the follicle in any section. When the sections were incubated with excess unlabelled probe (Fig. 2.4c), there was some nonspecific binding of the labelled probe in areas of the surface epithelium, the basement membrane and the interstitium. No hybridization signal was detected in sections for which the probe or the digoxigenin antibody had been omitted (data not shown), or when the neomycin transferase oligonucleotide was used as a probe (Fig. 2.4d).

Confocal microscopy was used to perform densitometry to compare KL expression in ovarian follicles of PMSG-primed (Fig. 2.5a,b,d) and untreated (Fig. 2.5c) animals. An arbitrary scale of 0 to 240 units of alkaline phosphatase reaction product was used in which various intensities were colour coded (Fig. 2.5e). Fig. 2.5a,b are adjacent sections which

Figure 2.5

Colour densitometry of KL mRNA expression using *in situ* hybridization.

Photographs are of confocal microscopic images of 6 μm sections through whole ovaries from 28-day-old animals. Sections are from PMSG-primed (a,b,d) or unprimed (c) ovaries hybridized with a KL probe (b,c,d) or a neomycin transferase probe (a). e: The scale shown illustrates the range in which various intensities of alkaline phosphatase reaction product were colour coded. Strong hybridization signals (red and yellow) are found in the cumulus granulosa cells and in the mural granulsoa cells closest to the oocyte (solid arrows). Less hybridization (blue and green) occurs with increased distance from the oocyte and the antral surface (open arrows). (a,b: ×26; c,d: ×130).


a
b
c
d
e

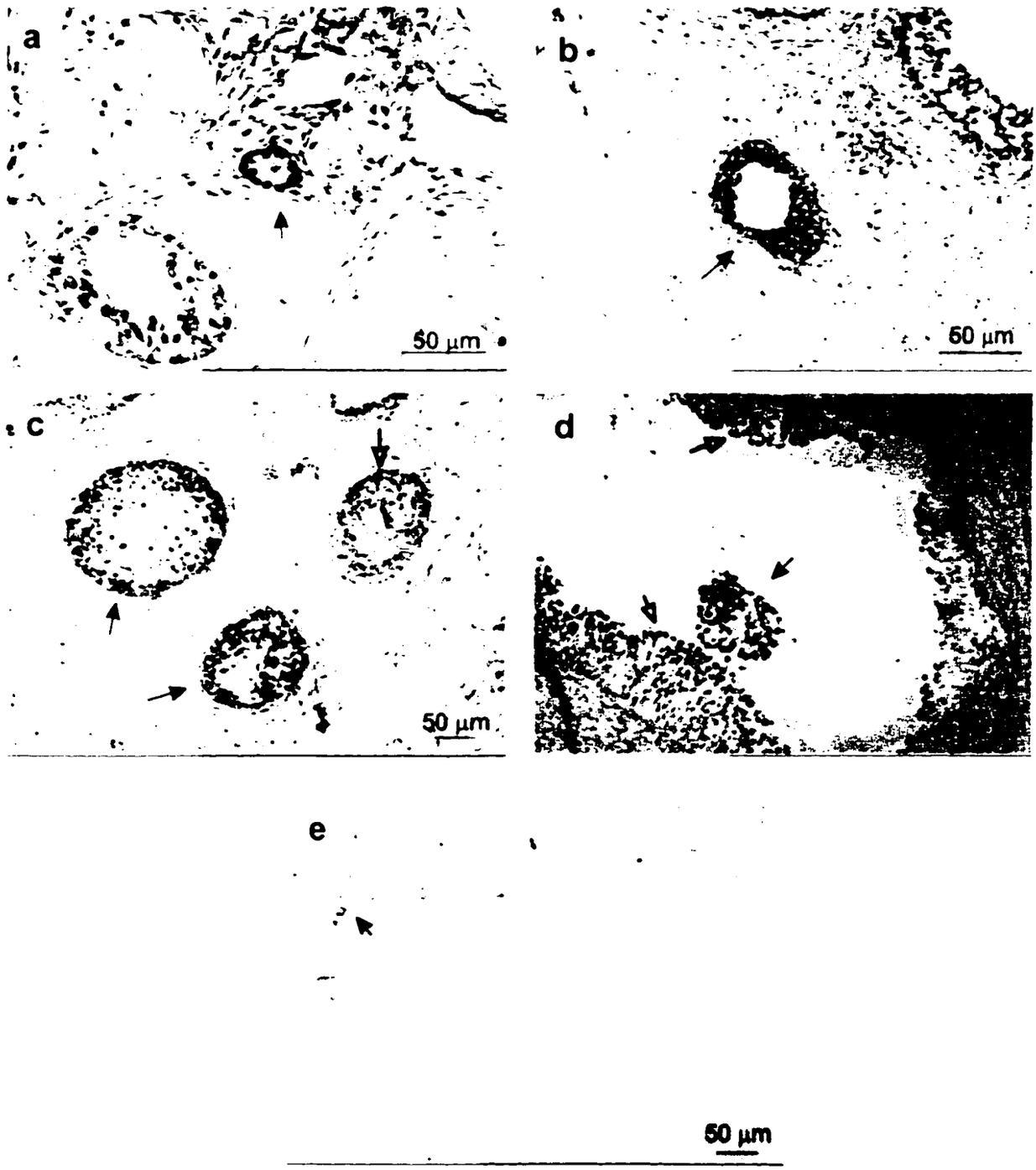
have been hybridized with neomycin transferase and KL probes, respectively. KL mRNA expression was seen specifically in the granulosa cells of the ovarian follicle as seen by the red and yellow colour (Fig 2.5b). There was higher red and yellow in the cumulus granulosa cells and in the mural granulosa cells closest to the oocyte. Less hybridization signal (indicated by blue and green colours) occurs with increased distance from the oocyte and with increased distance from the antral surface. When comparing KL mRNA expression in antral follicles from PMSG-primed (Fig. 2.5d) and untreated (Fig. 2.5c) rats, there was a greater amount of KL transcripts in the primed follicle as seen by higher amounts of red and yellow colour.

To determine the changes in KL protein expression with follicular development, immunohistochemistry was performed on sections of ovaries from 28-day old rats stimulated with PMSG. The antibody used was against the extracellular domain of KL protein and so would detect both isoforms of KL. A positive signal for KL protein was seen as a dark brown precipitate. KL was detected in the cuboidal granulosa cells of primary follicles located in the centre of the ovary (rete ovary) (Fig. 2.6a) and in all early preantral follicles (Fig. 2.6b). KL continued to be present in the granulosa cells of the late preantral/early antral follicles (Fig. 2.6b), however with formation of the antrum, KL protein was present predominantly in the cumulus granulosa cells and the mural granulosa cells lining the antral cavity (Fig 2.6d). Occasionally some diffuse brown staining was evident in the antral cavity, possibly due to soluble KL produced by the follicular granulosa cells. In some cases large preantral follicles with very little KL staining were also observed (Fig. 2.6c). As a negative control, some ovary sections were taken through the immunohistochemistry

Figure 2.6

Immunolocalization of KL protein in PMSG-primed rat ovaries

Photomicrographs are of bright-field images 6 μm sections through whole ovaries from 28 day old animals treated with PMSG. Sections were exposed to antibodies against the extracellular domain of KL (a-d) and brown staining represents positive KL protein. Representative follicles at primary (a), early preantral (b), late preantral/early antral (c) and antral (d) stage of development are indicated with arrows. In c, a late preantral/early antral follicle with very little KL staining is indicated with an open arrow. In d, cumulus (solid arrows) and mural granulosa cells (open arrows) are indicated, respectively. Primary antibody was excluded (e) from the procedure and nonspecific binding was seen in some regions of the interstitium (arrow).


a
50 µm
b
60 µm
c
50 µm
d
e
50 µm

procedure without being exposed to primary antibodies and are shown in Fig 2.6e. No staining was evident in the granulosa cells of this section indicating that the staining seen in sections with antibodies was due to specific antigen-antibody interactions. Some non-specific staining was seen in the interstitium and the basement membrane.

2.3.4 *In vivo* hormonal regulation of KL expression in the ovary

The results of the *in situ* hybridization studies indicated that the abundance of KL mRNA in the granulosa cells of prepubertal rats increased in response to PMSG stimulation. To investigate this finding more quantitatively, KL expression was assessed in whole ovaries and in the granulosa cells isolated from untreated and PMSG-primed animals. In whole ovary RNA (Fig. 2.7), there was a moderate increase ($p<0.05$) in KL expression with PMSG treatment. However, when northern blot analysis was done with RNA extracted from granulosa cells of hormone-treated and untreated animals (Fig. 2.7), a similar but more dramatic effect was seen ($p<0.01$). When the band intensities were normalized for loading differences using α-tubulin band intensities, a 3.1 ± 0.2 fold increase in KL expression was seen 40 hr after PMSG treatment compared to untreated (control) granulosa cells (which confirms the qualitative increase seen with the *in situ* hybridization results).

To determine if the increase in KL mRNA expression in response to PMSG treatment occurs due to a direct effect of the gonadotropin or is an indirect effect mediated by increased estradiol production by granulosa cells, animals were treated with DES, an estrogen analogue that stimulates granulosa cell proliferation (Goldenburg *et al.*, 1972). Northern blot analysis was done with granulosa cell RNA from untreated, DES-primed and PMSG-primed rats (Fig. 2.8)

Figure 2.7

Northern blot analysis of KL mRNA in control and PMSG-treated ovaries and granulosa cells

Total RNA of ovaries and freshly isolated granulosa from untreated and PMSG-primed rats were probed with a ^{32}P-labelled 2.06 kb KL cDNA probe (A). Gel electrophoresis was done with 15 μg of total RNA in each lane and total RNA samples from NIH 3T3 fibroblasts and P19 embryonal carcinoma cells were included in the northern blot analysis to serve as positive and negative KL mRNA controls, respectively. The blot was then stripped and re-probed with ^{32}P-labelled α-tubulin cDNA (B). To quantitate the effect of hormone treatment, intensity of the bands on the autoradiographic film was estimated using densitometry and the ratio of KL/α-tubulin values were standardized to levels seen in untreated ovary or granulosa cell samples which were arbitrarily set to 1.0. A histogram illustrating the ratio of KL/α-tubulin values is shown in C where each bar represents mean ± S.E.M. KL/α-tubulin values from at least 5 experiments and statistical significance was determined using a t test. Bars with * are significantly different from the respective control.

* $p<0.05$

** $p<0.01$

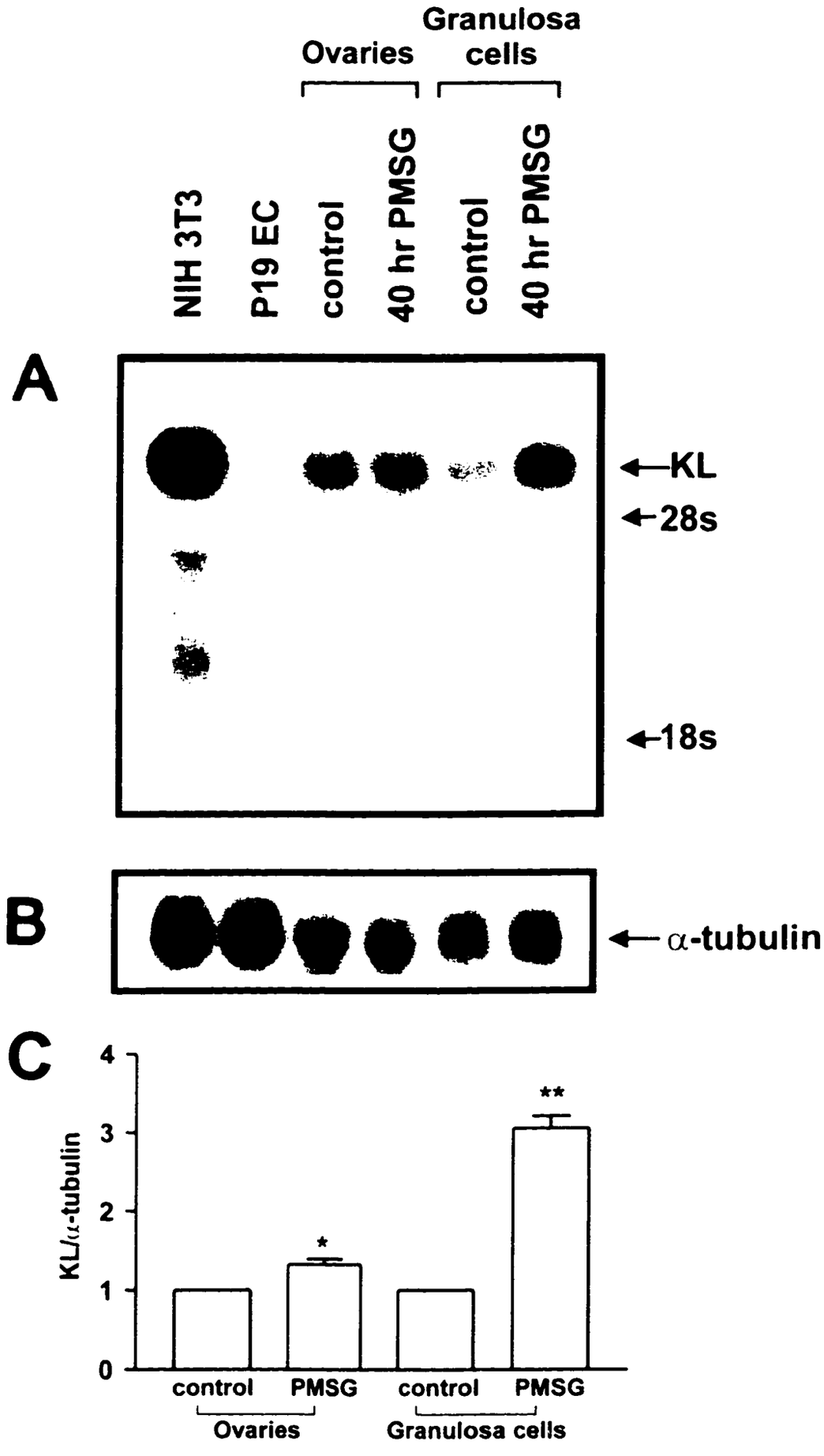
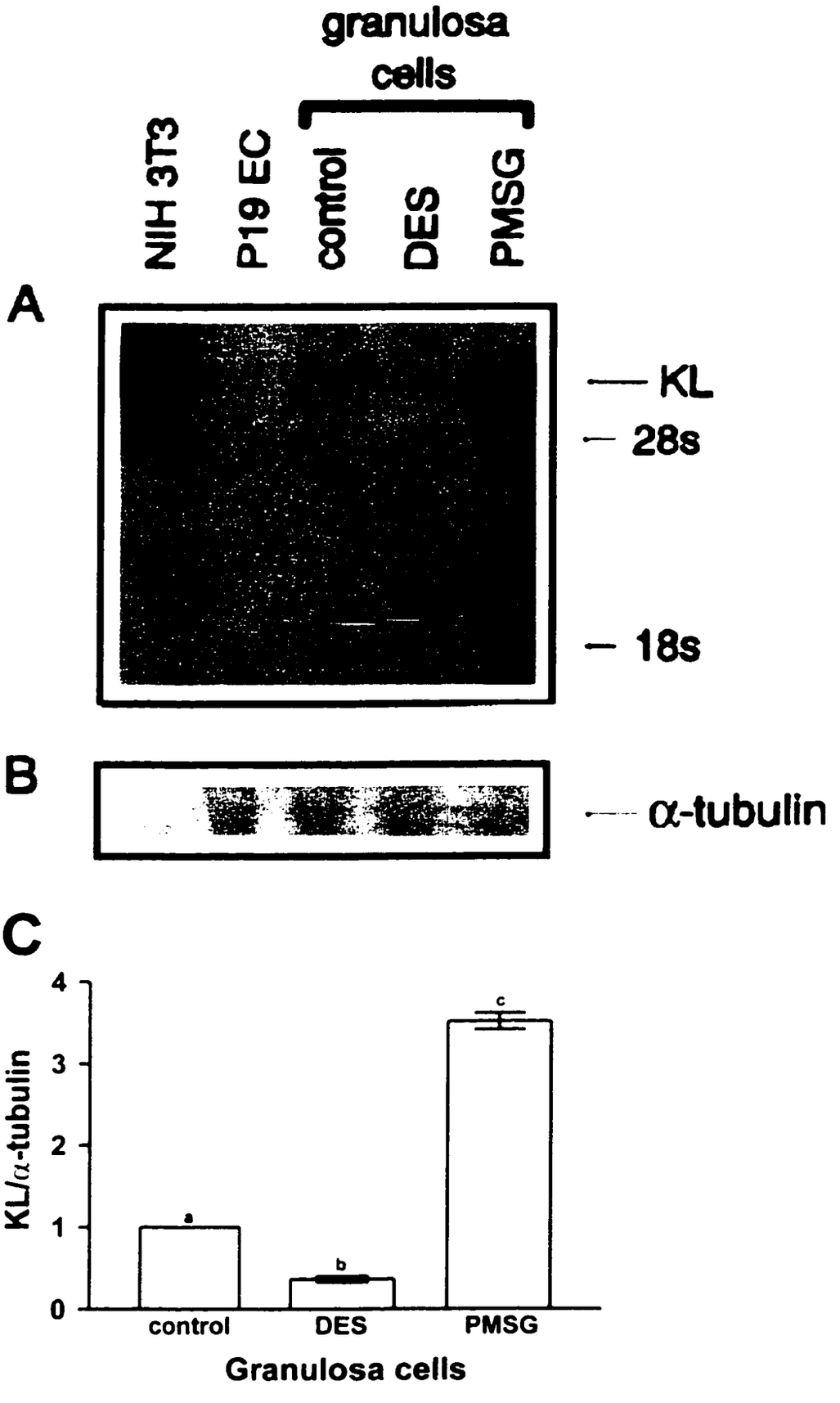

Granulosa cells
Ovaries
NIH 3T3
P19 EC
control
40 hr PMSG
control
40 hr PMSG
A
KL
28s
18s
B
α-tubulin
C
KL/α-tubulin
4
3
2
1
0
*
**
control
PMSG
control
PMSG
Ovaries
Granulosa cells

Figure 2.8

Northern blot analysis of KL mRNA in granulosa cells of control, *in vivo* estrogen- and gonadotropin-treated rats

Total RNA of granulosa cells from untreated, DES- and PMSG-primed rats were probed with a ^{32}P-labelled 2.06 kb KL cDNA probe (A). Gel electrophoresis was done with 15 μg of total RNA in each lane and total RNA samples from NIH 3T3 fibroblasts and P19 embryonic carcinoma cells were included in the northern blot analysis to serve as positive and negative KL mRNA controls, respectively. The blot was then stripped and re-probed with ^{32}P-labelled α-tubulin cDNA (B). Intensity of the bands on the autoradiographic film was estimated using densitometry, and the ratio of KL/α-tubulin values were standardized to levels seen in untreated granulosa cell samples which were arbitrarily set to 1.0. Histograms illustrating the mean ratio of KL/α-tubulin values of two experiments are shown in C and error bars represent the maximum and minimum data points. Statistical significance was determined using analysis of variance and when significant effects were observed, Dunnett's test was used for multiple comparisons. Bars indicated with different letters are significantly different ($p > 0.01$).


granulosa cells
NIH 3T3
P19 EC
control
DES
PMSG
A
KL
28s
18s
B
α-tubulin
C
KL/α-tubulin
4
3
2
1
0
a
b
c
control
DES
PMSG
Granulosa cells

The increase in KL expression that occurred following PMSG injection was not seen in the granulosa cells of DES-primed rats, and in fact there was a 3-fold decrease in KL transcript abundance in estrogen-treated rats.

2.4 Discussion

The experiments reported here demonstrate that Kit receptors are localized to the surface of rat oocytes from antral follicles, and that transcripts and protein of the ligand for the Kit receptor are found in the granulosa cells of rat ovaries, notably in the cumulus granulosa cells that enclose the oocyte. With increasing age of the rat, there were variations in KL expression within the ovary with high levels in the ovaries of neonatal rats and slightly lower levels in the ovaries of prepubertal rats. Treatment of the animals with PMSG before isolation of the ovaries resulted in an increase in the relative abundance of KL mRNA detected in the granulosa cells, suggesting that the production of this growth factor is regulated, directly or indirectly, by gonadotropins. Since KL expression decreased in ovaries from rats treated with DES, estrogen probably does not mediate the gonadotropin-stimulated increase in KL mRNA production.

We report here that Kit receptors are expressed on the surface of rat oocytes, which supports the localization of Kit seen in mouse (Manova et al., 1990; Horie et al., 1991) and human (Smikle *et al.*, 1996a) oocytes. Although there is a report of spontaneous Kit mutations arising in Sprague-Dawley rats (Tsujimura et al., 1991) our results are the first to demonstrate Kit expression in any rat tissues.

We have demonstrated the presence of a single KL mRNA transcript of 6.5 kb in total RNA preparations of ovaries isolated from neonatal and prepubertal rats. These findings are in agreement with all other reports of KL expression in mouse (Matsui et al., 1990; Motro and Bernstein, 1993; Manova et al., 1993), ovine (Gentry et al., 1996; Tisdall et al., 1997) and human ovaries (Laitinen et al., 1995). Manova *et al.*, (1993) demonstrated that there was significant expression of KL in the perinatal ovary; however they were not able to detect KL mRNA expression in ovary samples of mice 5 and 8 days after birth. KL mRNA expression was once again detected in whole ovary samples isolated from mice two weeks of age and onwards, at levels of expression similar to those seen in perinatal mice. We demonstrated that there are substantially higher amounts of KL mRNA expression in whole ovaries from neonatal compared to prepubertal rats. We have not investigated KL expression in whole ovaries taken from rats at any other age. Although both rats and mice are rodent species, there are some differences in development seen in perinatal ovaries of these two species. At the time of birth, primordial follicle organization is completed in the mouse ovary and in the most part the oocytes have become meiotically arrested in the dictyate phase of meiosis prophase I. However, in rats this process of primordial follicle organization and meiotic prophase I is not complete until the third day after birth. A possible explanation of higher KL expression around the time of birth in rats may be that KL is involved in the initiation of the arrest of oocytes in meiotic prophase I.

Previous studies have shown that, in preantral follicles in mice, KL transcripts are present in the granulosa cells (Horie et al., 1991; Motro et al., 1991; Keshet et al., 1991). In this study, we have extended this observation to rat ovaries and observed KL mRNA and

protein in primary, preantral and the more mature antral follicles. We have demonstrated that although granulosa cells are the site of KL expression in the primary and preantral follicles, after antral follicle formation, the cumulus granulosa cells that enclose the oocyte are the primary sites of KL production (both mRNA and protein), with a clear decrease in KL production in the granulosa cells with increased distance from the oocyte and antrum. This localization of KL to the cumulus cells in late antral follicles suggests that KL may be important in the regulation of oocyte function, notably the maintenance of meiotic arrest, a function that has been shown to be regulated by granulosa cells and their products (reviewed in Eppig, 1993). The follicular localization we have demonstrated is in agreement with that shown in ovine follicles, where KL mRNA is evident in the granulosa cells from primary to antral follicles and KL is expressed in both cumulus and mural cells of antral follicles (Tisdall et al., 1997). However, our studies with respect to antral follicle localization of KL are in direct contrast with previous studies using mouse ovaries that show KL expression only in the more peripheral mural granulosa cells in antral follicles, and not in the cumulus granulosa cells (Motro and Bernstein, 1993; Manova et al., 1993). This difference in the distribution of KL expression between mice and rats may suggest species-specific differences in the factors that regulate KL expression as well as differences in the function of KL at this stage of follicular development.

A recent study has demonstrated that KL expression in mouse granulosa cell monolayers can be increased if they are co-cultured with oocytes (Packer et al., 1994). There is considerable evidence to demonstrate that oocytes secrete factors that regulate several granulosa cell functions, including cumulus expansion (Vanderhyden et al., 1990; Salustri et

al., 1990; Buccione et al., 1990; Singh et al., 1993), proliferation (Vanderhyden et al., 1992), follicular structural integrity (Vanderhyden et al., 1992), and steroidogenesis (Vanderhyden et al., 1993). Vanderhyden et al (1992) have shown previously the prolonged effects of oocyte-secreted factors on rat cumulus granulosa cells compared to mouse cumulus cells, although the cause of the maintained sensitivity to the factors is still unknown. Even after complete removal of the oocyte from oocyte-cumulus cell complexes, rat cumulus cells maintained their ability to perform FSH-stimulated, oocyte-dependent activities for several hours. Given the species-specific differences in the follicular localization of KL expression, it is possible that rat oocytes continue to secrete a factor that helps to maintain KL expression in cumulus granulosa cells throughout follicular development.

Examination of the relative expression of KL in the ovary using both *in situ* hybridization and northern blot analysis revealed that the number of KL mRNA transcripts in granulosa cells is increased by PMSG treatment. Rossi et al. (1993) have demonstrated that KL mRNA is increased in cultured Sertoli cells, the male homolog of granulosa cells, in the presence of FSH. Various developmental steps during the growth and maturation of both male and female germ cells are strictly regulated by hormonal stimulation of the surrounding somatic cells. The identification of a hormonally-regulated factor that has a biological effect on germ cells is therefore a finding of major importance to the understanding of the mechanisms of somatic cell regulation of germ cell development.

A study has found that the cAMP analog, dibutyryl cAMP, increased KL expression in granulosa cells of prepubertal mice (Packer et al., 1994). In the experiments described here, exposure of the ovaries to PMSG before the isolation of granulosa cells resulted in a

significant increase in the abundance of KL mRNA transcripts, suggesting that gonadotropins are physiological regulators of granulosa cell KL production and that cAMP mediates the gonadotropic stimulus. PMSG contains both FSH- and LH-like activity, and it is not clear from our experiments which of the gonadotropic activities is responsible for stimulating KL expression. It is well established that estradiol is a major product of FSH-stimulated granulosa cells in antral follicles (Gore-Langton and Armstrong, 1988) and, therefore, we investigated estradiol as a potential mediator of the gonadotropin-stimulated increase in KL mRNA production. DES, like PMSG, stimulates follicle growth, but in contrast to PMSG, formation of the fluid-filled antrum and differentiation of granulosa cells into cumulus and mural granulosa cell subpopulations do not occur (Goldenburg et al., 1972). DES priming caused a decrease in the abundance of KL transcripts, suggesting that the stimulation of KL expression in granulosa cells by gonadotropins does not involve estrogenic activity.

These studies demonstrate that KL is highly expressed in ovaries of neonatal and prepubertal rats. Localization studies in prepubertal rats demonstrated that Kit is expressed on the plasma membrane of oocytes and that KL is produced by the cumulus granulosa cells and to a lesser extent the mural granulosa cells. KL expression is upregulated with *in vivo* PMSG-treatment but is independent of estradiol stimulation. The localization of Kit and KL within the follicle suggests a role for Kit/KL interactions in oocyte function.

CHAPTER 3 - KL AND KIT INTERACTIONS REGULATE OOCYTE MEIOSIS

3.1 Introduction

The expression of KL and Kit in the rat ovary has been demonstrated and discussed in detail in the previous chapter. Granulosa cells, the somatic cells that surround the oocyte, are the only site of KL production in developing and antral rat follicles. KL is produced predominantly in those granulosa cells that are closest to the oocyte within the rat antral follicle. In addition, we have localized Kit to the plasma membrane region of oocytes isolated from antral follicles. The complementary localization seen with Kit and KL suggests a potential role for Kit/KL interactions in oocyte function. Furthermore, strong evidence for the importance of a functional receptor-ligand interaction in the ovarian follicle is provided by phenotypes of mice with milder mutations at the *Mgf* locus (see Table 1.2). Oocytes are present in the ovaries of these animals, albeit in reduced numbers, but follicular development is arrested early in growth, indicating the need for the presence of the ligand for oocyte and follicular development.

Kit and KL interactions have been shown to result in a wide variety of cellular responses: cell proliferation, survival, migration, adhesion, and in some cases, differentiation and maturation (see Table 1.3). The best characterized of these cellular responses is the effect of Kit stimulation on the cell cycle. Many of the cell types examined, including progenitor hematopoeitic cells (Metcalf and Nicola, 1991), mast cells (Tsai et al., 1991), primordial germ cells (Matsui et al., 1991) and type A spermatogonia (Rossi et al., 1993), respond to activation

of the Kit receptor by stimulating the mitotic cell cycle. In contrast to these proliferative effects, an inhibition of the cell cycle by Kit has been reported in breast cancer and melanoma cells (Huang et al., 1996; Nishida et al., 1996). Oocytes are unique cells compared to other cells of the body in that oocytes are not mitotically active but rather meiotically active and, therefore, activation of Kit must result in some function that is unique to the oocyte.

From late embryogenesis to the time of the preovulatory surge of gonadotropins that triggers ovulation, oocytes are arrested at the diplotene stage of meiosis I of the cell cycle. During oocyte growth, the oocyte remains meiotically arrested while it increases in diameter to reach its full-grown size. Normal oocyte growth and the acquisition of developmental capabilities require this maintenance of meiotic arrest. Indeed, disruption of meiotic inhibition within the follicle can, under certain circumstances, result in parthenogenetic activation of the oocyte and the formation of intrafollicular teratomas (Eppig, 1977; Furuta et al., 1995). When oocytes are removed from antral follicles and placed into culture they will spontaneously resume meiosis (Pincus and Enzmann, 1935), leading to the hypothesis that meiosis-inhibiting factors found within follicles maintain the oocytes in a meiotically-arrested state until the time of ovulation. A number of intrafollicular molecules produced by granulosa cells have been shown to delay or inhibit spontaneous oocyte maturation and have been discussed in detail in section 1.9.

KL mRNA is abundantly expressed in the granulosa cells of rat antral follicles, and granulosa cells in antral follicles actively maintain the oocyte in meiotic arrest (reviewed in Masui and Clarke, 1979; Eppig, 1993). Although low levels of KL are produced in granulosa cells of less developed follicles, the oocytes in these follicles are meiotically-incompetent.

Oocytes acquire meiotic competence in antral follicles and, coincidently, KL expression increases 3.5-fold with antral follicle development. Therefore, given the pattern and regulation of KL expression in antral follicles, and the known function of granulosa cells in the maintenance of meiotic arrest, the specific aim of the study described in this chapter was to determine the role of KL in the regulation of meiosis in fully-grown oocytes. To test this objective oocytes isolated from antral follicles were stimulated with KL and assessed for GVBD and Pb formation, two morphological indicators of oocyte meiotic maturation. Also converse experiments were done in which Kit receptor expression was inhibited by antisense oligonucleotide injections and the effect on GVBD was assessed. The effect of cAMP on KL-induced inhibition of meiosis was also examined.

3.2 Materials and Methods

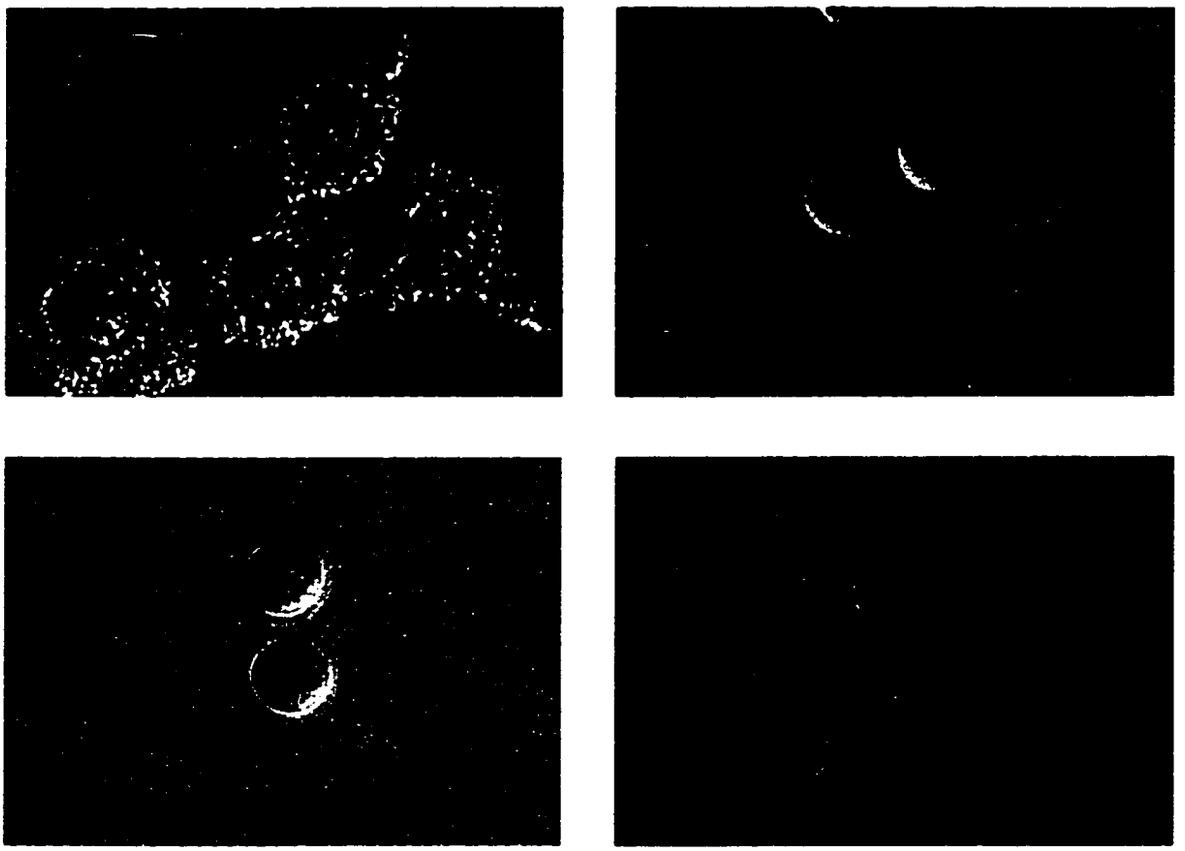
3.2.1 Oocyte Collection and Culture

Ovaries were collected from 40 hr PMSG-primed and untreated animals and placed in WAY/BSA containing either 0, 50 or 500 ng/ml KL (generously provided by Immunex, Seattle, Washington). OCC were collected (Fig 3.1a) and stripped of their cumulus granulosa cells, as discussed previously. Groups of 30-65 denuded oocytes were cultured in Falcon culture dishes (Becton Dickinson) in 200 µl drops of WAY/BSA containing 0, 50 or 500 ng/ml KL covered with equilibrated mineral oil. Light mineral oil (Fisher), 200 ml, was equilibrated with 20 ml WAY/BSA by stirring for 4 days, with one media change after 2 days. After allowing the media to settle, the equilibrated oil was

Figure 3.1

Photomicrographs of the morphological indicators of meiotic maturation

In order to assess oocyte meiotic maturation in rodent oocytes, oocyte-cumulus cell complexes are isolated from antral follicles of rat ovaries (a) and stripped of their granulosa cells to yield denuded oocytes (b). Once removed from follicles, oocytes will spontaneously resume meiosis and this resumption can be assessed within 2-4 hr of culture by germinal vesicle breakdown as seen by the dissolution of the nuclear envelope (c) and the formation of the first polar body within 12 hr of the start of the culture (d). B. Vanderhyden, 1987



sterilized by filtration through a 0.45 μm filter. Oocytes were assessed for GVBD (Fig. 3.1b,c) every half hour for the first 4 hr and then at 24 hr after incubation. Oocytes were assessed for Pb formation (Fig. 3.1d) at various intervals up to 24 hr.

In some experiments the specificity of KL action on the Kit receptors was determined using rat antibodies against the extracellular portion of the mouse Kit receptor (ACK2; kindly provided by Dr. S.-I. Nishikawa, Kumamoto, Japan). ACK2 has been used extensively to neutralize Kit receptors in testes and melanocytes (Yoshinaga et al., 1991; Nishikawa et al., 1991). Oocytes from PMSG-primed rats were preincubated in 0.2 μl ACK2 in 200 μl WAY/BSA for 1 hr. This quantity of ACK2 is capable of saturating the receptors of 2×10^5 mast cells (Nishikawa, personal communication). In these experiments the preincubation medium also contained 50 μM 3-isobutyl-1-methyl-xanthine (IBMX; Aldrich Chemical Co., Milwaukee, WI), a cyclophosphodiesterase inhibitor which prevents the degradation of cAMP and prevents oocytes from prematurely undergoing meiotic maturation. After the preincubation period, the oocytes were washed twice in WAY/BSA to remove IBMX and cultured for 24 hr with (500 ng/ml) or without KL and GVBD was assessed every half hour for 4 hr and then at 24 hr.

3.2.2 Inhibition of Kit receptor expression by intraoocyte microinjection

a) Oocyte microinjection

OCC from 40 hr PMSG-treated rats were collected in WAY supplemented with 5% FBS and 250 μM IBMX. This concentration of IBMX was determined in preliminary experiments to be suboptimal for the maintenance of meiotic arrest in rat oocytes cultured

for 24 hr. OCC were washed twice in WAY/FBS/IBMX and then placed in 100 µl of media under mineral oil. Using an Eppendorf injector 5242 and an inverted Leitz microscope equipped with Leitz micromanipulators, approximately 2-5 pl of oligonucleotides (antisense or missense) in microinjection buffer or buffer alone was delivered to each cumulus-enclosed oocyte. Oocytes injected in the presence of their surrounding cumulus cells have enhanced survival rates (Paules et al., 1989). After injection, each group of complexes was washed twice in media and cultured at a concentration of 50 OCC per 100 µl of media under oil for 4 hr for subsequent immunodetection of Kit (as described in section 2.2.2) or for 20-24 hr for assessment of GVBD as follows. After the 20-24 hr incubation, OCC were placed in WAY/FBS/IBMX and repeatedly pipetted to remove the cumulus granulosa cells and were scored for GVBD. To confirm meiotic competence of the oocytes, the immature oocytes (with an intact germinal vesicle) were cultured for a further 24 hr in 100 µl of WAY/FBS and assessed for GVBD. Reported values of GVBD were adjusted to reflect only competent oocytes, although in all cases the percentage of competent oocytes was >97%.

b) Oligonucleotides

A 25 base pair oligonucleotide (5'GGA TGG ATG GCG GAG ACG GCT CCC C 3') antisense to nucleotides 120-146 of the murine Kit cDNA (Qiu et al., 1988) was synthesized. This sequence is complementary to a region of Kit mRNA close to the start codon and thus would presumably prevent the translation of the Kit mRNA to protein. Missense oligonucleotides (5' CTG GCT GAC CTG CCC CAC GTG GAC C 3') were also synthesized and included all the nucleotides of the antisense oligonucleotide, but in random

order. This missense oligonucleotide had no homology to any known sequences registered with Genbank. Both these oligonucleotides were HPLC purified, dried under vacuum and resuspended in microinjection TE buffer (10 mM Tris-HCl, 0.15 mM EDTA, pH 7.5) at a concentration of 100 ng/µl.

c) Immunocytochemical detection of Kit on microinjected oocytes

To determine the presence of Kit before and after microinjection, oocytes were stripped of cumulus cells by repeated pipetting and then exposed to antibodies to detect Kit receptors according to the procedure described in section 2.2.2.

3.2.3 Effect of KL and cAMP on spontaneous meiotic maturation

To determine the effect of KL and cAMP on spontaneous meiotic maturation, denuded oocytes were isolated from rat ovaries in WAY/BSA in the presence of 250 µM IMBX as described above and cultured in groups of 40-50 oocytes in Falcon culture dishes (Becton Dickinson) in 100 µl drops of WAY/BSA containing 50 ng/ml KL (generously provided by Immunex, Seattle, WA) and/or 300 µM dibutyryl cyclic AMP (dbcAMP; Sigma) covered with equilibrated mineral oil. Oocytes were assessed for GVBD every hour for the first 3 hr and after 24 h. To determine meiotic competence, these oocytes were then washed 3 times in WAY/BSA, and cultured in 100 µl of WAY/BSA for an additional 24 hr and assessed for GBVD. All values presented have been adjusted to include only competent oocytes, although in all cases the percentage of competent oocytes was > 97 %.

3.2.4 Data presentation and statistical analysis

Results were graphed using the mean percentage ± S.E.M. of oocytes undergoing GVBD or Pb formation. All oocyte culture experiments were performed at least 4 times. Effect of replicate and treatment were examined by analysis of variance using arcsin transformation of the proportions, and Fischer's protected least-squares was used to detect differences using a computer software called "StatView".

3.3 Results

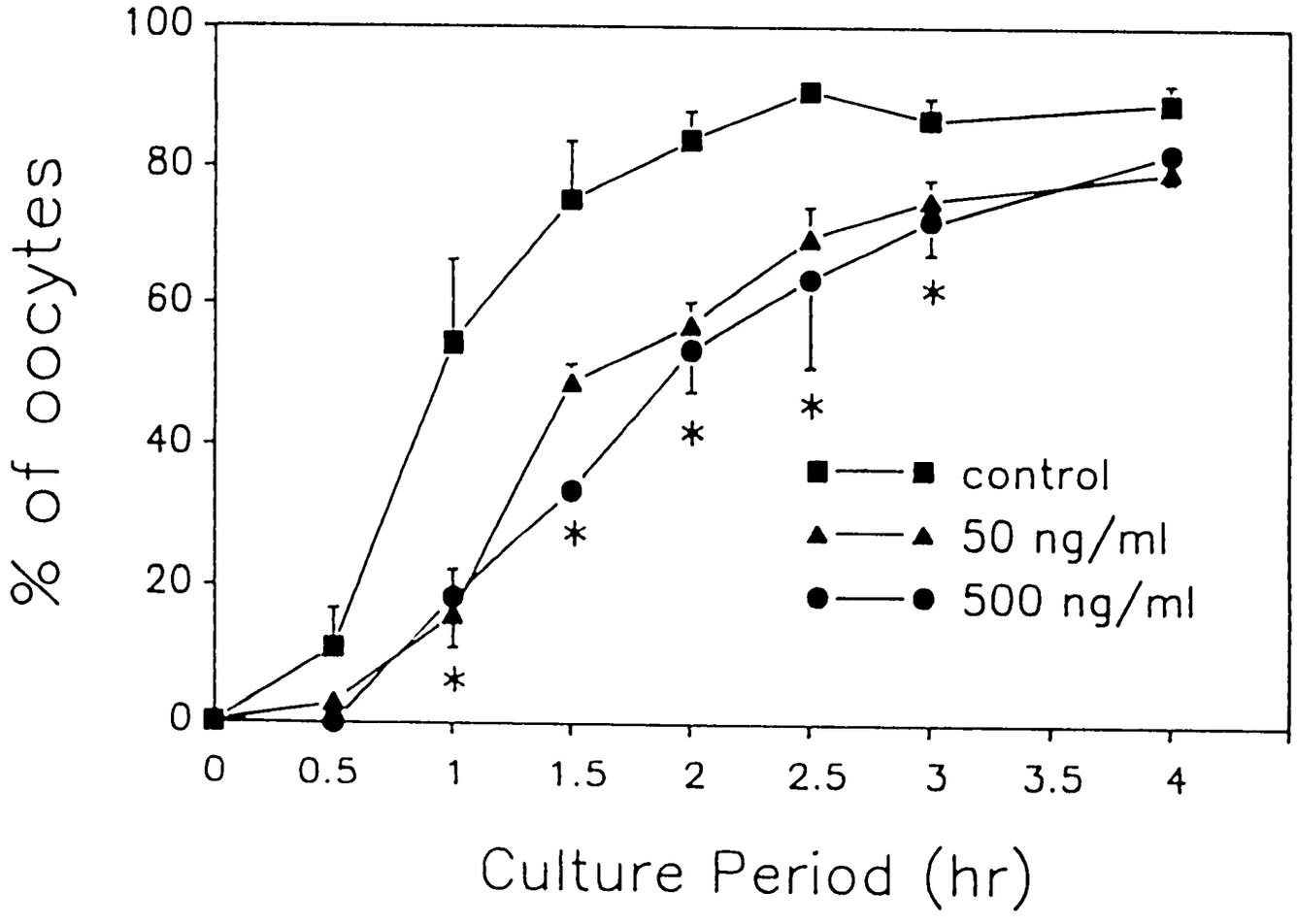
3.3.1 Effect of soluble KL on spontaneous meiotic maturation of oocytes

Studies were done to investigate a possible functional role for KL in oocyte meiotic regulation by culturing oocytes from PMSG-primed rats in the presence of KL and assessing the rate of spontaneous meiotic maturation over a 24 hr period. Fig. 3.2 illustrates the percentage of oocytes undergoing GVBD when cultured for 4 hr in 0, 50 or 500 ng/ml KL. At each time point, the presence of KL at either concentration resulted in a significant decrease in the proportion of oocytes undergoing GVBD ($p<0.05$). The mean time required for 50% of the oocytes in each group to achieve GVBD was estimated from the data presented and was greater in KL-containing groups: control, 0.86 hr; 50 ng/ml, 1.53 hr; 500 ng/ml, 1.88 hr. There were no significant differences between the responses to the two concentrations of KL. A similar delay by KL was obtained using oocytes from untreated animals; however, the effect was less pronounced and

Figure 3.2

The effect of the presence or absence of KL on GVBD in fully-grown oocytes

Effect of KL on the timing of GVBD in oocytes isolated from PMSG-primed rats. Each data point represents mean ± S.E.M. observations made with a total number of 124-185 oocytes from 4 experiments. Comparisons between treatments and control data points were done within each time point and * indicates significant differences ($p<0.05$).


100
80
60
40
20
0
% of oocytes
0
0.5
1
1.5
2
2.5
3
3.5
4
Culture Period (hr)
control
50 ng/ml
500 ng/ml
*
*
*
*
*

was significant only at 1 hr after the beginning of culture (data not shown). After 24 hr of culture, there were no significant differences between the treatments in the proportion of oocytes that had undergone GVBD: control, 95% (n= 178); 50 ng/ml, 94% (n=125); 500 ng/ml, 98% (n=121).

After oocytes had been cultured in media alone (control) or in the presence of KL (50 or 500 ng/ml) for 7 hr, Pb formation was monitored for an additional 7 hr period (Fig. 3.3). At a concentration of 500 ng/ml, KL prevented more oocytes from undergoing Pb formation at every time point from 10 to 14 hr of culture although this effect did not achieve statistical significance. Thus the time required for 50% of the oocytes that had already achieved GVBD to form Pb was greater in KL-containing groups: control, 11.94 hr; 50 ng/ml, 13.81 hr; 500 ng/ml, 13.75 hr.

To demonstrate the specificity of the KL-mediated inhibition of spontaneous meiotic maturation, the Kit antibody, ACK2, was used in some experiments. By itself, the addition of ACK2 had no effect on the rate of spontaneous oocyte maturation (Fig. 3.4). When oocytes that were previously treated with ACK2 were cultured in KL-containing WAY/BSA (500 ng/ml), the percentage of oocytes that underwent GVBD was not significantly different from control oocytes, except at 0.5 hr of culture. Oocytes cultured with KL alone experienced a delay in undergoing GVBD. Fifty percent of control and ACK2 + KL-treated oocytes had undergone GVBD after 1.25 and 1.15 hr, respectively. In contrast, in the presence of KL alone, it took 2.06 hr for 50% of the cultured oocytes to undergo GVBD.

Figure 3.3

The effect of the presence or absence of KL on Pb formation in oocytes that had undergone germinal vesicle breakdown

Effect of KL on the timing of first Pb extrusion in oocytes that had undergone GVBD. Oocytes were isolated from PMSG-primed rats, and were cultured for up to 14 hr. Each data point represents mean ± S.E.M. observations made with a total number of 105-175 oocytes from 4 experiments. Comparisons between treatments and control data points were done within each time point and groups were not found to be significantly different.

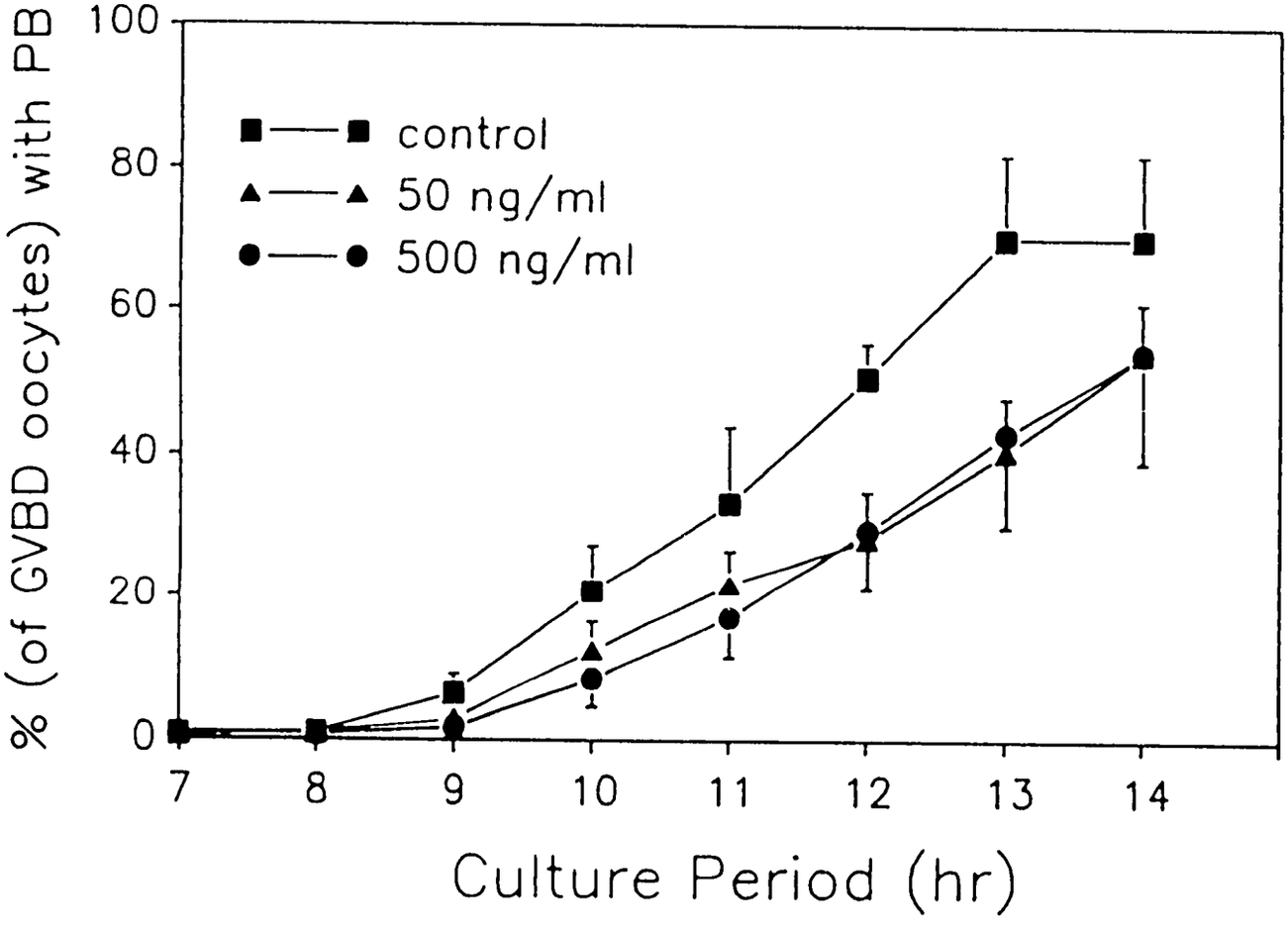
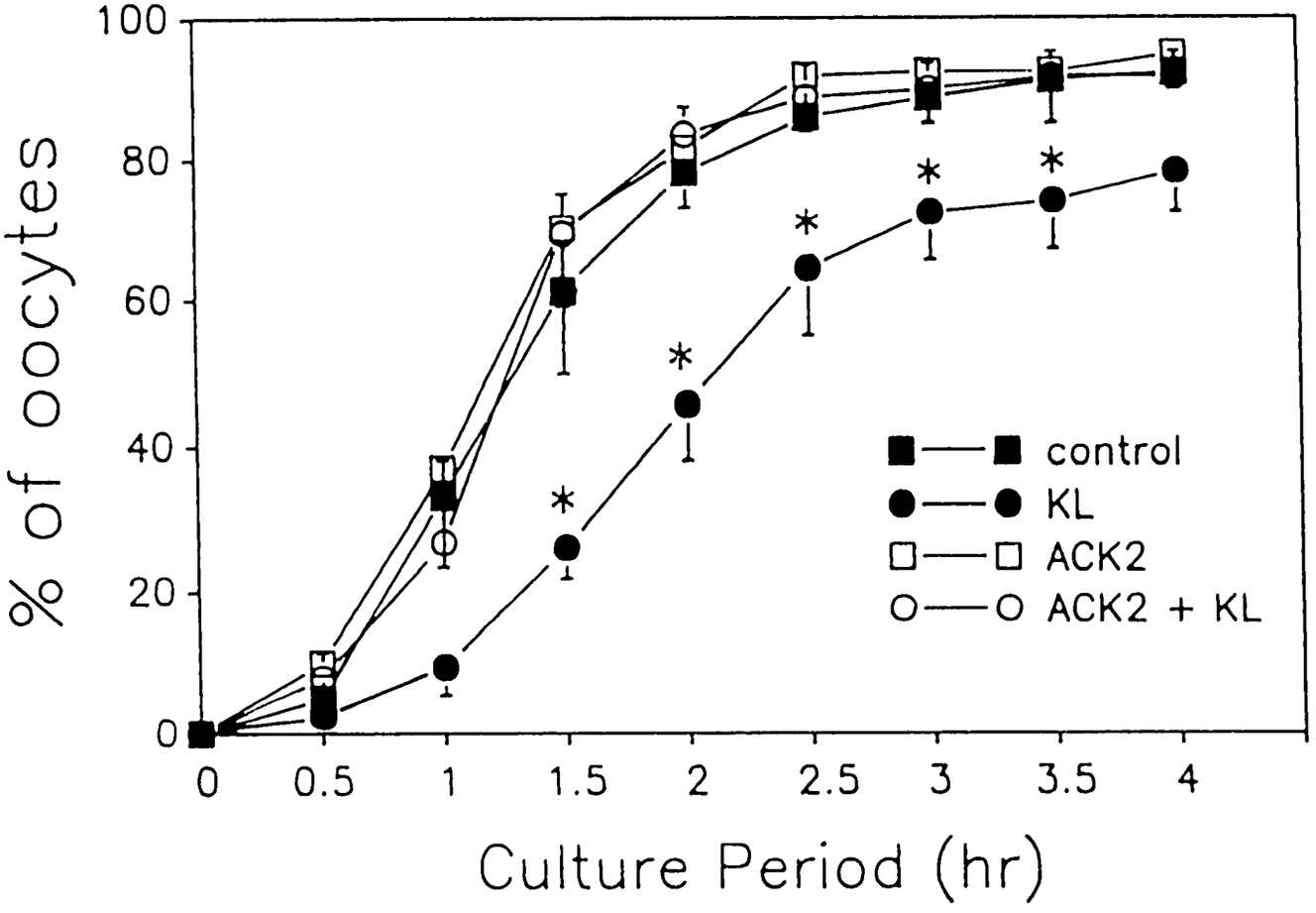

% (of GVBD oocytes) with PB
100
80
60
40
20
0
control
50 ng/ml
500 ng/ml
7
8
9
10
11
12
13
14
Culture Period (hr)

Figure 3.4

KL-induced inhibition of GVBD occurs through interactions with the Kit receptor

Effect of KL (500 ng/ml) on the timing of germinal vesicle breakdown in oocytes pretreated for 1 hr with ACK2, an antibody to the extracellular domain of the Kit receptor and then cultured for 4 hr. 3-isobutyl-1-methyl-xanthine (IBMX) was present in the preincubation medium to prevent premature meiotic maturation. Oocytes were isolated from PMSG-primed animals in 4 experiments; each data point represents mean ± S.E.M. observations made with a total number of 71-208 oocytes. Comparisons between treatments and control data points were done within each time point and * indicates significant differences ($p<0.05$).


100
80
60
40
20
0
% of oocytes
0
0.5
1
1.5
2
2.5
3
3.5
4
Culture Period (hr)
control
KL
ACK2
ACK2 + KL
*

3.3.2 Effect of Removal of Kit on Oocyte Meiotic Arrest

To further investigate the importance of Kit/KL interactions in maintaining rat oocytes in meiotic arrest, expression of Kit receptors was inhibited by the microinjection of antisense oligonucleotides and spontaneous meiotic maturation was assessed. However, in order to confirm that microinjection of antisense oligonucleotides had caused a decrease in the levels of Kit on the oocyte surface, immunofluorescence was performed 4 hr after microinjection in some groups of oocytes. Whole-mounted oocytes are shown in Fig. 3.5. A strong signal for Kit is seen on the plasma membrane of all rat oocytes that had not been microinjected (Fig. 3.5a) or had been microinjected with missense oligonucleotides (Fig. 3.5d). However, oocytes which had been injected with antisense oligonucleotides showed substantially less staining, although it was still membrane associated (Fig. 3.5b,c). Transmitted light photomicrographs of microinjected oocytes are shown below the fluorescence photographs. Of the total OCC injected, 33 % of the antisense-injected oocytes had almost no staining (as seen in Fig. 3.5b), and the rest had minimal staining (as in Fig. 3.5c). Thus antisense oligonucleotide injections resulted in an overall decrease in Kit receptor levels in rat oocytes.

Once we had determined that microinjection of antisense oligonucleotides inhibited the expression of Kit on the surface of oocytes, oligonucleotides were microinjected into OCC and the oocytes were assessed for GVBD after 20-24 hr in IBMX-containing media.(Fig 3.6) Uninjected (control) oocytes, were cultured in parallel with oocytes microinjected with antisense oligonucleotides, missense oligonucleotides or buffer alone. The concentration of

Figure 3.5

Microinjection of oligonucleotides antisense to Kit mRNA ablates Kit receptor expression on oocytes

Detection of Kit receptors on rat oocytes using immunofluorescence and anti-Kit antibodies. Kit was detected on the plasma membrane of uninjected (a) or missense oligonucleotide-injected oocytes (d), but little or no Kit was detected after microinjection of antisense oligonucleotides (b,c). a', b', c' and d' are transmitted light photomicrographs of the oocytes pictured in a, b, c and d, respectively. Results are representative observations made in 3 independent experiments.

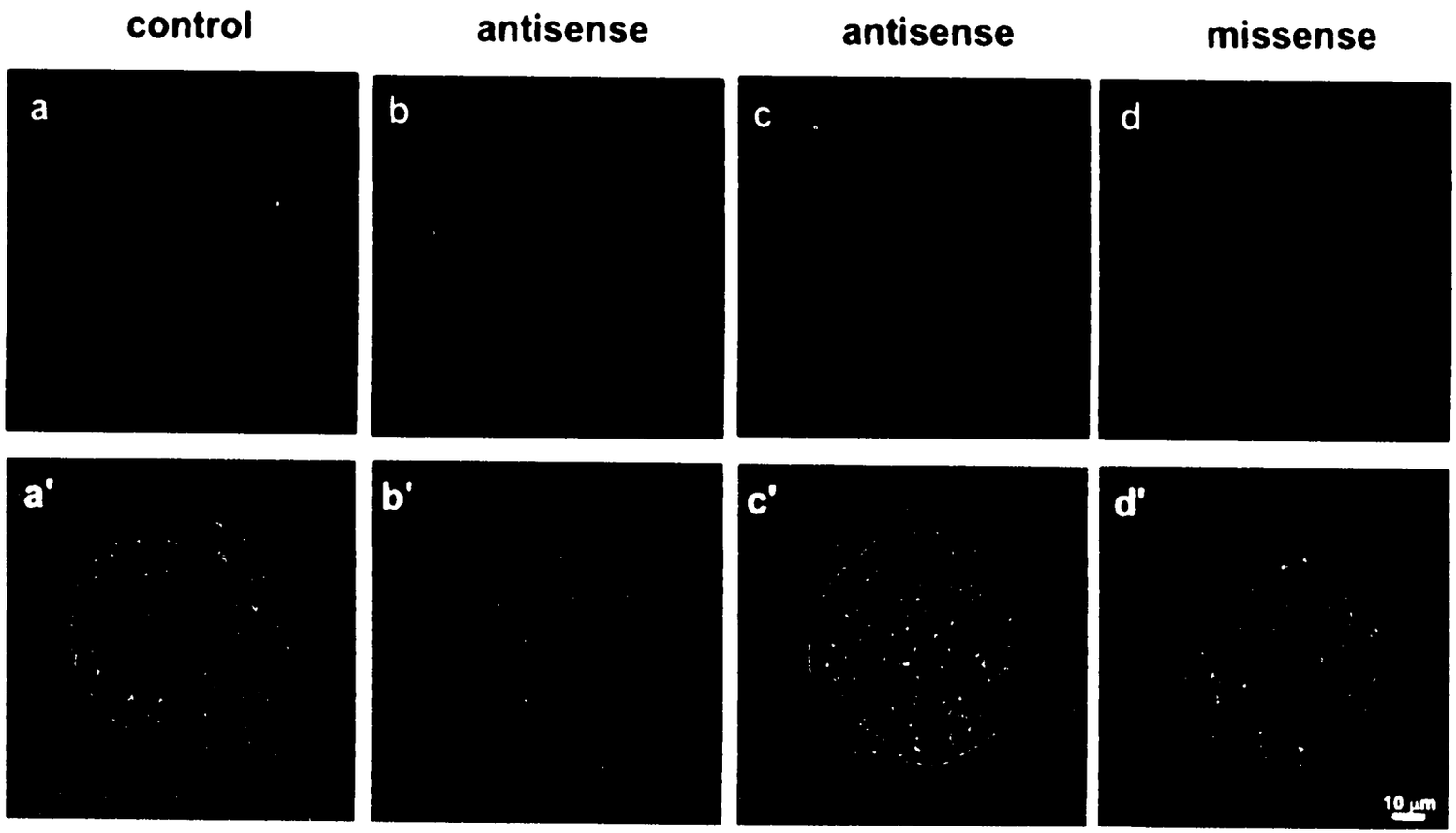
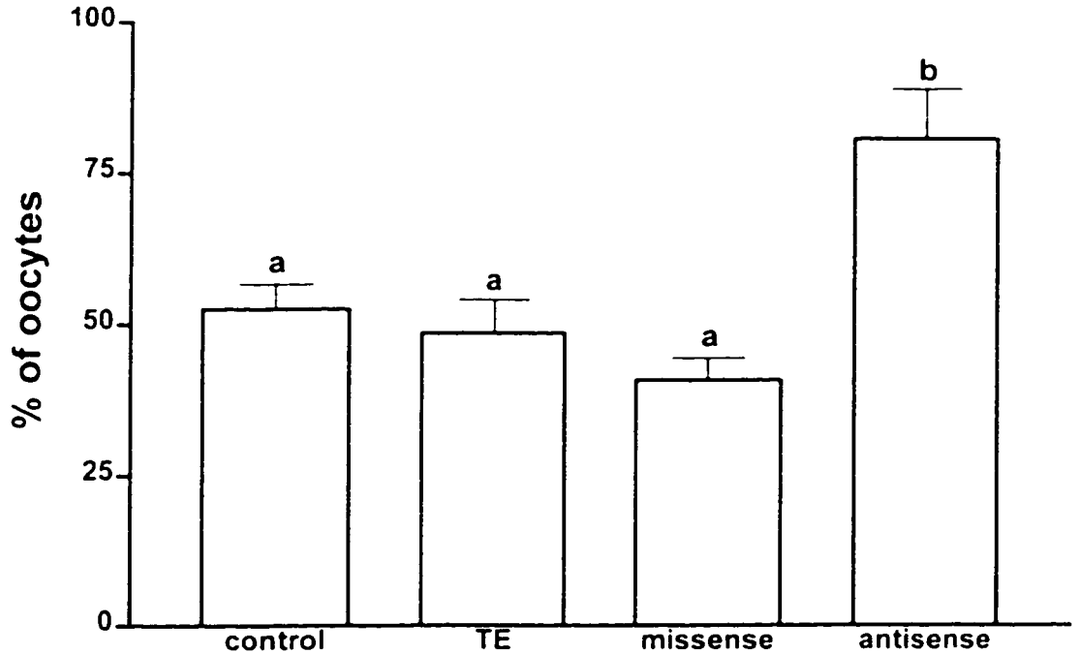

control
antisense
antisense
missense
a
b
c
d
a'
b'
c'
d'
10 µm

Figure 3.6

Effect of removal of Kit receptors on GVBD in oocytes

Germinal vesicle breakdown in oocytes cultured for 24 hr after microinjection of buffer alone (Tris-EDTA, TE), oligonucleotides that are antisense to Kit mRNA, or missense oligonucleotides. Each bar represents mean ± S.E.M. from 7 experiments with a total of 98-363 oocytes/treatment. To determine the effect of replicate and treatment, analysis of variance was done on arcsin transformed data and differences were detected using Fischer's protected least-squares. Bars indicated with different letters are significantly different ($p<0.05$).


100
75
50
25
0
% of oocytes
a
a
a
b
control
TE
missense
antisense

IBMX in the culture system was sufficient to maintain 48.4 ± 5% (n=363) of control oocytes in meiotic arrest (Fig. 3.6). In contrast, 30% more oocytes had undergone GVBD following injection with oligonucleotides antisense to the Kit receptor mRNA, as compared to oocytes that were injected with missense oligonucleotides or buffer alone ($p<0.05$).

3.3.3 Effect of KL and cAMP on Meiotic Maturation

To examine whether the inhibitory effects of cAMP and KL on oocyte meiosis are mediated via a shared signal transduction pathway, granulosa-free oocytes were cultured in the presence of KL and/or dbcAMP, a membrane-permeable analogue of cAMP (Henion et al, 1967). In previous experiments, 50 ng/ml of KL caused maximal inhibition of GVBD during the first 4 hr of culture and this concentration was again used. Dibutyryl cAMP treatment at maximum doses greater than 1.0 mM is able to completely inhibit oocyte maturation (Magnusson and Hillensjo, 1977), and thus potentially may override the transient inhibition seen with KL treatment. Therefore, a suboptimal dose of dbcAMP was chosen by culturing oocytes for 3 hr in media alone (control) or in the presence of 75 to 600 μM dbcAMP (Fig. 3.7). The dbcAMP concentration of 300 μM was chosen for the combination experiment because it maintained 50% of the total number of oocytes in meiotic arrest over the 3 hr culture period. To determine the effect of dbcAMP on the KL-induced delay in GVBD, oocytes were cultured in 50 ng/ml of KL and/or 300 μM dbcAMP for 24 hr with assessments at every hour for the first 3 hr. After 2 and 3 hr in culture, KL and dbcAMP both resulted in significantly ($p<0.05$) fewer oocytes undergoing GVBD compared to untreated oocytes. The response of the oocytes to KL + dbcAMP was not significantly different from that of oocytes in dbcAMP alone at either time point although there was a

Figure 3.7

Dose-response of cAMP analogue-stimulated inhibition of spontaneous meiotic maturation

Germinal vesicle breakdown in oocytes cultured for 2 or 3 hr in the absence or presence of various doses of dibutyryl cAMP (75, 150, 300 and 600 μM). Each bar represents mean ± S.E.M. made with a total of 61-123 oocytes isolated from PMSG-primed animals in 3 experiments. Dashed line indicates 50 % of oocytes.

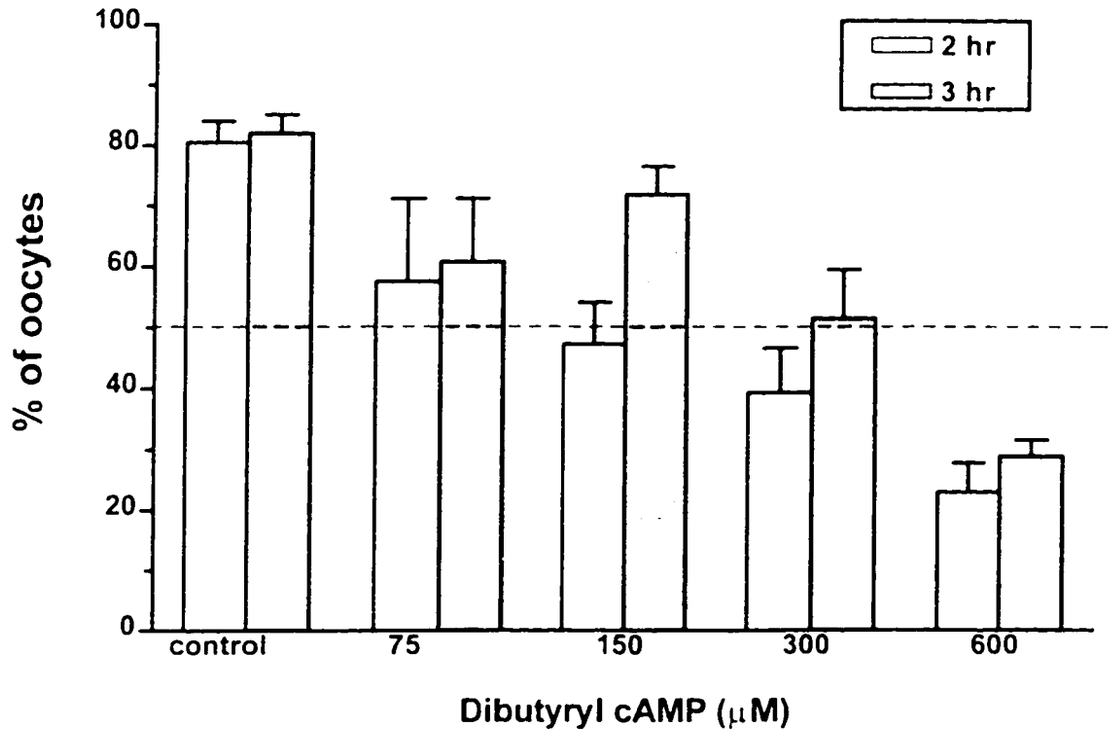
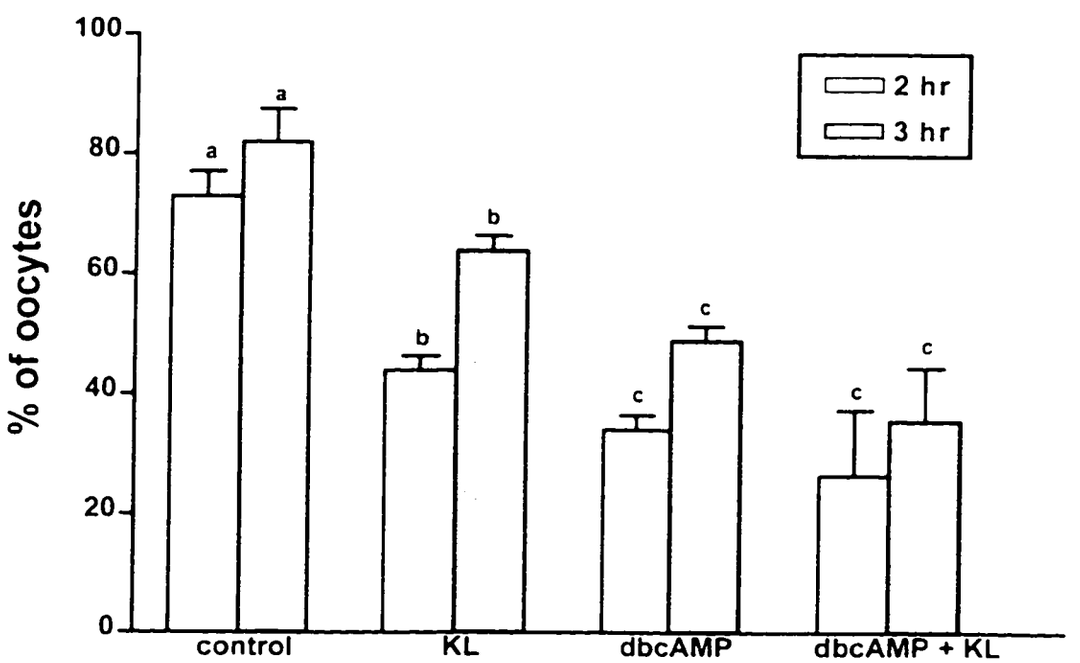

100
80
60
40
20
0
% of oocytes
2 hr
3 hr
control
75
150
300
600
Dibutyryl cAMP (μM)

Figure 3.8

Effect of a combination treatment of KL and cAMP on GVBD

Germinal vesicle breakdown in oocytes cultured for 2 or 3 hr in the presence or absence of 50 ng/ml KL and/or 300 μM dibutyryl cAMP. Each bar represents mean ± S.E.M. from 5 experiments with a total of 97-168 oocytes/treatment. To determine the effect of replicate and treatment, analysis of variance was done on arcsin transformed data and differences were detected using Fischer's protected least-squares. Bars indicated with different letters are significantly different ($p<0.05$).


100
80
60
40
20
0
% of oocytes
2 hr
3 hr
a
a
b
b
c
c
c
c
control
KL
dbcAMP
dbcAMP + KL

trend towards greater inhibition of meiosis in the presence of both additives compared to either alone (Fig. 3.8).

3.4 Discussion

The experiments reported here demonstrate that KL caused a transient delay in spontaneous meiotic maturation of fully-grown oocytes isolated from antral follicles. Conversely, by preventing Kit receptor expression on oocytes by the microinjection of antisense oligonucleotides, more oocytes resumed meiosis. In addition, KL treatment was unable to enhance the inhibitory actions of cAMP on oocyte meiosis.

In several investigations with Kit receptor-bearing cell types (hematopoeitic cells, mast cells, primordial germ cells, male germ cells), KL was found to act as a survival and proliferative agent (Dolci et al., 1991 and 1991b; Godin et al., 1991; Matsui et al., 1991; Tsai et al., 1991; Metcalf and Nicola, 1991; Rossi et al., 1993). Consequently, one might have expected KL to stimulate meiotic maturation in oocytes. On the contrary, oocytes cultured in the presence of KL showed a significant, although transient, decrease in the rate of meiotic maturation, as was seen by delayed GVBD and Pb extrusion, although it is quite reasonable to suspect that the delayed formation of polar bodies by the oocytes is simply a reflection of the earlier delay in GVBD. These effects were a specific response to the interaction of KL with Kit receptors, and could be prevented by the addition of ACK2 to immunoneutralize the Kit receptors. It appears then that, as in many cells, the function of Kit in oocytes may be regulation of the cell cycle; however, the regulation by KL in the follicle operates to oppose the progression of the cell cycle. This proposed function as a negative regulator of growth

in certain tissues is supported by observations that Kit expression is lost in breast (Natali et al., 1992a) and testicular germ cell tumours (Strohmeyer et al., 1991).

It has been demonstrated previously that the cumulus granulosa cells mediate the signals which inhibit meiotic maturation (Tsafriri and Channing, 1975; Gwatkin and Anderson, 1976). Cumulus-enclosed oocytes undergo meiotic maturation more slowly than cumulus-free oocytes (Vanderhyden and Armstrong, 1990). In addition, numerous factors have been shown to inhibit meiotic maturation *in vitro*, including oocyte maturation inhibitor (Tsafriri and Channing, 1975; Tsafriri et al., 1976; Tsafriri et al., 1977), dibutyryl cyclic adenosine monophosphate (Masui and Clarke, 1979; Schultz et al., 1983; Eppig and Downs, 1984; Schultz, 1986), hypoxanthine (Downs et al., 1985; Eppig et al., 1985) and Müllerian inhibiting substance (Takahashi et al., 1986; Ueno et al., 1988). We have shown that cumulus granulosa cells are the primary site of KL production in the ovary and that KL inhibits spontaneous meiotic maturation, suggesting that KL may be another granulosa cell-derived regulatory factor that participates in the maintenance of intrafollicular meiotic arrest. Give that gonadotropins stimulated KL expression, it can be postulated that the low levels of gonadotropins that are present throughout follicular development may stimulate sufficient quantities of KL to maintain the growing oocyte in meiotic arrest.

In our experiments we observed a significant delay in rat oocyte maturation in response to KL; however, the effect was transient. There are several possible reasons why the effects of KL in this system are transient. First, the stability of KL in these culture conditions is not known, so perhaps the transient inhibition reflected an unstable stimulus. Recombinant KL which is unglycosylated had been used in these experiments; possibly the

lack of posttranslational modifications directly, or indirectly through the inability of recombinant KL to form non-covalently associated dimers, contributed to a decreased stability in culture. Second, exposure of the oocytes to KL in culture, or the culture conditions themselves, may have caused a desensitization, perhaps through Kit receptor down-regulation, that rendered the oocytes incapable of a sustained response to KL. It has already been demonstrated using mouse mast cells that Kit receptor is sensitive to ligand-mediated internalization and that the internalized ligand-receptor complexes are polyubiquinated and thus most likely targeted for lysosomal degradation (Yee et al., 1993; Miyazawa et al., 1994).

A third possible reason for the transient effects of KL in this culture system lies in the nature of the ligand. The close proximity of the cells that produce the ligand (granulosa) to the cell that bears the receptor (oocyte) suggests that the oocyte might be more receptive to the membrane-bound form of the ligand. The affected phenotype seen with *Mgf*^{tm1} best exemplifies the biological importance of membrane-anchored KL (Table 1.2), where *Mgf*^{tm1} mutant mice are sterile animals lacking coat colour, and suffer from severe anemia, despite the presence of soluble KL (Brannan et al., 1991; Flanagan et al., 1991). Furthermore, in the testis membrane-associated KL has a quality of activity that can not be replaced by larger doses of soluble KL (Allard et al., 1996). Evidence for the importance of membrane-associated KL will be discussed in more detail in section 5.1.

When cumulus-enclosed oocytes were microinjected with antisense oligonucleotides complementary to Kit mRNA, two observations were made: 1) there was drop in Kit receptor levels expressed on oocytes as detected by immunofluorescence, and 2) there was

a decrease in the number of oocytes maintained in meiotic arrest. Antisense oligonucleotides have been used previously to demonstrate the involvement of protein kinases in oocyte maturation (O'Keefe et al., 1989). It has been shown that microinjection of antisense oligonucleotides causes a decrease in the amount of complementary mRNA (targeted RNA) and presumably prevents the synthesis of new protein. In our experiments we observed little or no Kit expression in antisense oligonucleotide-injected oocytes. This complete and rapid loss of Kit within 4 hr after microinjection of antisense oligonucleotides was not unexpected since the half-life of Kit in human and mouse somatic cells is less than 3 hours (Yee et al., 1993; Miyazawa et al., 1994; Ogawa et al., 1995).

To determine the effect of loss of Kit receptor on oocyte meiosis, two conditions were maintained: first, microinjection and subsequent culture were done with intact oocyte-cumulus cell complexes as the cumulus granulosa cells serve as the source of KL for the oocytic Kit receptor. This allowed us to determine the effect of ablation of Kit on spontaneous oocyte meiotic maturation in which the three dimensional structure of the complex was maintained, and there was a continued supply of KL in the form (transmembrane and/or soluble) found *in vivo*. Thus the maintained presence of granulosa cells presumably encouraged activation of Kit receptors. Secondly, since oocytes in culture will spontaneously resume meiosis, IBMX was included in both the microinjection and culture media at a concentration at which 50% of control (uninjected) oocytes were maintained in meiotic arrest. Under these conditions, the microinjection of oligonucleotides antisense to Kit mRNA resulted in an overall decrease in oocytic Kit receptor levels which, in turn, relieved the meiotic arrest in these oocytes.

Although there are no published reports demonstrating an inhibition of oocyte meiotic maturation due to KL/Kit interactions in any other species, recent studies provide circumstantial evidence to suggest that lack of KL may result in the premature maturation of oocytes in antral follicles. Tisdall et al. (1997) demonstrated that in the sheep ovary although KL continued to be expressed in the mural granulosa cells of some large atretic follicles, the cumulus granulosa cells showed a reduction in KL expression. The down-regulation of KL in the cumulus cells around the oocyte in large atretic antral follicles may indicate that lack of KL/Kit interactions are involved in the process of meiotic reactivation which often occurs when the oocyte degenerates (Engle, 1927). In addition, Yoshida and coworkers (1997) demonstrated that when mice were injected intravenously with ACK2 to neutralize Kit function, there was a dramatic decrease in the survival of large antral follicles. Here, the authors suggested that the inactivation of Kit resulted in negative effects on oocyte development, such as the premature meiotic maturation of intrafollicular oocytes which, in turn, resulted in the degeneration of the antral follicles. Although not definitive, these observations suggest that, in sheep and mice, KL/Kit interactions may also play a role in the maintenance of meiotic arrest during follicular development.

Although this study demonstrated that Kit and KL interactions inhibit spontaneous meiotic maturation of oocytes, a number of molecules found within the follicle have also been shown to regulate oocyte meiosis in culture. It is apparent that the maintenance of meiotic arrest is a complex process involving numerous factors whose importance may lie in their developmental expression or their specific interactions with other components of the system. It is possible, therefore, that KL may interact with other ovarian factors within the

follicle in a synergistic manner *in vivo*. In most systems examined to date, KL has limited activity by itself, but acts synergistically with a variety of other cytokines. In studies using mast cells and hematopoeitic precursors, the presence of cytokines, particularly the interleukins (Tsuji et al., 1991; Kirshenbaum et al., 1991; Broxmeyer et al., 1991; Muench et al., 1992), potentiates the effects of KL on cell proliferation. Although interleukins-1 and -6 are also produced by granulosa cells (Barak et al., 1992; Gorospe et al., 1992; Gorospe and Spangelo, 1993), our preliminary experiments indicate that interactions between these cytokines and KL do not result in a significant potentiation of oocyte meiotic inhibition (data not shown).

Cyclic AMP is clearly an important regulator of meiosis and we tested the possibility that KL acts in concert with cAMP to regulate spontaneous meiotic maturation. However, when oocytes are cultured in the presence of both dbcAMP and KL, the response of the combined treatment was not different from dbcAMP alone, and thus KL was not able to enhance the inhibitory effects of dbcAMP. These results provide the first evidence of ligand-tyrosine kinase receptor interaction involved in the maintenance of meiotic arrest and suggest that its signal transduction is independent of cAMP. Thus within the follicle there may be at least two distinct mechanisms by which the oocyte is maintained in arrest. Indeed the possibility of redundant pathways for meiotic inhibition was raised also in the microinjection experiments. When culture media concentrations of IBMX were used that inhibited the resumption of meiosis in 100% of uninjected oocytes, oocytes microinjected with Kit antisense oligonucleotides could not undergo GVBD. Furthermore, although oocyte meiosis normally resumes when intraoocyte cAMP levels drop (Aberdam et al., 1987), oocytes can

be maintained in meiotic arrest despite a drop in intraoocyte cAMP if tyrosine phosphorylation is maintained with the phosphatase inhibitor, vanadate (Morla et al., 1989). Although these authors interpreted their results as suggesting that a tyrosine-phosphorylated mediator (possibly $p34^{cdc2}$) acts downstream of cAMP, an alternative interpretion may be that another tyrosine-phosphorylated mediator (possibly Kit?) acts independently of cAMP.

It should be noted, however, that the combined effect of KL+dbcAMP caused considerably fewer oocytes to undergo meiosis compared with dbcAMP alone, although this difference did not achieve significance because of the variability of response to the combined treatment. Therefore, the possibility that Kit and cAMP share a common effector molecule(s) cannot be ruled out. Indeed, the perturbation of cAMP-maintained arrest by ablation of Kit expression using antisense oligonucleotides suggests that this interpretation is quite feasible. Whether Kit is a tyrosine-phosphorylated mediator that acts independently of cAMP or whether these two pathways converge on common effector molecules remains to be determined.

To date, studies to identify Kit signal transduction pathways have been performed in cells that respond to KL stimulation by activating the cell cycle (section 1.3.1). The molecular basis of the inhibition of the cell cycle seen with oocytic Kit activation has not been determined. However, phosphorylated Kit receptors have been demonstrated in lysates prepared from mouse ovaries and the KL-stimulated phosphorylation of Kit in spermatogonia has also been demonstrated (Dym et al., 1995). Many of the signal transduction molecules shown to mediate Kit signal in other cell types are also known to be present in oocytes. Raf, Mek and Map kinases p42 and p44 have been identified in mouse

oocytes (Harrouk and Clarke, 1995) along with PLCγ1 (Sette et al., 1997). Ras proteins (Gibb et al., 1989), GAP (Pomerance et al., 1996), and the p85 subunit of PI3K (Klippel et al., 1994) have not been investigated in mammalian oocytes although their expression has been reported in Xenopus oocytes and the microinjection of SH2 domains of these molecules resulted in an inhibition of G_2 to M transition (Aroca et al., 1996). Interestingly, microinjection of SH2 domains of PI3K was able to both stimulate and inhibit Xenopus oocyte meiotic maturation depending on the region from which the SH2 domains were derived, and hence PI3K serves as an example of a molecule that is able to have opposing effects on the cell cycle, at least *in vitro*. In addition, KL-stimulated inhibition of oocyte maturation may require a slightly different subset of molecules to transduce this signal or molecules which function differently in this milieu. For example, KL-induced adhesion, cell migration and differentiation in mast cells require the activation of PKC (Blume-Jensen et al., 1995; Vosseller et al., 1997), whereas PKC activity is not required for KL-stimulated mast cell proliferation (Blume-Jensen et al., 1993; 1994 and 1995).

Interestingly, activation of PKC is associated with an inhibition of meiotic maturation. PKC stimulation using the active phorbol ester 12-O-etra-decanoyl phorbol-13-acetate (TPA) caused the inhibition of both GVBD and Pb formation in mouse oocytes (Bornslaeger et al., 1986b; Lefevre *et al.*, 1992). Very little is known about the interactions of molecules involved in the PKC signal transduction pathway and cell cycle regulators; however, PKC stimulation inhibits specific changes in oocyte phosphoprotein metabolism (Bornslaeger et al., 1986b). In addition, TPA stimulation did not inhibit the maturation-associated decrease in oocytic cAMP levels, and inhibition of cAMP-dependent protein

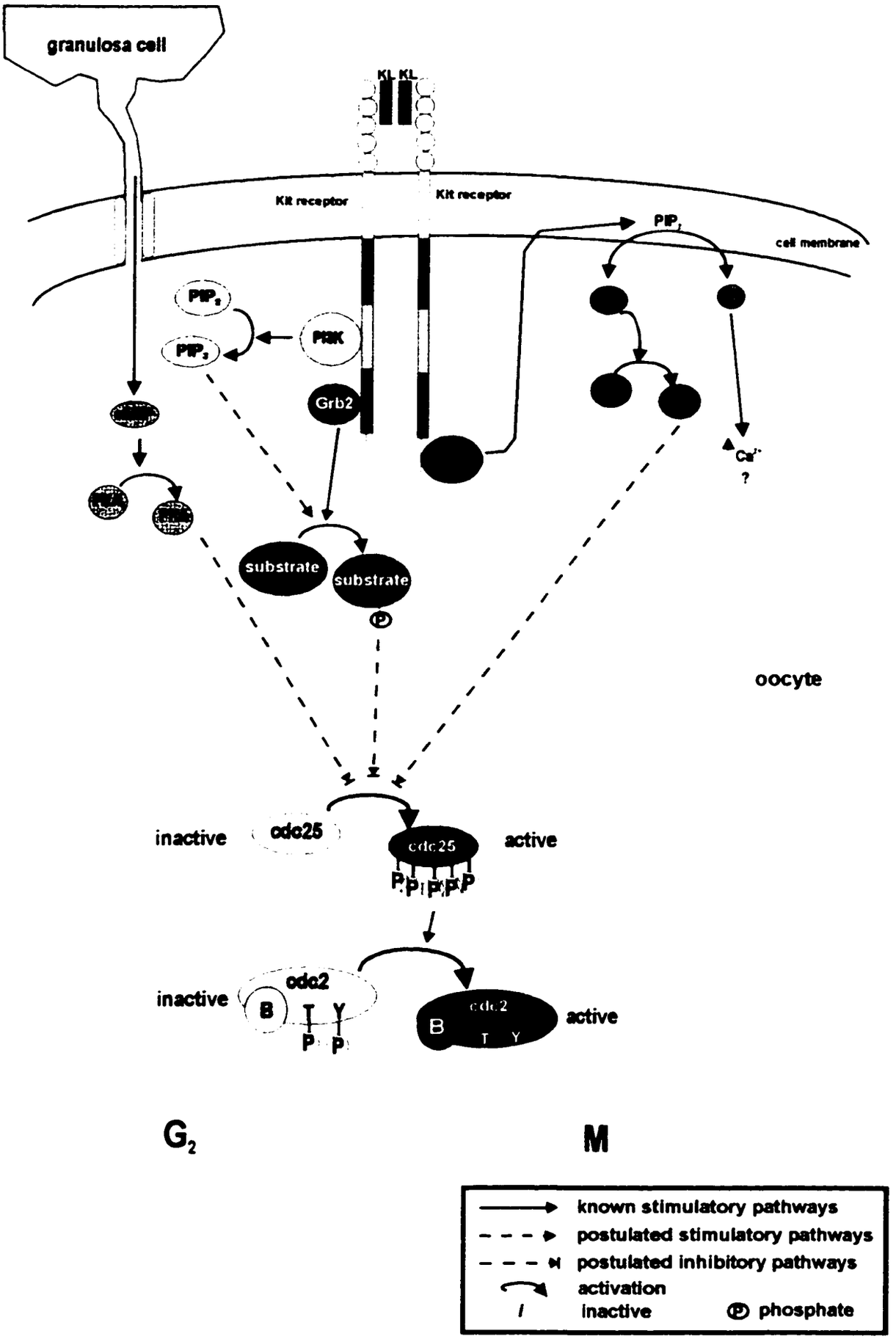
kinase (PKA) failed to induce GVBD in the presence of TPA implying that PKC stimulation may occur either downstream of PKA activity or independent of the PKA pathway (Bornslaeger et al., 1986b). Although the degree of inhibition was different between PKC- and Kit-stimulated inhibition of oocyte maturation, both inhibited GVBD and Pb in a cAMP-independent manner. Since Kit signal transduction for some cellular responses requires PKC activation and, PKC activation inhibits oocyte meiotic maturation, we propose that the effect of KL on oocyte maturation may be mediated, in part, by signal transduction through PKC stimulation. A second possibility is that Kit activation occurs independently of PKC stimulation but both act on a common cell cycle regulatory molecule(s) to inhibit G_2 to M transition.

Since $p34^{cdc2}$ is a universal regulator of the cell cycle, Kit signal transduction may ultimately affect the phosphorylation state of $p34^{cdc2}$. Goren and Dekel (1994) have demonstrated the presence of a vanadate-sensitive tyrosine phosphorylated molecule with cdc25 activity which regulates the activity of $p34^{cdc2}$. This molecule may be the common target of both cAMP-mediated and Kit-mediated inhibition in G_2 to M transition (Fig 3.9). Furthermore, meiosis-specific molecules may also be involved in the modulation of Kit and KL interactions. *Ayk1*, a mammalian serine-threonine kinase expressed specifically in oocytes of antral follicles and late pachytene (meiotically-active) spermatocytes was the first mammalian gene to be identified having high homology to genes involved in meiotic cell cycle progression in both yeast and Drosophila (Yanai et al., 1997). In addition, as yet unidentified meiosis-specific molecules may be involved in the transduction of Kit signal to the oocyte nucleus.

Figure 3.9

Possible signal transduction pathways involved in inhibition of G_2 to M transition

KL stimulation of Kit receptor results in the phosphorylation of the Kit receptor which in turn activates two signal transduction cascades: the first via phosphorylations of substrates known to be involved in the activation of Kit in other cells (PI3K and Grb2), and the second via an activation of the PKC pathway. Activation of PKA, although independent of Kit signalling, is an important pathway also involved in the maintenance of meiotic arrest.


granulosa cell
KL KL
Kit receptor
Kit receptor
PIP2
cell membrane
PIP2
PIP3
PI3K
Grb2
Ca2+
?
substrate
substrate
P
oocyte
inactive
cdc25
cdc25
active
P P P P P
inactive
cdc2
B
T Y
P P
B
cdc2
T Y
active
G2
M
known stimulatory pathways
postulated stimulatory pathways
postulated inhibitory pathways
activation
inactive
P phosphate

These studies demonstrate that Kit/KL interactions inhibit the spontaneous maturation of the oocyte and may be important regulators in the intrafollicular maintenance of oocyte meiotic arrest.

CHAPTER 4 - CHANGES IN KL AND KIT EXPRESSION IN RESPONSE TO HCG STIMULATION

4.1 Introduction

In most mammals, the resumption of meiosis *in vivo* is triggered by a surge in LH levels. The involvement of LH in oocyte maturation has been demonstrated in cultured fully developed follicles (Eppig, 1993) and abrogation of this gonadotropin surge prevents the maturation of the oocyte (reviewed in Tsafriri et al., 1982). Although oocytes do not express LH receptors, FSH stimulation induces the expression of LH receptors on granulosa cells of preovulatory antral follicles (Lawrence et al., 1980). Therefore, LH most likely induces oocyte maturation via an indirect action which is mediated by follicular granulosa cells. One hypothesis is that LH induces a dismantling of the intercellular communication between oocytes and granulosa cells, specifically by the breakdown of heterologous gap junctions (Dekel, 1988). In this way LH does not provide a direct stimulatory signal for oocyte maturation but rather acts to relieve the inhibitory influences of granulosa cells. The finding that metabolic coupling in oocyte-cumulus cell complexes is reduced to 50 % of its initial level prior to the apparent resumption of meiosis in cultured cumulus enclosed rat oocytes lends support to this hypothesis (Dekel, 1988). According to this hypothesis, gonadotropin-induced GVBD is therefore initiated in the same way as spontaneous oocyte maturation; namely, by the withdrawal of granulosa cell produced, maturation inhibitory influences on the oocyte.

Another hypothesis proposes that LH induces the production of a positive GVB-inducing signal in granulosa cells that is then transmitted to the oocyte. Experiments with cultured cumulus enclosed oocytes suggest that a positive stimulus is generated within cumulus cells in response to ligand treatment that acts upon the oocyte to stimulate GBVD in spite of a continued presence of inhibitory influences (Downs et al., 1988; Fagbohun and Downs, 1991). However, perhaps the most compelling evidence to support this hypothesis is provided by recent findings which demonstrate that gonadotropin stimulation of oocyte-cumulus cell complexes results in the secretion of a heat-stable meiosis-activating substance which is capable of inducing meiosis in denuded oocytes in the presence of hypoxanthine (Byskov et al., 1995; Byskov et al., 1997). Therefore, although there are several factors produced by granulosa cells capable of contributing to the maintenance of oocyte meiotic arrest, the mechanism by which the LH surge stimulates oocyte meiotic maturation *in vivo* has not been fully elucidated.

Based on the localization of KL within antral follicles and its activity *in vitro*, it is possible that KL may be an important ovarian factor that maintains the oocyte in meiotic arrest in antral follicles. In this chapter the role that KL/Kit interactions play in the regulation of oocyte meiotic maturation is further examined. The main focus of this section is to determine if the resumption of oocyte meiosis in response to the LH surge is associated with a decrease in Kit receptor levels or a change in granulosa cell KL expression. To test this hypothesis, experiments were performed to document the timing of rat oocyte meiosis in response to hCG. Once the timing of maturation was established, the expression of Kit receptors in rat oocytes after hCG stimulation but before the onset of meiotic maturation was

determined. KL mRNA levels were determined in total granulosa cells, as well as in cumulus granulosa cells after hCG stimulation. Using these data changes in Kit and/or KL expression were correlated with hCG-stimulated resumption in meiosis.

4.2 Materials and Methods

4.2.1 Timing of resumption of oocyte meiosis after hCG injection

Prepubertal Sprague-Dawley rats (26-28 day old) from the University of Ottawa animal colony were injected with 7.5 IU of PMSG and 40 hr later injected with 7.5 IU human chorionic gonadotropin (hCG; Sigma). This hormone treatment results in the presence of fully grown follicles at 40 hr after PMSG treatment. If PMSG-primed rats are treated with hCG, ovulation occurs 12 hr later (Walton and Armstrong, 1983). Animals were sacrificed at 0, 2, 3, 4, 5 and 6 hr post-hCG injection and ovaries placed in WAY media supplemented with 5% FBS and 500 μM IBMX. (This concentration of IBMX prevents the premature resumption of meiosis in meiotically-arrested rat oocytes). OCC were isolated from the 7-10 largest follicles of each ovary with the assumption that these were destined to ovulate. At the later time points the preovulatory follicles could be more easily distinguished because they were more vascularized. OCC were stripped of their granulosa cells and assessed for the presence or absence of the germinal vesicle.

4.2.2 Kit receptor expression before and after the administration of hCG

OCC were isolated from ovaries 40 hr after PMSG and 3 hr post hCG treatment. Oocytes were stripped of their granulosa cells and exposed to antibodies to detect Kit

receptors as described previously (section 2.2.2). In some cases oocytes were exposed to Kit peptides (Santa Cruz Biotechnology) to determine the specificity of the Kit antibodies.

4.2.3 KL expression in periovulatory follicles

PMSG/hCG treated rats were sacrificed at 0, 12, 24 and 40 hr post-PMSG treatment and 3, 6, 12, 24 and 48 hr post-hCG treatment. Ovaries were removed and either submerged in LiCl/urea for RNA extraction or put into WAY/FBS for granulosa/luteal cell isolation and subsequent RNA extraction. RNA was extracted from ovaries and freshly isolated granulosa cells using LiCl precipitation methods and, isolated RNAs were electrophoretically separated and blotted on Nylon membranes for northern blot analysis using procedures described in section 2.2.4.

4.2.4 Relative expression of membrane-bound and soluble KL in periovulatory granulosa cells

Reverse transcription polymerase chain reaction (RT-PCR) was used to determine the relative proportion of KL-1 and KL-2 transcripts in the same preparations of RNA that had been used for northern blot analysis as described above. RT reactions (21 µl total volume) were done using 1.5 µg aliquots of total RNA, and the following components were added: 1 x amplification buffer (Gibco BRL), 3.8 mM $MgCl_2$ (Gibco BRL), each dNTP at 0.95 mM (Boehringer Mannheim), 4.76 µg/ml Oligo $(dT)_{12-18}$ (Boehringer Mannheim), 0.45 U/µl RNase inhibitor (Boehringer Mannheim), 10 U/µl Superscript RT (Boehringer Mannheim). These reactions were incubated for 1 hr at 37°C and then heat inactivated at

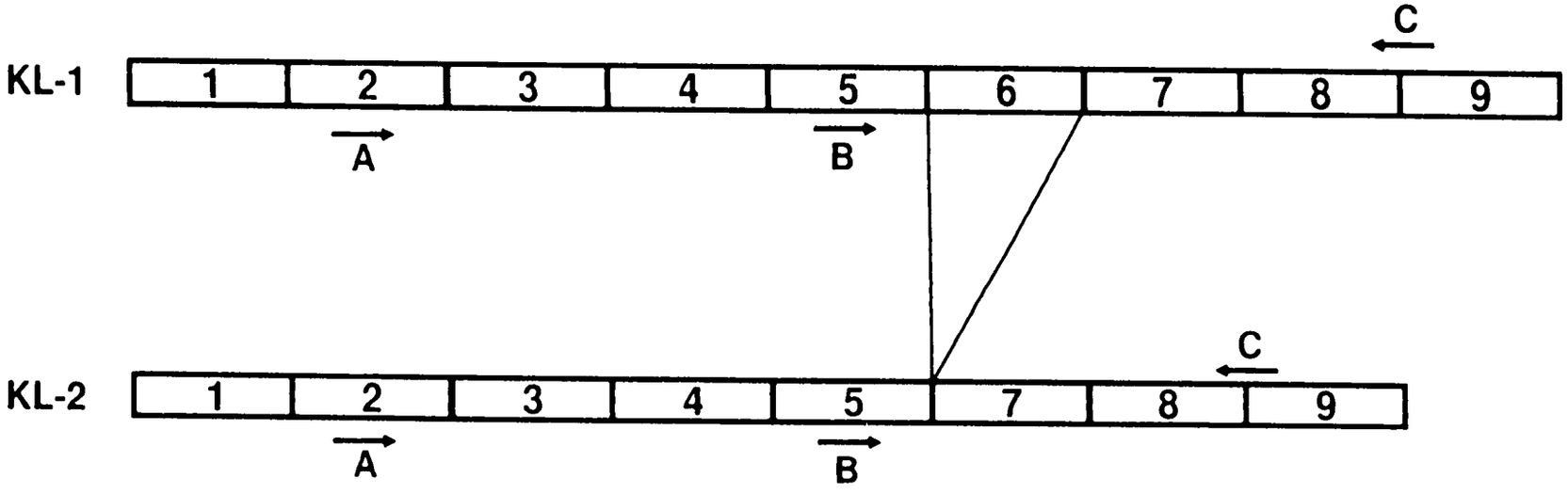
95°C for 5 min. PCR reactions (50 µl) were done in the same tube with the addition of the following components: 1 x amplification buffer, 1 mM $MgCl_2$, 0.2 pmoles/µl each KL primer (generously donated by AMGEN, Thousand Oakes, CA) and 0.02 U/µl TAQ polymerase (Gibco). Two separate pairs of primers were used, each pair spanning the alternatively spliced exon, so that both transcripts were amplified in our reactions (Fig. 4.1). Perkin-Elmer 2400 machines were used for the PCR reactions, using the following program: 95°C for 5 min, 3 cycles [95°C, 60°C and 72°C, at 2 min, 2 min and 3 min, respectively], 22 cycles [95°C, 60°C, and 72°C at 1 min each], then a 10 min 72°C final extension time. PCR products were then separated on a 1% NuSieve, 1% agarose gel. Some PCR reactions were done using water instead of RT sample to serve as a negative control. At least 4 replicates had been done for each time point.

To determine changes in KL mRNA expression in cumulus cells, OCC were isolated at 0, 1, 2 and 3 hr post-hCG injection of PMSG-primed rats and RNA was extracted from groups of 100 OCC as described previously (Temeles et al., 1994) with the exception that 1% linearized acrylamide (Gaillard and Strauss, 1990) was used to aid in RNA precipitation. To determine the relative changes in abundance of KL mRNA, an RT-PCR assay that uses exogenously added globin mRNA as an external standard (Temeles et al., 1994; Latham et al., 1994) was utilized and products amplified using KL specific primers (Fig. 4.1) were compared to those amplified by β-globin specific primers from the same RT reaction. For the primers that amplify KL and β-globin, the range of cycle numbers over which cDNA production was linear was determined by quantitating PCR products after staining with SYBR green (Molecular Probes Inc., Eugene, OR) at a dilution of 1:10,000 in distilled water.

Figure 4.1

Position of primers used in the amplification of KL cDNA.

KL-1 and KL-2 are two major mRNA isoforms of KL that arise from the differential splicing to include (KL-1) or exclude (KL-2) exon 6. Either upstream primers A or B were used with downstream primer C in PCR amplification of both KL cDNAs. Primer sequences and expected amplicon lengths are shown for each primer pair.



Primer sequence (5'-3')

A = ACT GCT CCT ATT TAA TCC TCT C
B = GTA TTT TCA ATA GAT CCA TTG A
C = CCA GTA TAA GGC TCC AAA AGC AA

Primer Pair	Product length
A and C	666 and 582 bp
B and C	287 and 203 bp

Gels were scanned using the blue fluorescence mode on a STORM 860 (Molecular Dynamics Sunnyvale, CA). Data was further analyzed using the ImageQuaNT software provided with phosphoimager. This RT-PCR assay can be used to determine relative abundance of KL to β-globin mRNA among different samples, but cannot be used to determine the absolute abundance of KL mRNA. In addition, endogenous β-actin was amplified to determine integrity of cellular RNA at the time of extraction and was used as a qualitative control only, as linearity was not determined for these primers.

For each group of OCC, the entire RNA sample (100 OCC) was used for RT (21 µl) reactions and done as follows: isolated RNA, 0.8 U/µl RNase inhibitor (Boehringer Mannheim), and 25 µg/ml Oligo $(dT)_{12-18}$ (Boehringer Mannheim) were denatured by heating at 65°C for 10 min and quick cooling on ice for 5 min, and then 1 x first strand buffer (Gibco BRL), 10 mM DTT (Gibco BRL), each dNTP at 2 mM (Boehringer Mannheim), and 10 U/µl Superscript RT (Boehringer Mannheim) were added to the denatured RNA/Oligo $(dT)_{12-18}$ mix and incubated for 1 hr at 37°C. After completion of the RT reaction, the RNA template was degraded by treatment with 0.08 U/µl RNase H for 10 min at 55°C. Amplification of KL or β-actin cDNA was done using 20 OCC equivalents or 10 OCC equivalents, respectively. PCR (50 µl) reactions were performed with the addition of the following components: 1 x amplification buffer, 1 mM $MgCl_2$, 0.02 U/µl TAQ polymerase (Gibco) and 0.2 pmoles/µl each KL primer (generously donated by AMGEN) or 0.2 pmoles/µl each β-actin primer (Clonetech Laboratories, Palo Alto, CA). Perkin-Elmer 2400 or 9600 machines were used for the PCR reactions, using the following program: 95°C for 5 min, 3 cycles [95°C, 60°C and 72°C, at 1 min each], 32 cycles [95°C, 60°C, and 72°C at

30 sec each], then a 10 min 72°C final extension time. Amplification of β-globin cDNA was done using 20 OCC equivalents with the addition of the following components: 1 x amplification buffer, 1.5 mM $MgCl_2$, 0.2 pmoles/μl each β-globin primer (Temeles et al., 1994; Latham et al., 1994) and 0.02 U/μl TAQ polymerase (Gibco). Perkin-Elmer 2400 or 9600 machines were used for the PCR reactions, using the following program: 95°C for 5 min, 27 cycles [95°C, 55°C, and 72°C at 30 sec each], then a 10 min 72°C final extension time. Rat mural granulosa cells were used as a positive control. At least 3 replicates were performed for each time point.

4.3 Results

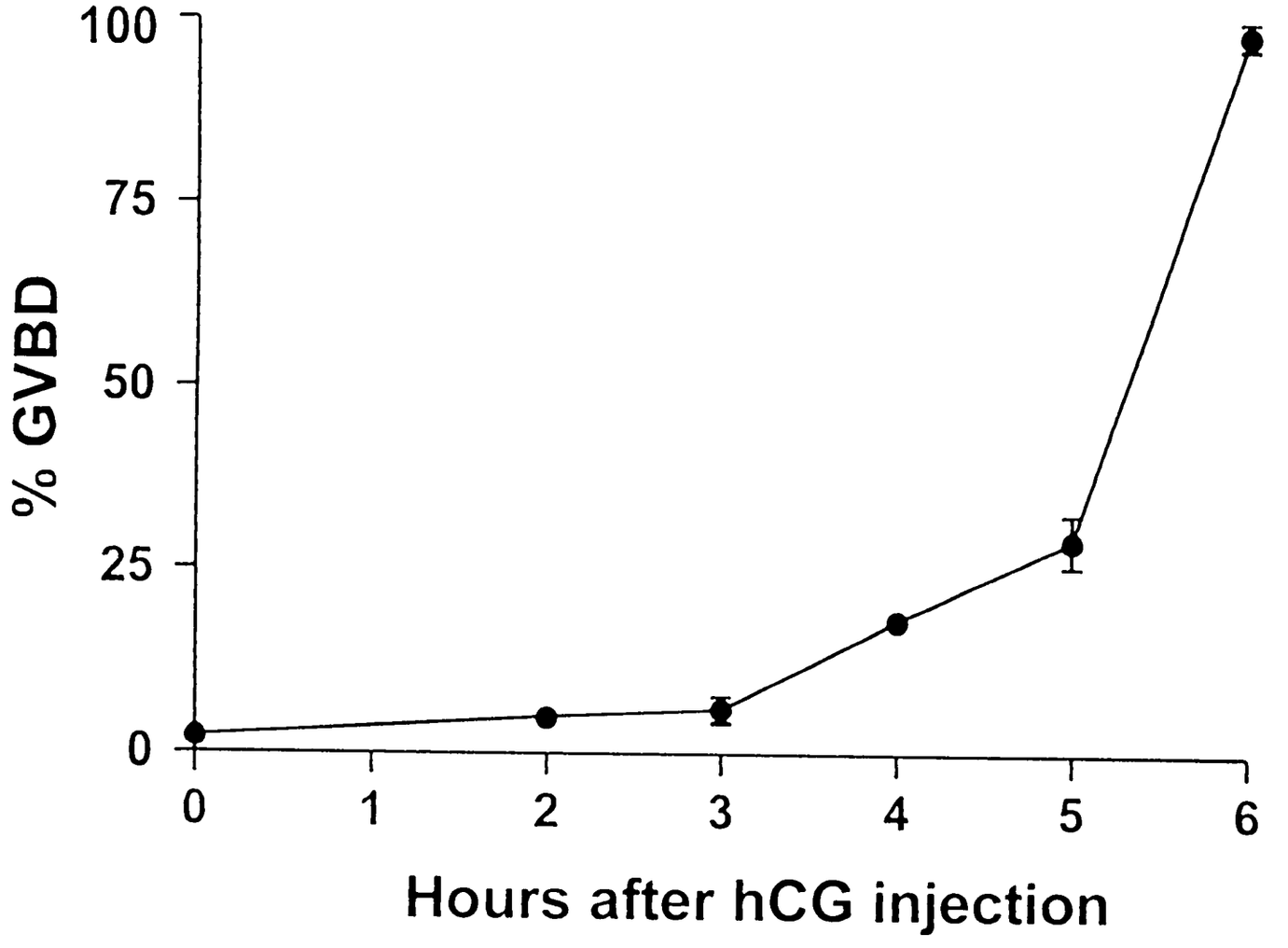
4.3.1 Timing of rat oocyte meiosis *in vivo*

To determine the timing of oocyte meiotic maturation in antral follicles after hCG administration, oocytes were isolated from the largest follicles at various time points after hCG stimulation and assessed for the presence or the absence of the germinal vesicle (Fig. 4.2). Oocytes isolated from preovulatory follicles 0-3 hr after hCG stimulation remained meiotically arrested with only 6.1 ± 1.8 % showing signs of GVBD. By 4 hr, 18.1 ± 0.5 % of oocytes had undergone GVBD and by 6 hr the percentage of oocytes maturing had reached 98 .2 ± 1.8%. Since no observable meiotic maturation was seen prior to 3 hr post-hCG treatment, close attention was paid to KL and Kit expression within the first 3 hr after hCG stimulation

Figure 4.2

Timing of GVBD in rat follicles after hCG treatment

Prepubertal rats were treated with PMSG and hCG. The 7 to 10 largest follicles were disrupted 0, 2, 3, 4, 5 and 6 h post-hCG treatment, oocyte-cumulus cell complexes were collected and oocytes were assessed for GVBD. Values presented are the mean ± S.E.M. of 3 experiments using a total of 54-116 oocytes per time point. On time points where variability is small, error bars are not evident as they are within the symbol.


100
75
50
25
0
% GVBD
0
1
2
3
4
5
6
Hours after hCG injection

4.3.2 Effect of hCG stimulation on Kit levels

The LH surge is the trigger for the resumption of oocyte meiosis in fully-grown preovulatory follicles in most mammals. To determine if the meiosis-inducing actions of LH could be attributed, at least in part, to alterations in the expression of Kit receptors, immunofluorescent staining was performed to localize Kit receptors in rat OCC before and 3 hr after *in vivo* administration of hCG. Similar levels of Kit receptors were present in oocytes before and after hCG treatment (Fig. 4.3a ,b), indicating that the LH surge does not inhibit the expression of Kit. The specificity of Kit antibodies was confirmed by competing the Kit antibodies with 10-fold greater concentration of Kit peptides (Fig. 4.3c,d). All staining could be competed with Kit peptides indicating that all staining seen with the Kit antibodies was specifically due to interactions with Kit receptors.

4.3.3 Effect of hCG on granulosa cell KL expression

KL expression was assessed in whole ovaries and in the granulosa cells isolated from untreated rats and from animals at various times after treatment with PMSG and hCG, with particular interest in the expression of KL within 3 hr after hCG stimulation. In whole ovary RNA (Fig. 4.4), there was a moderate, but significant ($p<0.01$) increase in KL expression with hormone (PMSG/hCG) treatment which peaked 6 hr after hCG treatment. Levels of KL mRNA expression returned to those of untreated animals at subsequent time points. When the band intensities were normalized for loading differences using α-tubulin band intensities, it was determined that 6 hr post-hCG treatment there was a 2.4 ± 0.1 fold increase in KL expression in whole ovary RNA extracts compared to KL expression in untreated (control) ovary samples. When northern blot analysis was done with RNA extracted from granulosa

Figure 4.3

Immunofluorescent detection of Kit receptor levels in oocytes isolated 40 h after PMSG injection or 3 h after hCG injection of PMSG-primed rats

Similar levels of Kit are detected before (a) and 3 hr after (b) *in vivo* hCG administration. To test the specificity of the Kit antibodies, a competition reaction was set up where oocytes were incubated simultaneously with the Kit antibodies and Kit peptides in 10 fold excess (c). The bright field photomicrograph of this oocyte (c) is also shown (d).

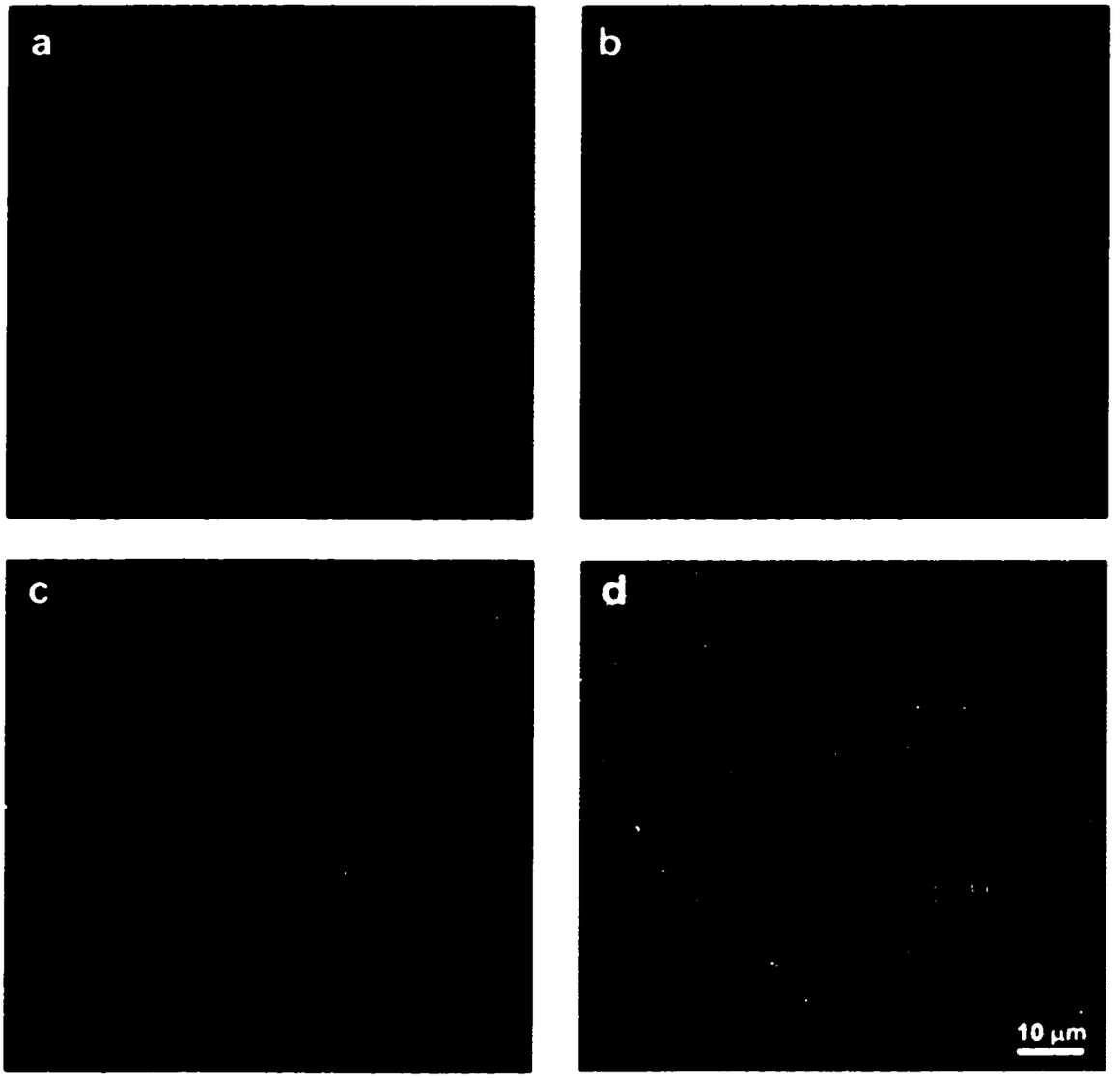
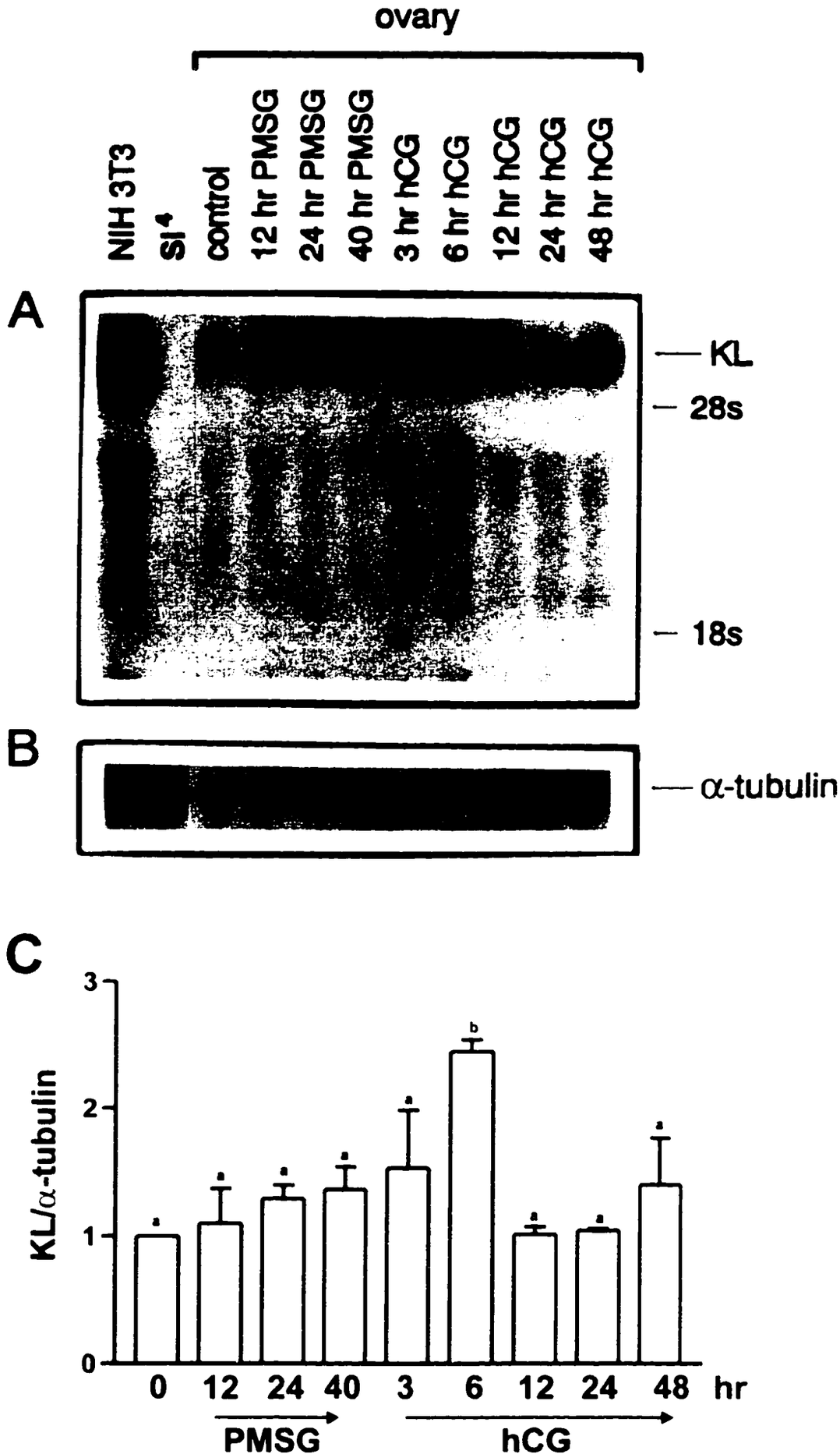

a
b
c
d
10 µm

Figure 4.4

Northern blot analysis of KL in rat ovaries after PMSG and hCG treatment

Total RNA of ovaries from untreated (control) and hormone-primed (PMSG and hCG) rats were probed with a ^{32}P-labelled 2.06 kb KL cDNA probe (A). NIH 3T3 and *Sl*^{d} cells served as positive and negative controls, respectively. The blot was re-probed with ^{32}P-labelled α-tubulin cDNA(B). To quantitate the effect of hormone treatment, the intensity of the bands on the autoradiographic film was estimated using densitometry and the ratio of KL/α-tubulin values were standardized to control levels which were arbitrarily set to 1.0. A histogram illustrating the differences in KL/α-tubulin ratios where each bar represents the mean ± S.E.M. values of 3 experiments is shown in C. Bars indicated with different letters are significantly different (p<0.01).


ovary
NIH 3T3
SI⁴
control
12 hr PMSG
24 hr PMSG
40 hr PMSG
3 hr hCG
6 hr hCG
12 hr hCG
24 hr hCG
48 hr hCG
A
KL
28s
18s
B
α-tubulin
C
KL/α-tubulin
3
2
1
0
b
a
0
12
24
40
3
6
12
24
48
hr
PMSG
hCG

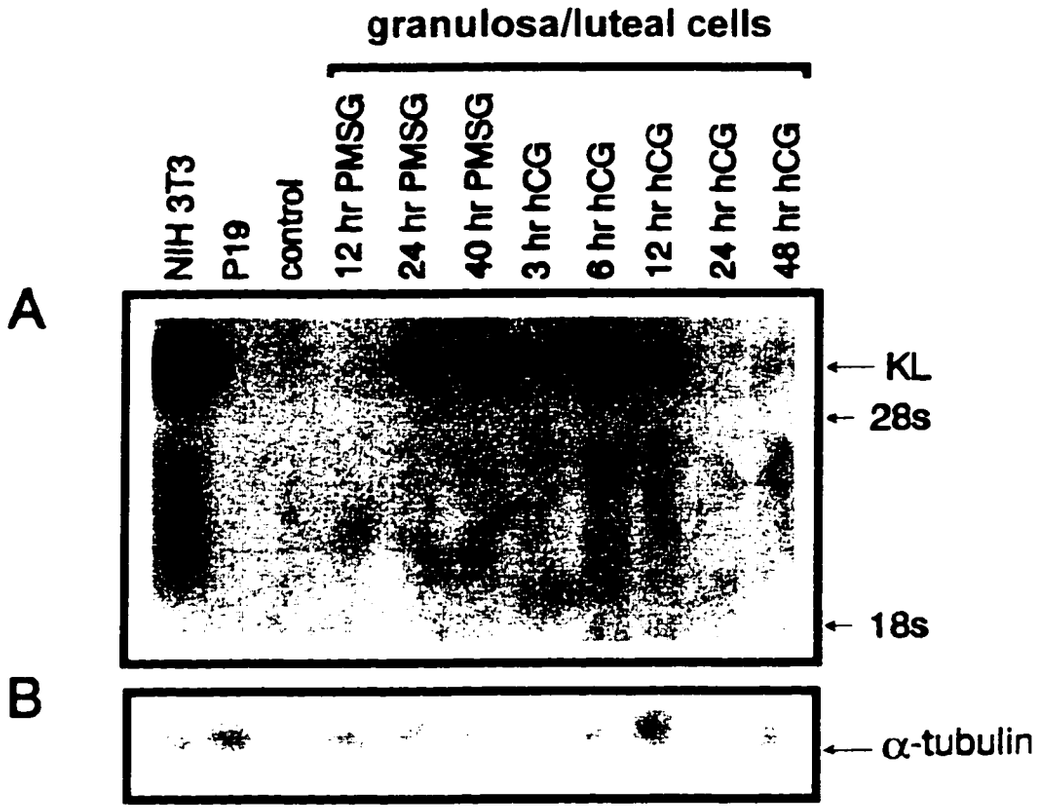
or luteal cells of hormone (PMSG/hCG)-treated and untreated animals (Fig. 4.5), a similar but more dramatic trend was seen ($p<0.01$). When the band intensities were normalized for loading differences using α-tubulin signal, a 3.3 ± 0.2 fold increase in KL expression was seen 40 hr after PMSG treatment compared to untreated animals. The increase in KL expression peaked to levels that were 7.1 ± 0.5 fold higher at 6 hr post-hCG treatment compared to levels seen in RNA extracts from untreated (control) granulosa cells. A significant effect of hCG was seen when comparing KL expression before (40 hr PMSG) and after 6 hr hCG treatment ($p<0.01$). Twenty-four hours after hCG stimulation KL expression returned to levels seen in control granulosa-cell luteal samples. No changes in KL mRNA levels were evident when comparing signal before (40 hr PMSG) and 3 hr after hCG stimulation.

Although there were dramatic alterations in the total amount of follicular KL mRNA levels in response to hCG stimulation, these changes did not occur until 6 hr after hCG stimulation and, therefore, are unlikely to mediate the resumption of meiotic maturation. To determine if there was a change in the form of KL presented to the Kit receptor, the relative abundance of the KL-1 and KL-2 transcripts was determined using RT-PCR on total RNA isolated from granulosa cells using KL primers (Fig 4.1) which span the alternatively spliced exon 6. PCR conditions were used that maintained the amplification of cDNA encoding KL-2 in the linear range to ensure that results accurately reflect the differences between levels of KL-1 and KL-2 transcripts within each RNA sample. In granulosa cell preparations from untreated ovaries and growing follicles (12, 24 and 40 hr after PMSG-treatment), there was a relative abundance of KL-2 transcripts compared to almost undetectable levels of KL-1

Figure 4.5

Northern blot analysis of KL in granulosa and luteal cell samples isolated from ovaries after PMSG and hCG treatment

Total RNA of granulosa cells and luteal cells from untreated (control) and hormone-treated (PMSG and hCG) rats were probed with a ^{32}P-labelled 2.06 kb KL cDNA probe (A). NIH 3T3 and *Sl*^{d} cells served as positive and negative controls, respectively. The blot was re-probed with ^{32}P-labelled α-tubulin cDNA (B). To quantitate the effect of hormone treatment, the intensity of the bands on the autoradiographic film was estimated using densitometry and the ratio of KL/α-tubulin values were standardized to control levels which were arbitrarily set to 1.0. A histogram illustrating the differences in KL/α-tubulin ratios where each bar represents the mean ± S.E.M. values of 3 experiments is shown in C. Bars indicated with different letters are significantly different (p<0.01). * indicates values that are significantly different from 40 hr PMSG treatment group (p<0.01).


granulosa/luteal cells
NIH 3T3
P19
control
12 hr PMSG
24 hr PMSG
40 hr PMSG
3 hr hCG
6 hr hCG
12 hr hCG
24 hr hCG
48 hr hCG
A
KL
28s
18s
B
α-tubulin

C

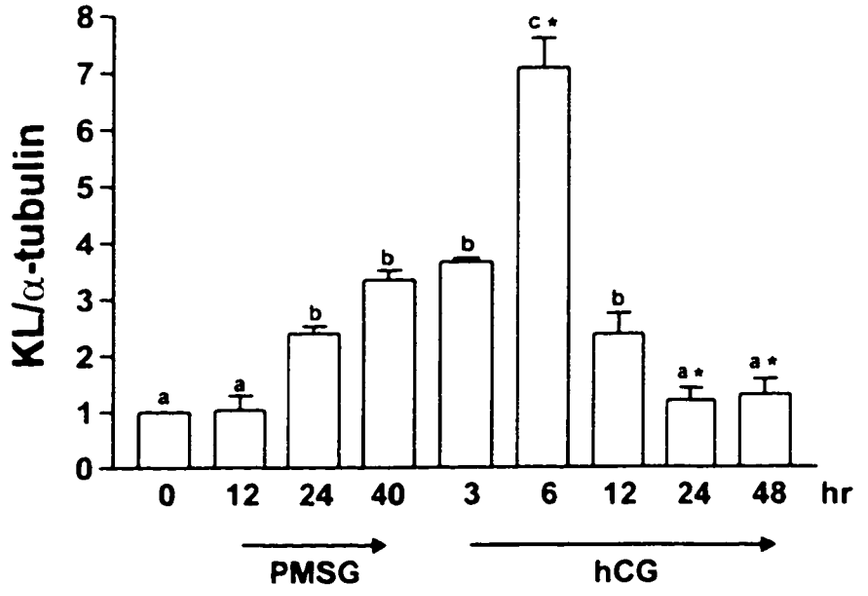
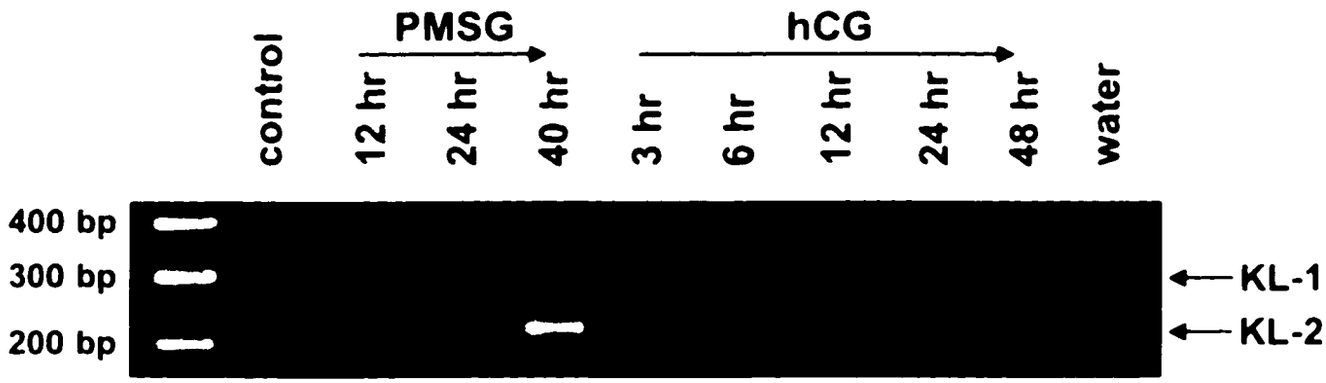

KL/α-tubulin
0
1
2
3
4
5
6
7
8
a
a
b
b
b
c*
b
a*
a*
0
12
24
40
3
6
12
24
48
hr
PMSG
hCG

Figure 4.6

Hormonal regulation of KL-1 and KL-2 mRNA expression in granulosa/luteal cells

Reverse transcription-polymerase chain reaction was performed on granulosa cells isolated from rats at 12, 24 and 40 h after PMSG injection, and at 3, 6, 12, 24 and 48 h after hCG injection of PMSG-primed rats. Granulosa cells from untreated rats were used as a control. KL primers (B and C)were used to amplify the KL-2 (203 bp) and KL-1 (287 bp) transcripts. Similar results were obtained with the second pair of PCR primers, A and C (data not shown).


PMSG
hCG
control
12 hr
24 hr
40 hr
3 hr
6 hr
12 hr
24 hr
48 hr
water
400 bp
300 bp
200 bp
KL-1
KL-2

transcripts (Fig. 4.6). However, treatment with hCG caused a distinct shift in the relative abundance of each transcript, with KL-1 transcripts increasing rapidly after hCG treatment until they predominated at later time points (Fig. 4.6). Thus in response to hCG, there is a shift from transcripts encoding for membrane-bound KL towards a greater abundance of transcripts that more readily yield soluble KL.

To determine if a similar shift in the expression of KL transcripts occurred in the cumulus cells, oocyte-cumulus cell complexes were isolated from rats at hourly intervals after hCG injection (Fig. 4.7). In complexes from PMSG-treated ovaries there was a relative abundance of KL-2 transcripts compared to lower levels of KL-1 transcripts, similar to the results found in mural granulosa cells (Fig. 4.7). However, within 2 hr after hCG treatment, transcripts for both KL-1 and KL-2 became undetectable and remained undetectable at 3 hr post-hCG, despite the integrity of β-actin and the success of the reverse transcription reactions, as demonstrated by the detection of rabbit β-globin.

4.4 Discussion

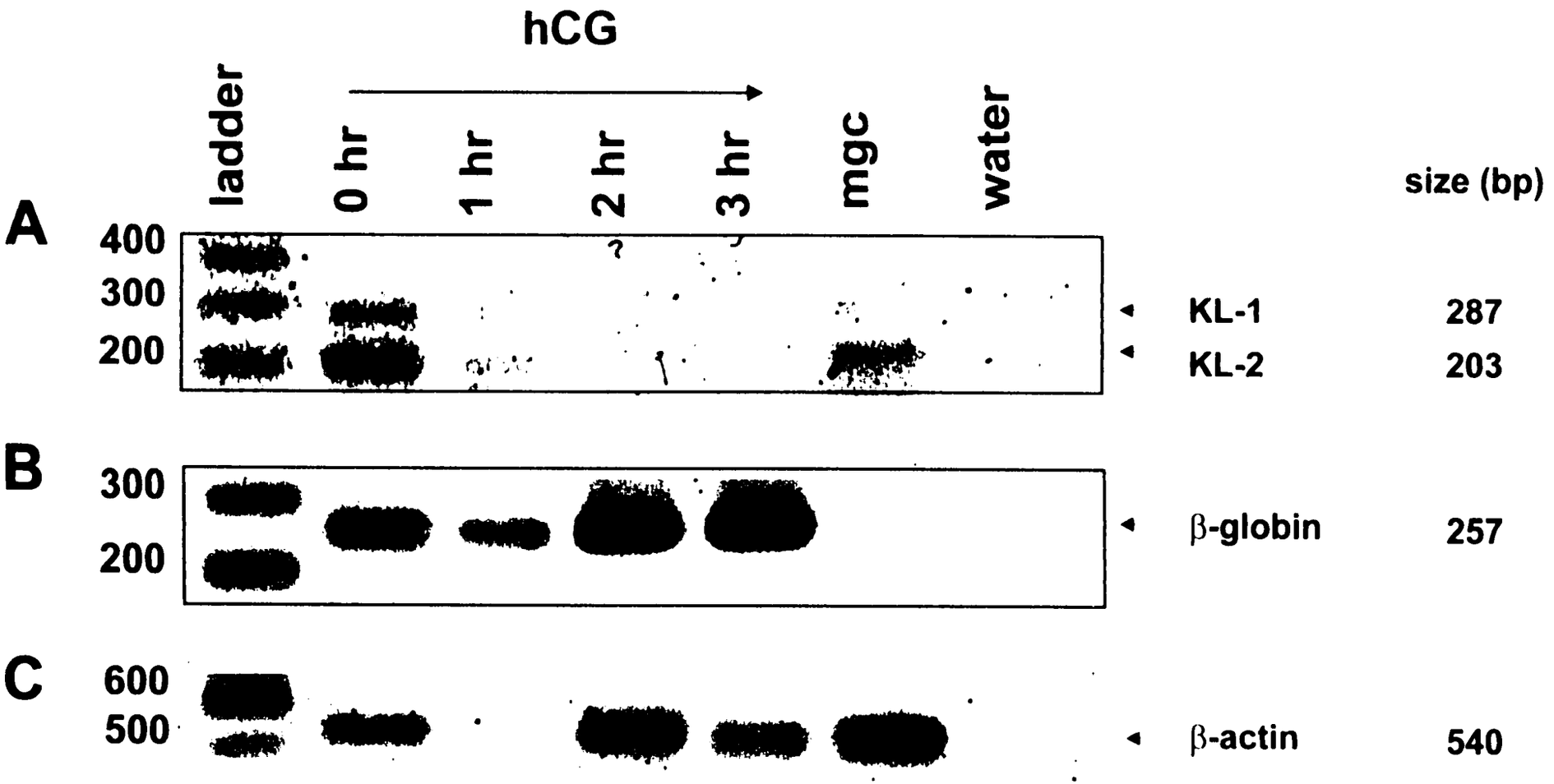
We have observed that, although Kit receptor levels in oocytes do not change in response to hCG stimulation, there is a shift in the form of KL transcripts present in mural granulosa cells from membrane-bound to more soluble KL after hCG stimulation and a loss of both forms of KL in the cumulus cells. The timing of KL mRNA downregulation in cumulus cells occurs prior to the onset of meiotic maturation in rat oocytes.

In most mammals, oocytes in preovulatory antral follicles are maintained in meiotic arrest until the surge in LH levels stimulates the resumption of oocyte meiosis (Tsafriri et al.,

Figure 4.7

Expression of KL in cumulus granulosa cells before and after hCG stimulation

Oocyte-cumulus cell complexes (OCC) were collected 0, 1, 2 and 3 hr after hCG treatment and utilized for RNA extraction and reverse transcription-polymerase chain reaction (RT-PCR). For each sample, a single RT reaction was used for PCR amplification of the following cDNAs: both forms of KL cDNA using primers B and C that span the alternatively spliced exon (A); rabbit β-globin cDNA, exogenously added to samples prior to RNA isolation as an external standard, using globin-specific primers (B); and, β-actin cDNA, endogenously present in the samples, using β-actin-specific primers (C). Rat mural granulosa cells (mgc) were used as a positive control. Note that in B, the last two lanes are water blanks.


hCG
ladder
0 hr
1 hr
2 hr
3 hr
mgc
water
size (bp)
A
400
300
200
KL-1
287
KL-2
203
B
300
200
β-globin
257
C
600
500
β-actin
540

1982). Although there appear to be several factors capable of contributing to the maintenance of oocyte meiotic arrest, all negative regulation must be removed or overridden by the LH surge. With regards to Kit and KL, it is possible that the meiosis-inducing effects of LH could be elicited by (a) a decrease in oocyte Kit expression, (b) a decrease in granulosa cell KL expression, or (c) altered biological activity of KL.

Previous work with mouse oocytes has shown that Kit receptors are present on the surface of oocytes at all stages of follicular development (Manova et al., 1990). Kit expression is highest in meiotically-arrested oocytes and this expression decreases after maturation (Horie et al., 1991). Our studies have demonstrated the presence of Kit receptor on fully-grown rat oocytes isolated from preovulatory antral follicles; however, unlike the mouse, the rat Kit receptor persists after hCG treatment. The continued presence of Kit receptor during oocyte maturation indicates that the LH surge probably does not inhibit the expression of Kit as a means of inducing meiosis.

KL mRNA levels in rat granulosa cells increase in response to hCG stimulation similar to findings in mice (Motro and Bernstein, 1993), indicating that meiotic resumption is probably not mediated by an overall decrease in follicular KL levels. The sharp increase in KL mRNA expression 6 hr after hCG treatment may be important in giving the oocyte developmental capabilities since higher levels of KL in follicular fluid are associated with greater successful outcome in IVF treatment (Smikle et al, 1996b). However, our results demonstrate a localized decrease in the expression of both forms of KL by the cumulus cells. This gonadotropin-induced loss of KL expression occurred 2 hr prior to the apparent

germinal vesicle breakdown in oocytes of preovulatory follicles, which supports at least a reasonable temporal association between the loss of KL with the resumption of meiosis.

A localized loss of KL mRNA in the cumulus cells has also been reported in ovine atretic follicles (Tisdall et al., 1997). The authors suggested that the lack of KL in the cumulus cells of atretic follicles may result in the premature maturation of the oocyte and, therefore, the authors speculated that the lack of KL/Kit interactions may be involved in the process of meiotic reactivation. In addition, although we have not been able to demonstrate any KL-stimulated inhibition of meiotic maturation using mouse oocytes in culture (our unpublished findings), Yoshida and coworkers (1997) demonstrated that immunoneutralization of Kit receptor induced preovulatory antral follicular atresia in follicles which would otherwise have ovulated. According to the authors' interpretation, the inactivation of Kit resulted in premature meiotic maturation of intrafollicular oocytes which, in turn, resulted in the degeneration of the antral follicles. In addition, the demonstration of decreased Kit receptors in meiotically mature oocytes would indicate that, in the mouse, lack of Kit/KL interactions may also occur in mouse follicles after the LH surge but the mechanism of action in the mouse may be a down regulation of Kit whereas in the rat it is the down regulation of the ligand.

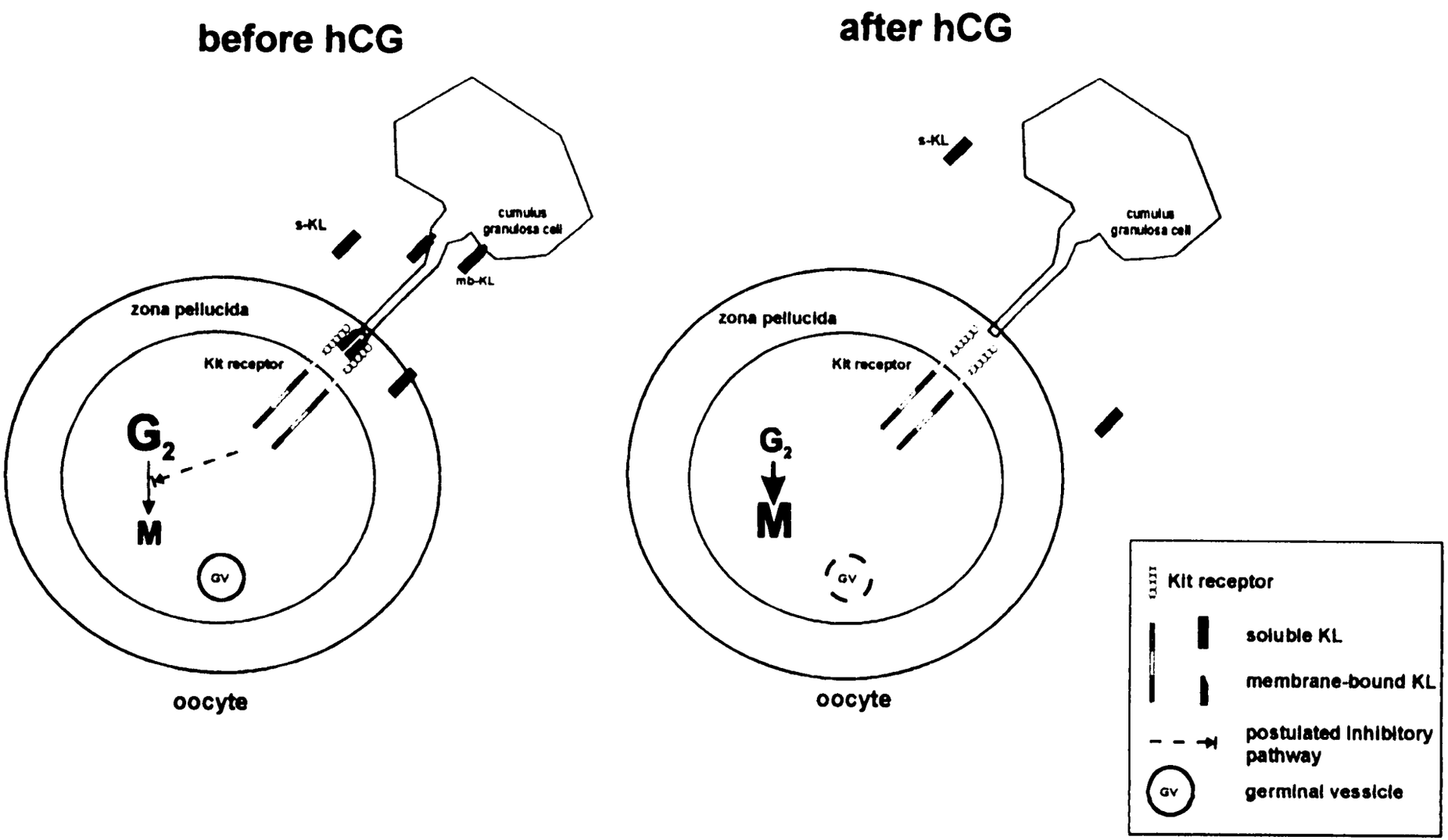
Both membrane-bound and soluble forms of KL are present in the mouse ovary (Manova et al., 1993). We report here that in response to hCG, there was a shift in steady-state transcript levels from membrane-bound KL to soluble KL in mural granulosa cells. Although there is evidence that there are age-specific changes in the relative amounts of transcripts encoding membrane-bound and soluble KL in both testes and ovaries (Manova

et al., 1993), this is the first evidence for a hormonally-induced shift. Both the membrane-bound and soluble forms of KL have been shown to be biologically active; however, in Sertoli cells (the male homolog of granulosa cells) membrane-bound KL is the more potent of the two forms (Tajima et al., 1991). The importance of membrane-bound KL is best exemplified in *Mgf*^{tm1d} mice, where only the soluble form of KL is expressed both pre- and postnatally, resulting in sterility due to deficient germ cell development. More recently, studies have shown that chemically-induced testicular atrophy resulted from a preferential loss of the transmembrane KL which was accompanied by a depletion of KL-dependent germ cells, despite the continued presence of soluble KL (Allard et al., 1996). These experiments indicate that, in the testis, membrane-bound KL has some level or quality of activity that cannot be replaced by soluble KL. If membrane-bound KL is also the more biologically active form in ovaries, then our results would indicate that there is proportionally more active KL in the follicle when oocytes are maintained in meiotic arrest and less active KL in the follicle when meiotic maturation has resumed (Fig 4.8). This scenario is further supported by the fact that addition of soluble KL was unable to completely block the progression of meiosis in cultured rat oocytes (Fig 3.2-3.4). Both soluble and membrane-bound KL have similar binding affinities for Kit (Flanagan and Leder, 1990); however, we can surmise that membrane-bound KL, due to the fact that it is anchored to the membrane, may prevent the subsequent down-regulation or internalization of activated Kit as has been demonstrated in mast cells (Yee et al., 1994). This may, in turn, maintain prolonged activity of the receptor which would have more potent effects on inhibiting oocyte meiosis. The relative activities

Figure 4.8

Model of KL expression in cumulus granulosa cells and its role in the reactivation of LH-stimulated meiotic maturation.

Prior to LH stimulation KL produced by cumulus granulosa cells acts on the Kit receptor and the activation of Kit receptors contributes to the maintained G_2 arrest of the oocyte. However, after the LH surge KL is no longer produced by the cumulus granulosa cells and there is a shift towards greater levels of soluble KL production by mural granulosa cells. Soluble KL unable to produce a sustained activation of Kit receptors and without which the oocyte undergoes G_2 to M transition.


before hCG
after hCG
s-KL
cumulus granulosa cell
mb-KL
zona pellucida
Kit receptor
G2
M
GV
oocyte
s-KL
cumulus granulosa cell
zona pellucida
Kit receptor
G2
M
GV
oocyte
Kit receptor
soluble KL
membrane-bound KL
postulated inhibitory pathway
GV
germinal vessicle

of membrane-bound and soluble KL for preventing the resumption of oocyte meiosis remain to be investigated.

These studies demonstrate that LH stimulation of oocyte meiosis may be mediated, at least in part, by a loss of KL expression in the cumulus cells and a reduction in the relative amounts of membrane-bound KL compared to soluble forms in the mural granulosa cells.

CHAPTER 5 - RELATIVE EFFECTS OF MEMBRANE-ASSOCIATED KL COMPARED TO SOLUBLE KL IN THE INHIBITION OF OOCYTE MEIOTIC MATURATION

5.1 Introduction

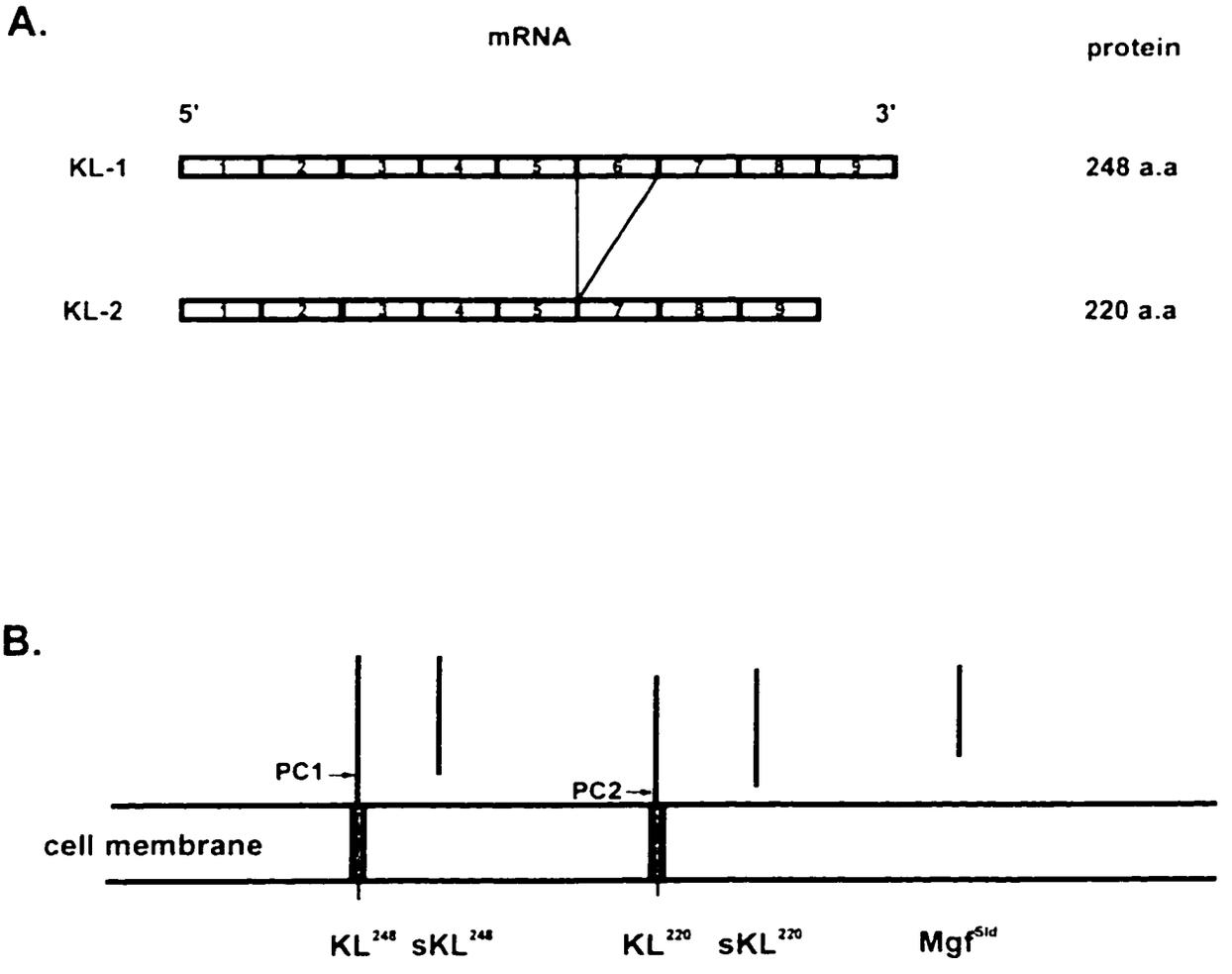
KL is expressed as either a membrane-bound or a soluble protein that arises from alternatively spliced mRNAs. Although 6 different transcripts have been reported (Arceci et al., 1992; Huang et al., 1992; Sharkey et al., 1992 and 1995), the two major mRNA species, KL-1 and KL-2 have been shown to be common to every tissue studied and show tissue specific patterns of expression (Huang et al., 1992). KL-1 and KL-2 yield membrane-associated proteins of 248 and 220 amino acids in length, respectively, which arise due to the inclusion or exclusion of exon 6 during post transcriptional RNA processing (Fig. 5.1). KL-1 is efficiently cleaved due to the presence of an 84 base pair exon (exon 6) which encodes a proteolytic cleavage site, and therefore the extracellular domain is released as a soluble product. However, KL-2 lacks this exon and hence remains more stably on the membrane (Huang et al., 1992; Majumdar et al., 1994). A second minor proteolytic cleavage site encoded in exon 7 may be used to release the extracellular domain of KL^{220} (Huang et al., 1992; Majumdar et al., 1994).

Several lines of evidence support the physiological importance of membrane-bound KL *in vivo*. The most compelling evidence is provided by the viable $Mgf^{Sl\ Sl\text{-}d}$ mutants in which the genomic regions encoding the transmembrane and cytoplasmic domains of KL are

Figure 5.1

Alternative splicing of KL mRNA

Two major mRNA species arise from the inclusion or exclusion of exon 6. The translation products for both KL-1 and KL-2 mRNA are shown along with the major proteolytic cleavage site located in the region encoded by exon 6 (PC1) and a minor proteolytic cleavage site encoded by exon 7 (PC2). The respective soluble forms of KL are illustrated along with the truncated form of KL associated with the *Mgf*$^{Sl\text{-}d}$ mutation. Based on Huang et al., 1992.



PC1 = major proteolytic cleavage site encoded within exon 6
PC2 = minor proteolytic cleavage site encoded within exon 7

deleted: these mice can only produce the secreted form of KL, but show all the pleiotropic defects seen in $Mgf^{Sl\ Sl}$ mutants (Brannan et al., 1991; Flanagan et al., 1991). A study of mast cell attachment to fibroblasts derived from $Mgf^{Sl\ Sl\text{-}d}$ mice showed that the extracellular domain of the membrane-associated form of KL was required to mediate this attachment (Adachi et al., 1992). Furthermore, Sertoli cells isolated from $Mgf^{Sl\ Sl\text{-}d}$ mice were unable to bind spermatogonia indicating that the membrane-associated form of KL serves a function in the adhesion of germ cells (Marziali et al., 1993), a process which is imperative for germ cell survival and development (reviewed in Means et al., 1980). In addition, transmembrane KL produced by fibroblasts transfected with KL-2 cDNA is able to support hematopoeisis for several weeks longer than soluble KL produced by fibroblasts transfected with KL-1 cDNA (Toksoz et al., 1992). Transmembrane KL is a more potent stimulator of *in vitro* PGC survival and proliferation compared to soluble KL (Dolci et al., 1991; Matsui et al., 1991; Pesce et al., 1993). In testes, membrane-bound KL is the more mitogenic form in its ability to induce the proliferation of Kit-bearing germ cells (Tajima et al., 1991; Allard et al., 1996). Based on this evidence, it is clear that membrane-bound KL has a quality of activity that can not be replaced by soluble KL. Both membrane-bound and soluble forms of KL are present in the rat ovary (Fig. 4.6); however, the relative importance of these proteins in regulating oocyte function has not yet been determined.

We have previously shown that the addition of soluble KL to cultured oocytes spontaneously inhibited oocyte meiotic maturation (Fig 3.2); however, this effect was quite short-lived and no differences in oocyte maturation were seen in the presence of KL after 4 hr in culture. Both mural and cumulus granulosa cells surrounding arrested oocytes produce

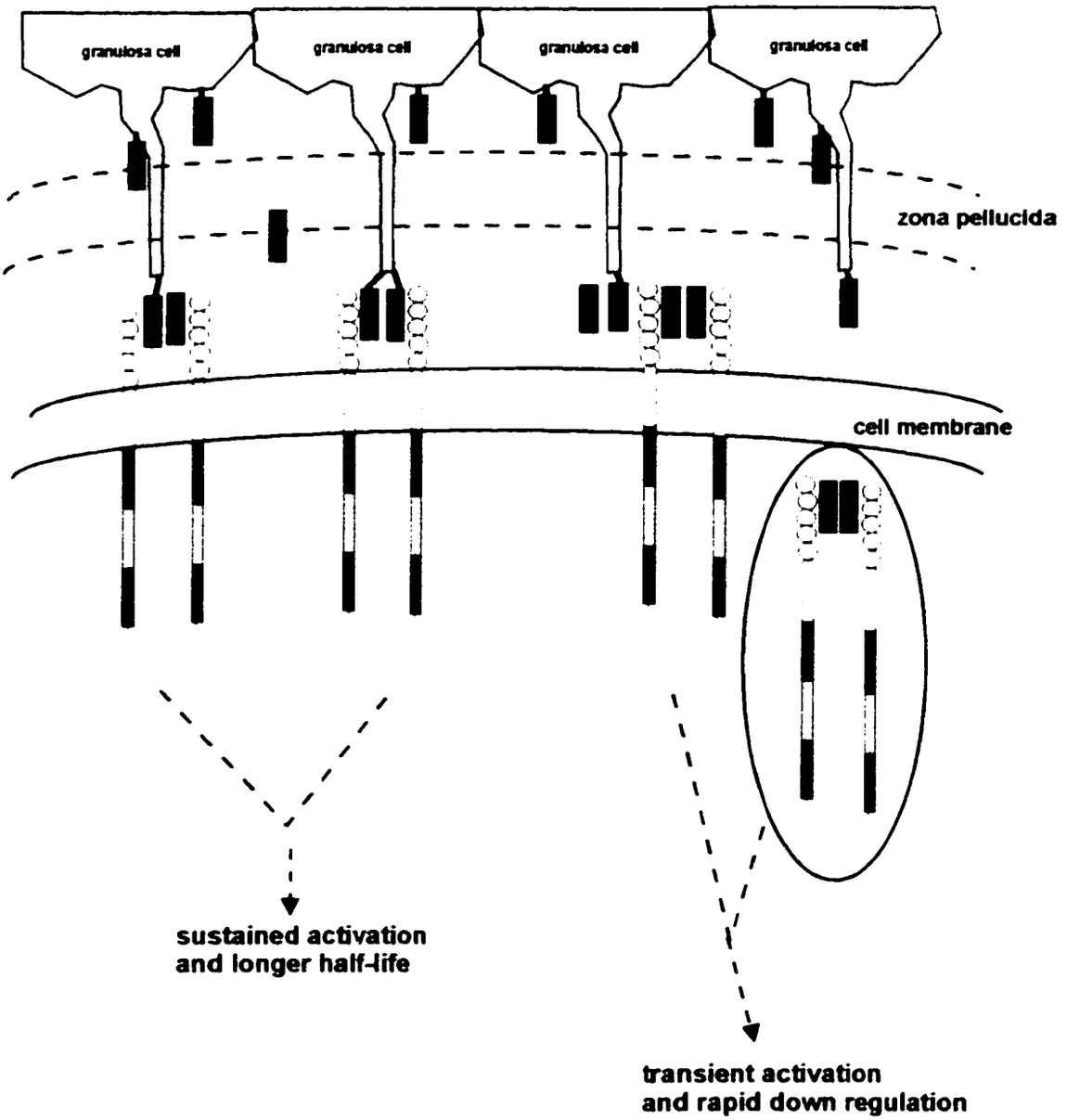
predominantly KL-2 transcripts, and therefore the majority of KL produced within the follicle is in the membrane-associated form. It is quite possible that the transmembrane form of KL is expressed on the cytoplasmic bridges that extend from the granulosa cells through the zona pellucida and synapse on the oocyte plasma membrane. This would allow interactions between transmembrane KL and oocytic Kit receptors (Fig. 5.2). Indeed, membrane-anchored KL induces a more persistent activation of Kit receptor kinase than the soluble form (Miyazawa et al., 1995; Kurosawa et al., 1996) and this interaction confers greater resistence of the Kit receptor to lysosomal degradation (Miyazawa et al., 1995). Consequently, it is reasonable to suspect that membrane-bound KL in granulosa cells may function as a more effective inhibitor of oocyte maturation compared to the soluble form by producing a more sustained signal (Fig 5.2).

We propose that KL in its transmembrane form is a more potent inhibitor of spontaneous oocyte meiotic maturation than soluble KL. To test this hypothesis, oocytes were stimulated with membrane-bound KL produced by mural granulosa cells and their rate of GVBD was compared to oocytes cultured without granulosa cells. Unfortunately these attempts were unsuccessful as KL mRNA expression is not maintained in cultured granulosa cells. As the last part of this study, follicular factors that increase KL mRNA expression in cultured granulosa cells were identified in order to determine granulosa cell culture conditions that maintain highest KL mRNA levels.

Figure 5.2

A postulated model of KL presentation to oocytic Kit receptors

Soluble KL produced by granulosa cells, diffuses through the zona pellucida and interacts with oocytic Kit receptors. Membrane-associated Kit may be expressed on the cytoplasmic extensions of granulosa cells. The close proximity of the granulosa cell plasma membrane and that of the oocyte enables interactions of the transmembrane ligand and receptor. Possible ligand dimer combinations and resulting Kit receptor activation are illustrated.


granulosa cell
granulosa cell
granulosa cell
granulosa cell
zona pellucida
cell membrane
sustained activation
and longer half-life
transient activation
and rapid down regulation
Kit receptor
soluble KL
membrane-bound KL

5.2 Materials and Methods

5.2.1 Effect of granulosa cells on GVBD in cultured oocytes

Granulosa cells were isolated 40 hr after PMSG-priming of prepubertal rats, dispersed by repeated pipetting through a Pasteur pipette and washed twice in WAY/BSA. Dispersed granulosa cells were seeded at a concentration of 1.0 x 10^6 cells/ml in 24-well plates (Falcon) in a total volume of 500 µl. Granulosa cells were incubated for 24 hr to allow cells to attach. On the next day granulosa cell monolayers were rinsed twice with fresh WAY/BSA to remove any non-adherent cells and were now ready for coculture with oocytes that had been isolated as follows. Oocytes were isolated from ovaries of rats 40 hr after PMSG injections, stripped of their granulosa cells in WAY/BSA/IBMX and exposed to Acid Tyrodes to remove the zona pellucida as described previously (see section 2.2.2). Zona-free denuded oocytes were washed twice with WAY/BSA and placed on top of monolayers of granulosa cells that had been plated for 24 hr. Using a dissecting scope, oocytes were monitored for GVBD for a total of 24 hr from the start of the coculture with assessments made every half hour within the first 4 hr of coculture. The proportion of oocytes having completed GVBD at 1.5 hr after the start of oocyte-granulosa cell coculture are presented as means of 5 experiments and error bars represent standard error of the mean.

5.2.2 KL mRNA expression in cultured granulosa cells

Granulosa cells were isolated from DES-primed ovaries in WAY/FBS and seeded at a density of 0.5 X 10^6 cells/ml of WAY/FBS in 100 mm tissue culture-treated dishes (Falcon). The next day, the cell monolayers were washed to remove any non-adherent cells

and replaced with either WAY/FBS or WAY/BSA supplemented with 5 μg/ml of insulin, transferrin, selenium suspension. Granulosa cells were then cultured alone or in the presence of the following additives: 17β-estradiol (E_2; Sigma), progesterone (P_4; Sigma) and testosterone (T; Sigma), each at a concentration of 0.5 μM, 120 ng/ml FSH (NIADDK-o FSH-18; NIH, Bethesda), 5 IU/ml hCG (Intervet), and 5, 10 and 20 ng/ml epidermal growth factor (EGF). Granulosa cells were also cultured in the absence or presence of 1 and 2 mM dibutyryl cyclic adenosine monophosphate (dbcAMP; Boehringer Mannheim), and 20, 100 and 500 μM 8-(chloroylphenylthio)-adenosine 3':5'-cyclic monophosphate (cp-cAMP; Sigma), two membrane-permeable analogues of cAMP. After either 24 or 48 hr of incubation, cells were harvested for RNA isolation. RNA was isolated using a standard LiCl precipitation method (Sambrook et al., 1989) or using RNeasy Kit (Qiagen, Santa Clarita, CA) according to manufacturer's instructions. Northern blots were prepared as described previously (section 2.2.4) and probed with P^{32}-labelled rat KL fragments described below.

5.2.3 Cloning of rat KL to generate rat KL probes

One microgram granulosa cell RNA that had been extracted from cells collected 40 hr after PMSG injections was used for reverse transcription and subsequent PCR-amplification of KL cDNA using KL-specific primers that span alternatively spliced exon 6 (generously donated by AMGEN) according to methods described in section 4.2.4. Briefly, primers A and C (Fig. 4.1) were used to amplify both 666 bp (KL-1) and 582 bp (KL-2) amplicons, and 10 μl (of 50 μl total) of the PCR products were used for the ligation of amplified cDNAs into the TA cloning sites of pCRII vector (Invitrogen, San Diego, CA)

(Fig 5.3). Ligations were done by incubating the linearized vector with PCR products in the presence of T_4 DNA ligase overnight at 14°C. The orientation of the inserted fragment was determined by enzyme digestions, as well as sequencing using Sp6 and M13 polymerases. Plasmids containing KL-1 cDNA sequences were pcrKL#21 and pcrKL#14 and plasmids identified as pcrKL#15 contained KL-2 cDNA. pcrKL#21 was used to generate probes for northern blot analysis as follows: The KL-1 cDNA fragments were retrieved using EcoRI digests; however, due to an internal EcoRI site, the 666 bp fragment was bisected to yield cDNAs of 370 and 296 bp. Both smaller fragments were gel purified together and used to generate P^{32}-labelled random primed probes to detect KL mRNA.

5.3 Results

5.3.1 Effect of granulosa cells on spontaneous oocyte maturation

Experiments were performed to determine the possible effects of membrane-associated KL on spontaneous oocyte maturation. Oocytes were cultured in the presence of granulosa cells isolated from PMSG-treated rats and served as the source of membrane-bound KL due to the production of high levels of KL-2 transcripts (Fig 4.7). In order to allow for interactions of membrane-bound KL on the surface of granulosa cells and Kit on the surface of the oocytes, oocytes were stripped of their granulosa cells and the zona pellucida was dissolved. Zona-free oocytes were cultured on monolayers of granulosa cells and as controls, groups of zona-free oocytes were cultured on plastic (without granulosa cells). Fig 5.4 illustrates the percentage of oocytes undergoing GVBD 1.5 hr after the start

Figure 5.3

Cloning of Rat KL cDNA

Rat KL cDNA was cloned using RT-PCR strategy and amplified fragments were subcloned into the TA cloning sites of commercially available pCRII vector (Invitrogen). The rat KL EcoRI fragments (370 and 296 bp) were isolated by gel purification, random-primed labelled and used as a probe for northern blot analysis.

The pcrKL#21 plasmid

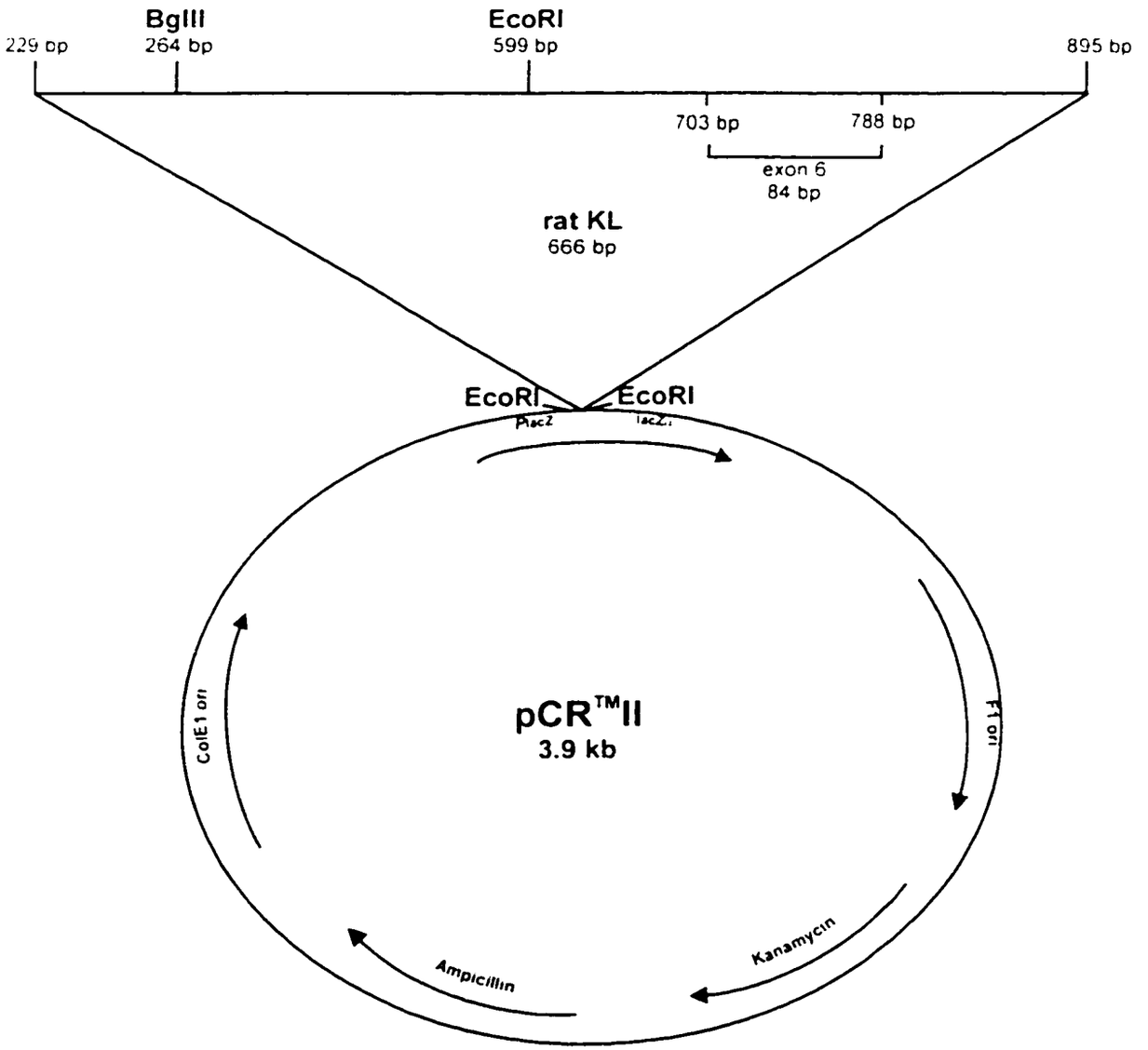
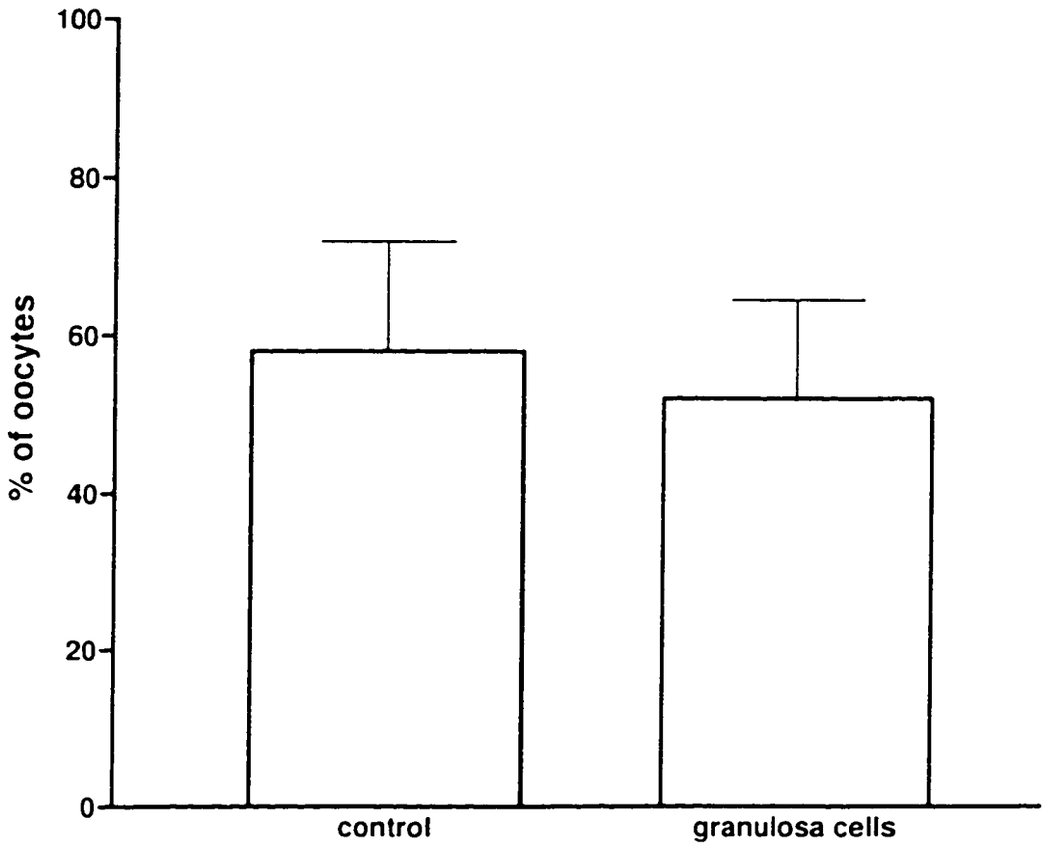


Figure 5.4

Effect of the presence or absence of granulosa cells on spontaneous oocyte maturation

Denuded, zona pellucida-free oocytes were cocultured directly on plastic (control) or on monolayers of mural granulosa cells from 40 hr PMSG-primed rats that were preincubated for 24 hr prior to the addition of oocytes. The proportion of total oocytes that had undergone germinal vesicle breakdown 1.5 hr after the start of the coculture is illustrated. Each data point represents the average of 78-90 oocytes ± S.E.M pooled from 5 experiments.


100
80
60
40
20
0
% of oocytes
control
granulosa cells

of the coculture. There was no significant difference between the proportion of oocytes undergoing GVBD on plastic and those on monolayers of granulosa cells, although the oocytes had attached to the monolayers of granulosa cells.

5.3.2 Effect of culture on KL expression in PMSG-primed granulosa cells

The lack of effect of granulosa cells on spontaneous oocyte meiotic maturation suggested that KL expression was decreased in granulosa cells from PMSG-primed rats when placed in culture. To determine KL mRNA expression in granulosa cells before and after culture, granulosa cells were isolated from rat ovaries 40 hr after PMSG-priming and were either flash frozen to be used for RNA extraction or cultured for 48 hr for subsequent RNA extraction. Northern blots were prepared from freshly isolated and cultured granulosa cell RNA samples. Taking differences in sample loading into consideration KL mRNA levels were 13.0 ± 0.4 fold lower in granulosa cells that had been cultured prior to RNA extraction, compared to those in which RNA was extracted immediately after isolation (Fig. 5.5). This decrease in KL mRNA levels occurred regardless of whether the culture was done in the presence or absence of 5% FBS (Fig. 5.5). To determine if there was a shift in the ratio of proportion of KL-1 and KL-2 mRNA content along with dramatic decreases in overall KL transcript levels, RT-PCR reactions using KL primers B and C (Fig 4.1) were performed on the granulosa cell RNA samples shown in Fig. 5.5 (Fig. 5.6). As expected, there was a predominance of KL-2 mRNA in RNA extracts of uncultured granulosa cells However, in those RNA samples extracted from cells that had been cultured prior to RNA isolation, there was a shift towards a greater expression of KL-1 compared to KL-2 mRNA.

Figure 5.5

Northern blot analysis of KL mRNA in granulosa cells before or after culture

Granulosa cells were isolated from 40 hr PMSG-primed rats. A portion of cells were flash frozen for total RNA extraction (0 hr) and the rest were cultured for a total of 48 hr in WAY/FBS (48 hr-a). In some cases granulosa cells were cultured for the second 24 hr in WAY/BSA (48 hr-b). Total RNA was extracted from fresh and cultured granulosa cells and probed with ^{32}P-labelled rat KL cDNA probes (A). Gel electrophoresis was done with 15 µg of total RNA in each lane and total RNA samples from NIH 3T3 and Sl^{4} fibroblasts were included in the northern blot analysis to serve as positive and negative KL mRNA controls, respectively. The blots were reprobed with ^{32}P-labelled 28s ribosomal marker cDNA (B). To quantitate the effect of culturing granulosa cells, the intensity of bands on the phosphoimager was estimated using ImageQuaNT software, and the ratio of KL/28s ribosomal mRNA values were standardized to uncultured (0 hr) levels which were arbitrarily set to 1.0 and are indicated. In C, a histogram illustrating the differences in KL/28s ribosomal mRNA ratios where each bar represents the mean values of 2 experiments is shown where error bars represent minimum and maximum data points. Bars indicated with different letters are significantly different (p<0.01).

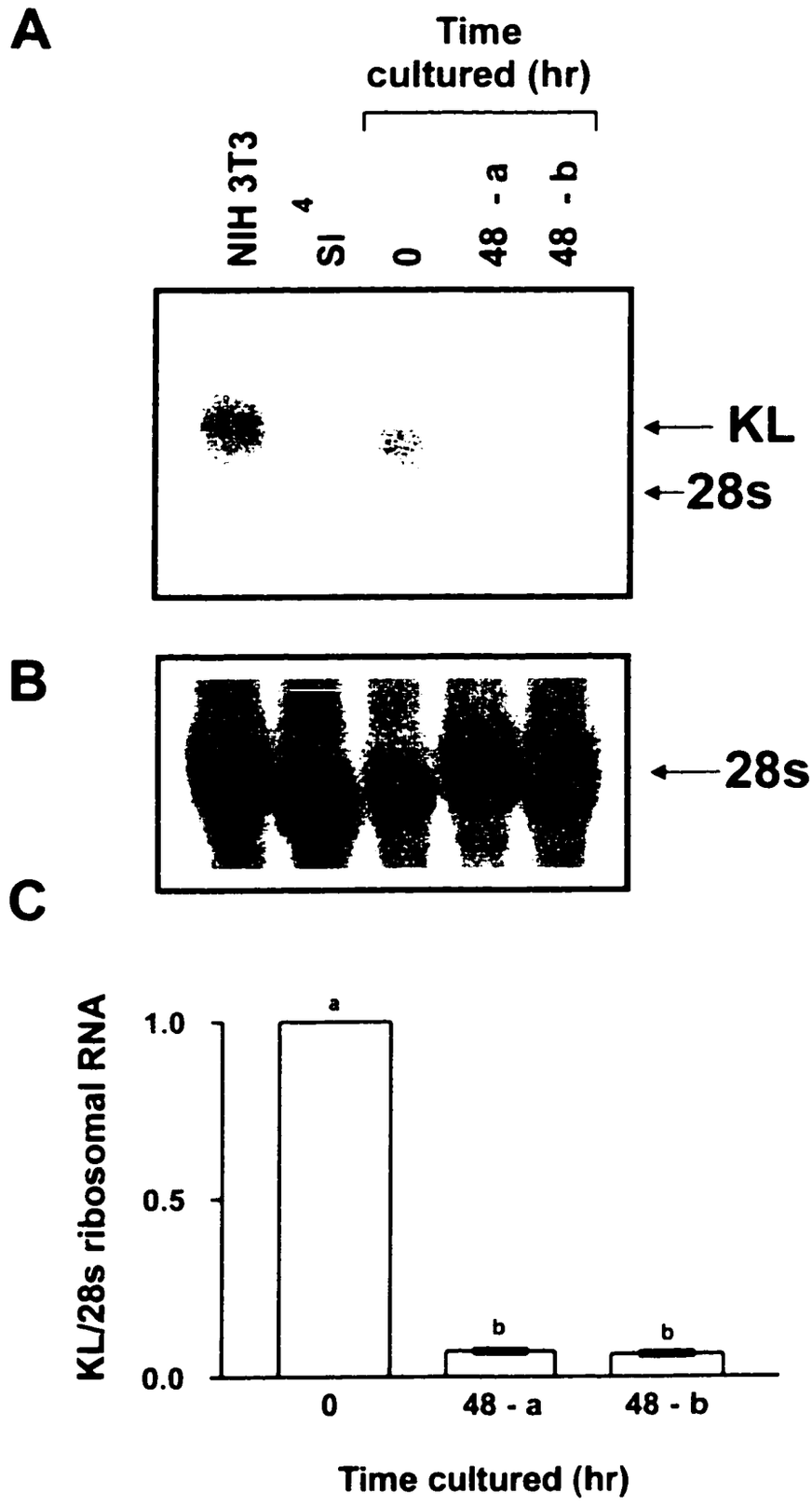
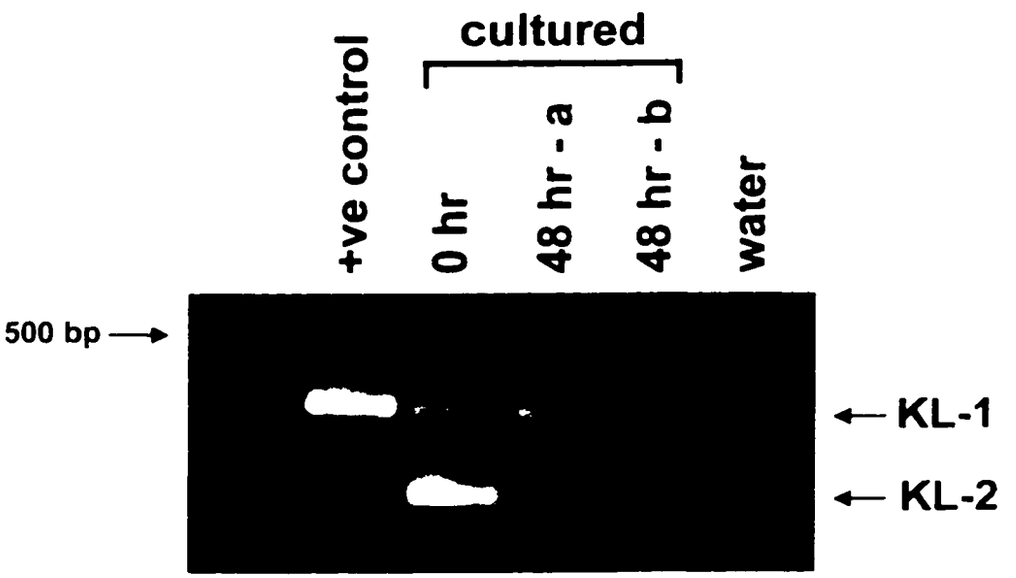

A
Time cultured (hr)
NIH 3T3
SI 4
0
48 - a
48 - b
KL
28s
B
28s
C
KL/28s ribosomal RNA
1.0
0.5
0.0
a
b
b
0
48 - a
48 - b
Time cultured (hr)

Figure 5.6

Reverse transcription polymerase chain reaction (RT-PCR) of granulosa cell RNA isolated before or after culture

Granulosa cells were isolated from 40 hr PMSG-primed rats. A portion of cells were flash frozen for total RNA extraction (0 hr) and the rest were cultured for 48 hr in WAY/FBS (48 hr-a). In some cases granulosa cells were cultured for the second 24 hr in WAY/BSA (48 hr-b). Total RNA was extracted from fresh and cultured granulosa cells and RT-PCR was performed with 1 μg total RNA using KL primers B and C. KL-1 (287 bp) and KL-2 (203 bp) represent the transcripts which when translated give rise to soluble KL and membrane-bound KL, respectively.


cultured
+ve control
0 hr
48 hr - a
48 hr - b
water
500 bp
KL-1
KL-2

5.3.3 Effect of ovarian factors on granulosa cell KL expression

To identify conditions in which KL expression could be up-regulated in cultured granulosa cells, granulosa cells were cultured in the absence or presence of various factors and KL mRNA expression was determined. Treatment of granulosa cells with either FSH (120 ng/ml) or hCG (5IU/ml) for 24 hr increased KL mRNA expression by 2.8 ± 0.1 or 2.2 ± 0.02 fold compared to untreated (0) granulosa cells, respectively (Fig 5.7 I and II). In addition in preliminary experiments, our results indicated that dbcAMP treatment of granulosa cells resulted in dramatic increases in KL mRNA expression at every concentration tested (0.5, 1.0 and 2.0 mM) to fold increases of 4.0, 3.7 and 2.4, respectively compared to untreated granulosa cells (Fig 5.8 I). A similar, but less dramatic increase in KL mRNA expression was seen when granulosa cells were treated with varying concentrations of cp-cAMP (Fig. 5.8 II). On the contrary, a 24 hr culture of granulosa cells in the presence of each of the three steroid hormones tested resulted in substantial decreases in KL mRNA expression in preliminary experiments so that there was a 2.6, 3.6 and 2.2 fold lower KL expression in the presence of E_2, P_4 and T, respectively, compared to untreated (control) granulosa cells (Fig 5.9). Treatment with EGF at any concentration did not affect KL mRNA expression (data not shown).

Figure 5.7

Northern Blot analysis of KL in DES-primed rat granulosa cells cultured in the absence or presence of FSH (I) or hCG (II)

Total RNA of DES-primed granulosa cells cultured for 24 hr alone or in the presence of (I) 120 ng/ml FSH, or (II) 5IU/ml hCG, and probed with a ^{32}P-labelled rat KL cDNA probe (A). Gel electrophoresis was done with 15 µg of total RNA in each lane and total RNA samples from NIH 3T3 and Sl^{4} fibroblasts were included in northern blot analysis to serve as positive and negative KL mRNA controls, respectively. The blots were reprobed with ^{32}P-labelled α-tubulin cDNA (B). To quantitate the effect of hormone treatment, the intensity of the bands on the phosphoimager was estimated using ImageQuaNT software, and the ratio of KL/α-tubulin values were standardized to control levels which were arbitrarily set to 1.0. A histogram illustrating the differences in KL/α-tubulin ratios where each bar represents the mean values of 2 experiments is shown in C. Error bars indicate maximum and minimum data points. * indicates values that are significantly different from untreated (0) samples.

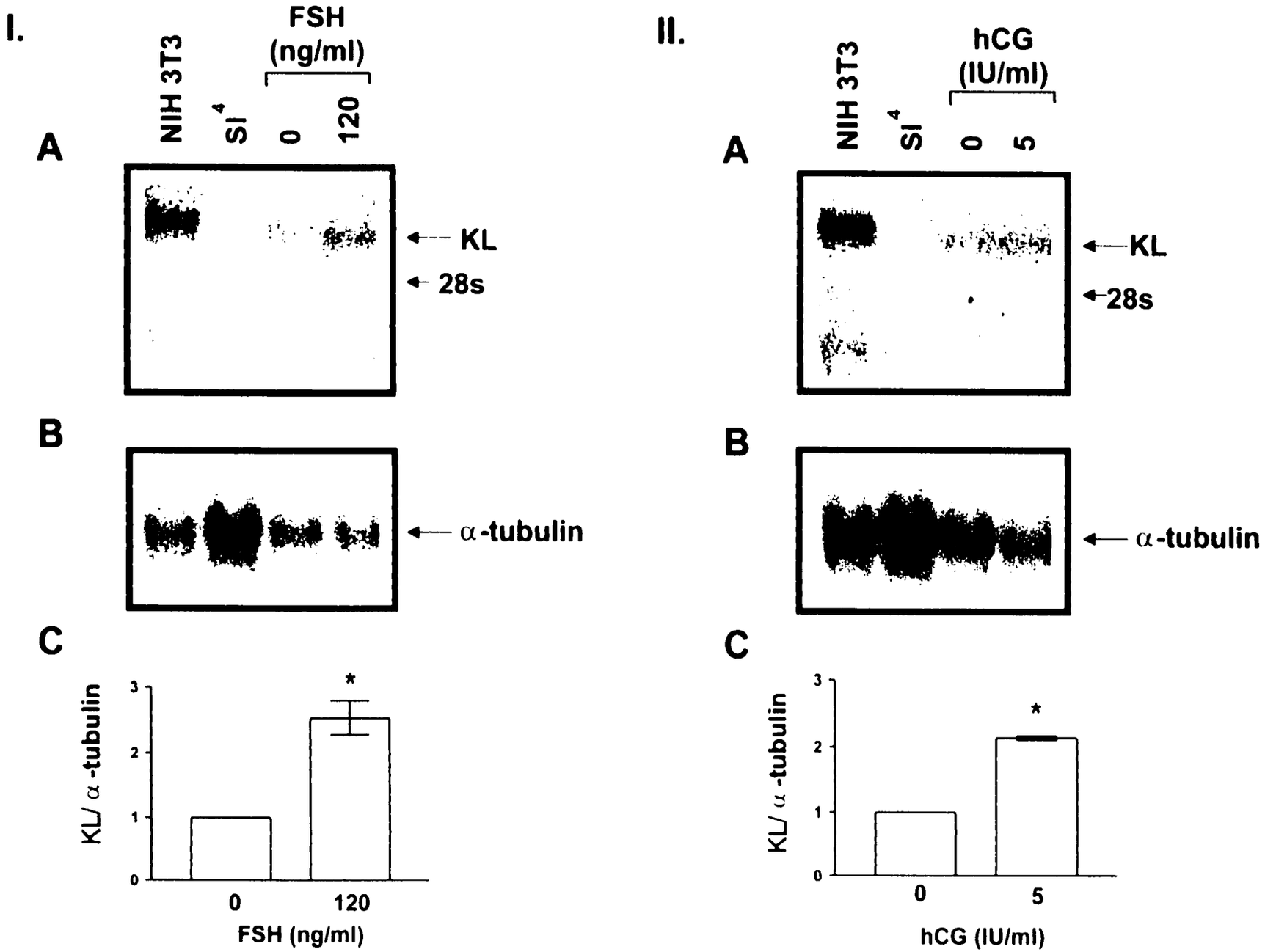

I.
NIH 3T3
SI4
FSH (ng/ml)
0
120
A
KL
28s
B
α-tubulin
C
KL/ α -tubulin
3
2
1
0
*
0
120
FSH (ng/ml)
II.
NIH 3T3
SI4
hCG (IU/ml)
0
5
A
KL
28s
B
α-tubulin
C
KL/ α -tubulin
3
2
1
0
*
0
5
hCG (IU/ml)

Figure 5.8

Northern Blot analysis of KL in DES-primed rat granulosa cells cultured alone or in the presence various concentrations of dbcAMP (I) or cp-cAMP (II)

Total RNA of DES-primed granulosa cells cultured for 48 hr alone or in the presence of 0.5, 1.0, 2.0 mM dbcAMP (I) or 20 and 100 μM cp-cAMP (II) and probed with a ^{32}P-labelled rat KL cDNA probe (A). Gel electrophoresis was done with 15 μg of total RNA in each lane and total RNA samples from NIH 3T3 and Sl^{d} fibroblasts were included in the northern blot analysis to serve as positive and negative KL mRNA controls, respectively. The blots were reprobed with ^{32}P-labelled α-tubulin cDNA (B). To quantitate the effect of treatment, the intensity of the bands on the phosphoimager was estimated using ImageQuaNT software, and the ratio of KL/α-tubulin values were standardized to control levels which were arbitrarily set to 1.0 and are shown in C (n=1).

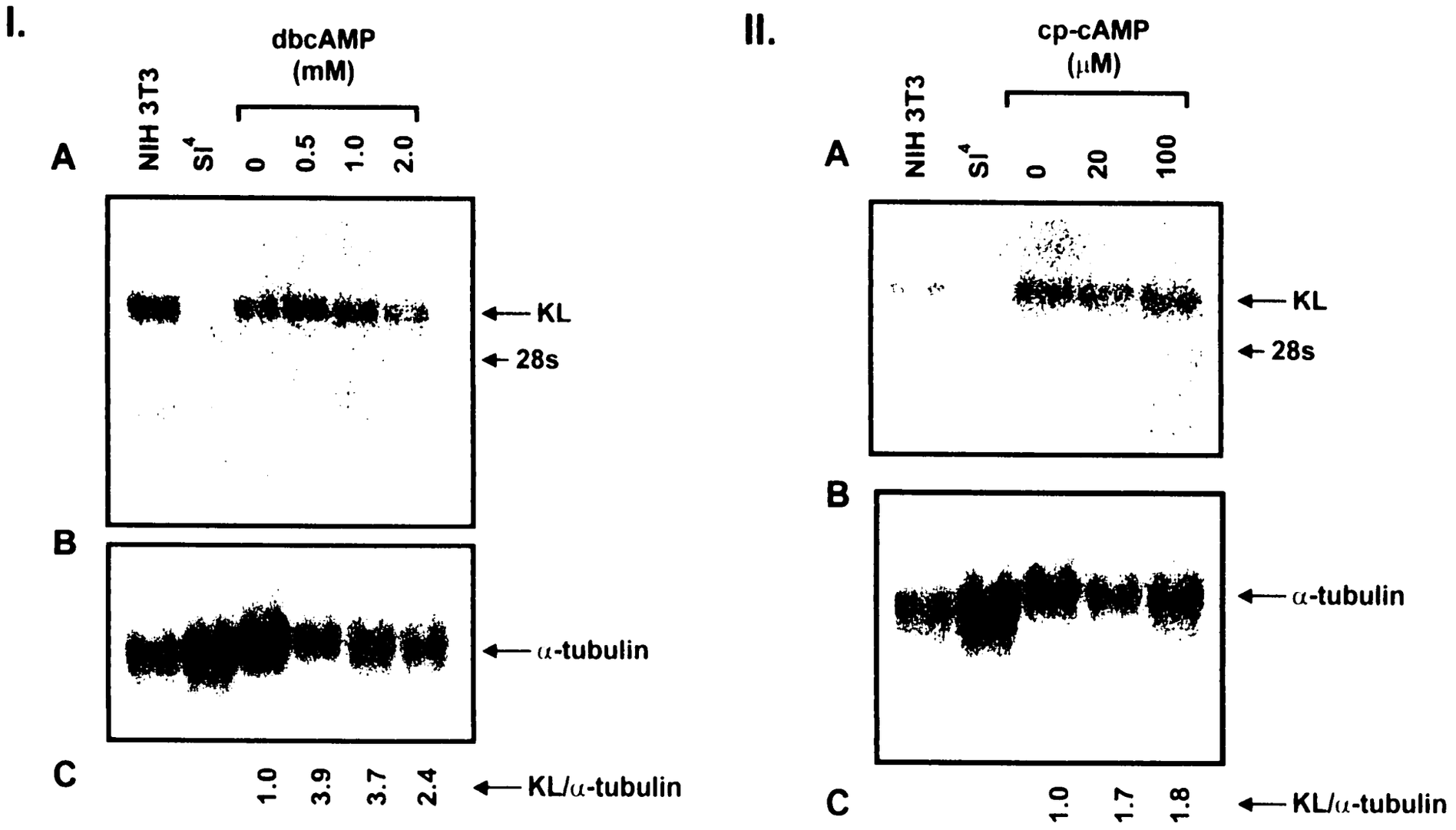
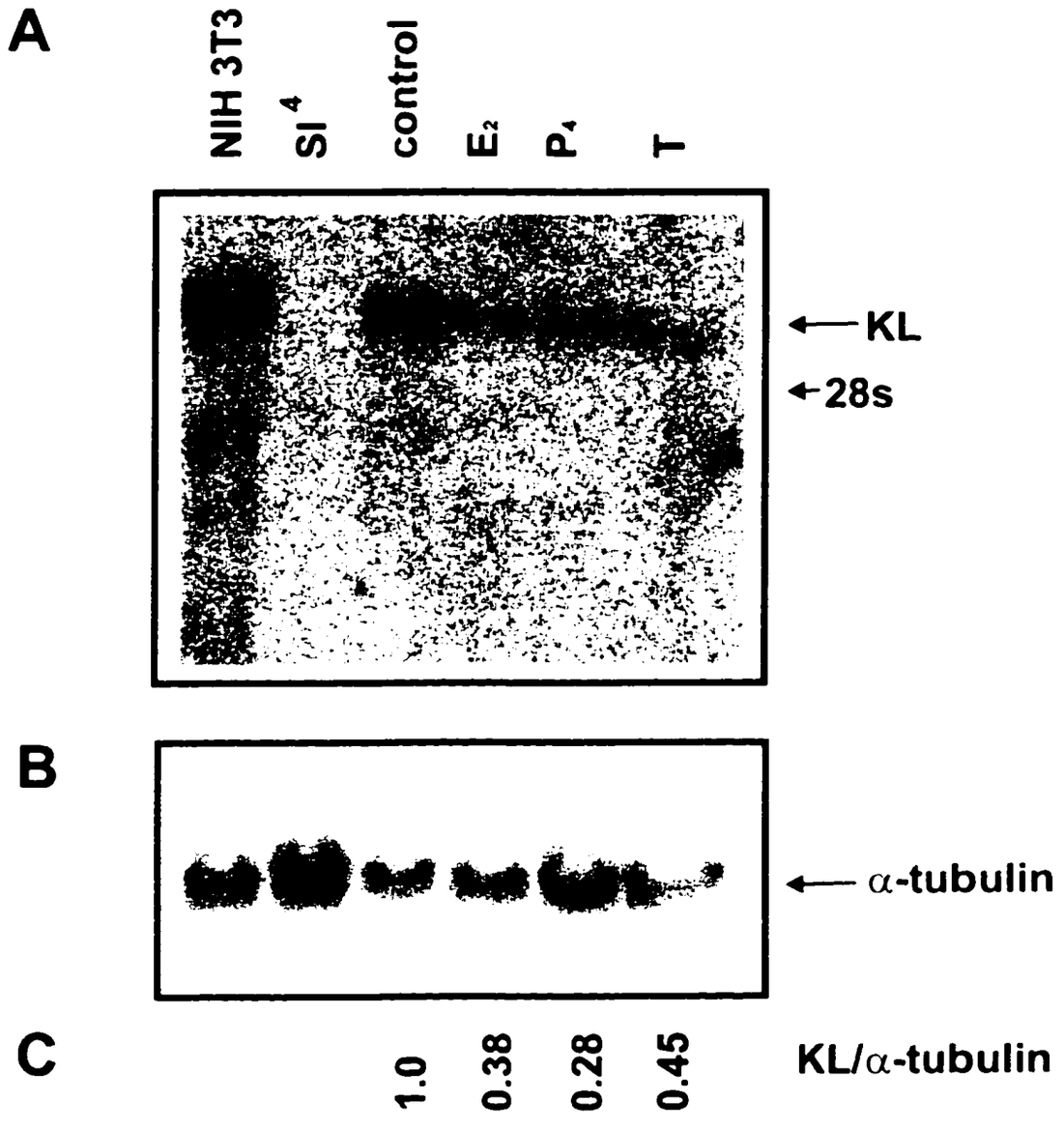

I.
dbcAMP
(mM)
NIH 3T3
SI4
0
0.5
1.0
2.0
A
KL
28s
B
α-tubulin
C
1.0
3.9
3.7
2.4
KL/α-tubulin
II.
cp-cAMP
(μM)
NIH 3T3
SI4
0
20
100
A
KL
28s
B
α-tubulin
C
1.0
1.7
1.8
KL/α-tubulin

Figure 5.9

Northern Blot analysis of KL in DES-primed rat granulosa cells cultured alone or in the presence steroid hormones

Total RNA of DES-primed granulosa cells cultured for 24 hr alone or in the presence of either E_2, P_4 or T (0.5 μM), and probed with a ^{32}P-labelled rat KL cDNA probe (A). Gel electrophoresis was done with 15 μg of total RNA in each lane and total RNA samples from NIH 3T3 and Sl^4 fibroblasts were included in the northern blot analysis to serve as positive and negative KL mRNA controls, respectively. The blots were reprobed with ^{32}P-labelled α-tubulin cDNA (B). To quantitate the effect of hormone treatment, the intensity of the bands on the phosphoimager was estimated using ImageQuaNT software, and the ratio of KL/α-tubulin values were standardized to control levels which were arbitrarily set to 1.0 and is shown in C (n=1).


A
NIH 3T3
SI4
control
E2
P4
T
KL
28s
B
α-tubulin
C
1.0
0.38
0.28
0.45
KL/α-tubulin

5.4 Discussion

In attempts to determine the relative importance of transmembrane KL on the inhibition of oocyte meiotic maturation the observation was made that there was a dramatic decrease in KL mRNA expression in cultured granulosa cells and an alteration in the proportion of KL-1 and KL-2 transcripts being produced. To identify culture conditions that upregulated levels of KL mRNA DES-primed granulosa cells were cultured with gonadotropins, membrane-permeable analogues of cAMP and steroid hormones. In the presence of gonadotropins or membrane-permeable analogues of cAMP KL expression increased in these otherwise low KL mRNA producing cells while steroid hormone treatment further decreased undifferentiated granulosa cell KL mRNA expression.

When mural granulosa cells were isolated from follicles and placed in culture, KL expression was dramatically downregulated and there was a switch from KL-2 to predominantly KL-1 production. This suggests that factors within the preovulatory follicle upregulate KL mRNA levels and influence the splicing of this transcript to enable the production of more membrane-associated KL as the final product. It is reasonable to suspect that the lack of expression of membrane-bound KL in cultured granulosa cells was the major contributing factor to the failed attempts to determine the relative biopotency of membrane-bound KL compared to soluble KL in the regulation of oocyte maturation.

Granulosa cells isolated from DES-primed ovaries produce low amounts of KL compared to those isolated from PMSG-primed ovaries (Fig. 2.8). DES-priming stimulates the follicles to increase in size due to the mitogenic effects of estrogen; however the granulosa cells remain largely undifferentiated and resemble those of large preantral follicles

(Goldenburg et al, 1972). We were interested in identifying the culture conditions in which granulosa cells would produce high levels of KL-2 mRNA. Cyclic AMP is one of the second messengers associated with the signal transduction of gonadotropin receptors (Gore-Langton and Armstrong, 1988) and cAMP analogues and gonadotropins have been shown to modulate KL mRNA expression in both granulosa and Sertoli cells. In our experiments stimulation of DES-primed rat granulosa cells with cAMP analogues dramatically increased KL mRNA expression. Consistent with our findings, an upregulation of KL mRNA or protein expression occurs when mouse undifferentiated granulosa cells and Sertoli cells were cultured with 1.0 mM dbcAMP (Rossi et al., 1991 and 1993; Tajima et al., 1993; Packer et al., 1994).

Gonadotropin treatment of cultured DES-primed granulosa cells results in a stimulation of KL mRNA expression, further emphasizing the role of KL as an intrafollicular gonadotropin mediator. These results are in agreement with our previous studies describing the effects of *in vivo* gonadotropin stimulation on follicular KL expression in which stimulation with PMSG and hCG resulted in increased KL mRNA expression in rat granulosa cells (illustrated in Fig 4.5). Our findings are the first to demonstrate that direct FSH stimulation increases KL transcript expression in cultured granulosa cells. In Sertoli cell-enriched cultures, stimulation with a similar concentration of FSH resulted in an increase in total KL mRNA expression and with this stimulation there was an equal induction of both KL-1 and KL-2 mRNAs (Rossi et al, 1993). Experiments to determine the effect of FSH stimulation on KL-1 and KL-2 mRNA in granulosa cells are in progress.

Motro and Bernstein (1993) demonstrated that hCG and the LH component of PMSG promoted KL mRNA expression in mural granulosa cells. In our experiments we also observed increases in KL mRNA expression with hCG stimulation; however, since LH receptors are not expressed in undifferentiated granulosa cells (May and Schomberg, 1984a; Eppig et al, 1997), there are two possible explanations for the hCG-stimulated response seen in our cultures. The first possibility is that DES-primed granulosa cells spontaneously acquired LH receptors sometime during the 24 hr incubation period and were then able to respond to hCG stimulation for the duration of the culture period. This explanation is unlikely because we did not observe increased KL expression in granulosa cells cultured for 48 hr in the presence of hCG (data not shown). The second, more likely possibility is that there may be small quantities of FSH in the hCG preparation and that increases in KL expression in granulosa cells were in response to the contaminating FSH rather than hCG.

In contrast to our findings, gonadotropin stimulation resulted in decreased KL mRNA steady-state levels in cultures of human granulosa-luteal cells (Laitinen et al., 1995). Granulosa-luteal cells were isolated from preovulatory human follicles and functionally resemble the granulosa cells found in late preovulatory follicles and early corpus luteal structures. The authors (Laitinen et al., 1995) attributed this discrepancy in gonadotropin-stimulated change in KL mRNA expression to species-specific differences. An alternative interpretation could be that the gonadotropin-stimulated decrease in KL expression may reflect the differentiation state of the granulosa-luteal cells. A gonadotropin-stimulated decrease in KL expression may serve as a signal for an as yet unidentified granulosa-luteal cell function.

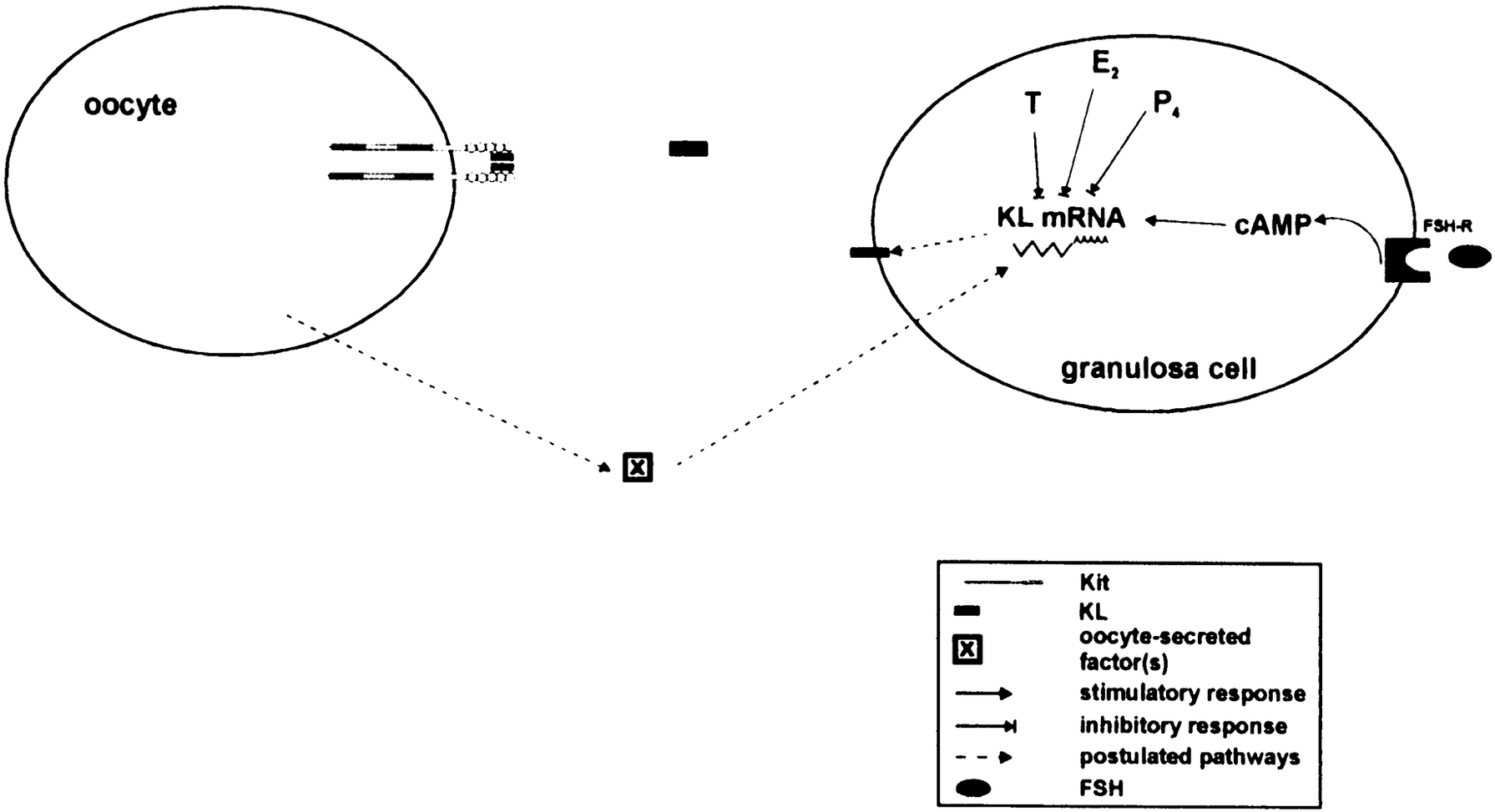
Interestingly, KL mRNA levels decrease when granulosa cells are cultured in the presence of steroids. *In vivo* estrogen treatment resulted in decreased KL expression (Fig. 2.8) and the present experiments are in agreement with this result. The decreases in KL mRNA expression seen with progesterone and testosterone treatment are novel findings. Typically, undifferentiated granulosa cells produce low levels of steroid hormones and only after differentiation do the granulosa cells produce large quantities of steroids (Gore-Langton and Armstrong, 1988). Exposure of undifferentiated granulosa cells within a growing follicle to high levels of steroids may indicate some sort of imbalance in cell function. Given that Kit and KL interactions play an important role in oocyte growth and early follicular development (section 1.7), downregulating KL may prevent or retard the further development of that particular possibly unhealthy follicle. On occasion follicles that do not express KL have been observed in rat ovary sections and this observation was also reported by Manova et al (1993) and Tisdall et al (1997) in mouse and ovine ovaries, respectively.

The identification of hormones that alter KL mRNA expression in cultured granulosa cells is an important finding and one can speculate that KL mRNA levels are regulated in a similar fashion within follicles (Fig. 5.10). In early follicular development there are increasing FSH levels and low steroid levels, conditions which promote KL mRNA production. Furthermore, Packer et al. (1994) demonstrated a moderate increase in KL expression when undifferentiated granulosa cells were cultured in the presence of denuded oocytes, presumably by the paracrine action of oocyte secreted factors. In this way, follicular KL mRNA levels increase and are maintained by the continued stimulation by FSH and granulosa cell communication with the oocyte (Fig. 5.10).

Figure 5.10

Model of KL mRNA regulation in growing follicles

FSH and oocyte secreted factors (Packer et al, 1994) cause an increase in KL mRNA expression in granulosa cells to presumably result in an increase in KL protein which can interact with Kit receptor located on oocytes. Steroid hormones cause a decrease in KL expression.


oocyte
E2
T
P4
KL mRNA
cAMP
FSH-R
granulosa cell
X
Kit
KL
oocyte-secreted factor(s)
stimulatory response
inhibitory response
postulated pathways
FSH

Rat mural granulosa cells from 40 hr PMSG-primed ovaries produce LH receptors (May and Schomberg, 1984b; Eppig et al., 1997). Within preovulatory follicles, mural granulosa cell produce predominantly estradiol (Gore-Langton and Armstrong, 1988); however, once in culture these cells spontaneously differentiate to become predominantly progesterone producing (Gore-Langton and Armstrong, 1988). When PMSG-primed granulosa cells are cultured, there is a shift from predominantly KL-2 mRNA to predominantly KL-1 mRNA production (Fig 5.6). Given that granulosa cells spontaneously differentiate in culture it is possible that spontaneous differentiation resulted in alterations in the form of KL mRNA produced. In addition gonadotropin-induced differentiation of rat granulosa cells also resulted in decreasing levels of KL-2 mRNA and increasing levels of KL-1 mRNA (Fig. 4.6). Moreover, human granulosa-luteal cells have proportionally much lower levels of KL-2 mRNA compared to KL-1 (Laitinen et al., 1995). Based on these findings we propose that the expression of KL-2 mRNA is associated with relatively undifferentiated cells and the loss of KL-2 (and/or gain of KL-1) mRNA serves as a marker for granulosa cell differentiation as mural granulosa cells proceed with their program of differentiation. This loss of KL-2 mRNA expression occurs after the appearance of LH receptors because granulosa cells in antral follicles prior to the LH surge have acquired LH receptors but continue to express predominantly KL-2 mRNA (Fig 4.6). Similar changes in the ratio of KL-1 and KL-2 are not seen in cumulus granulosa cells possibly due to the production of much lower levels of LH receptors (Peng et al., 1991; Camp et al., 1991; Whitelaw et al., 1992) or modulation of cumulus cell phenotype by the oocyte (Eppig et al., 1997). It has been previously proposed that the default program of differentiation is that of

the mural granulosa cell phenotype in preovulatory follicles with the expression of LH receptor as a marker for this phenotype. The oocyte in the preovulatory follicle averts this pathway of differentiation and hence modulates the phenotype of the granulosa cells closest to the oocyte to give rise to the cumulus granulosa cell phenotype (Eppig et al., 1997). Because both mural and cumulus granulosa cells down regulate KL-2 mRNA expression, lack of KL-2 mRNA may be associated with the differentiation of granulosa cells, in general. However, because hCG stimulation of mural granulosa cells results in increased KL-1 mRNA, this may serve as a marker for differentiated granulosa cells. Since cumulus granulosa cells do not express increased KL-1 mRNA levels after hCG stimulation, the production of KL-1 mRNA may not be associated with the cumulus cell phenotype. It is not clear whether the oocyte is involved in suppressing KL-1 mRNA expression in granulosa cells although it has been previously demonstrated that oocyte-secreted factors prevent LH receptor mRNA expression (Eppig et al., 1997).

These studies demonstrate that KL mRNA is not only dramatically reduced in cultured granulosa cells but also that these granulosa cells shift from producing more KL-2 to producing predominantly KL-1 mRNA. To determine the relative biopotency of membrane-bound versus soluble KL to inhibit oocyte maturation, it was first imperative to identify culture conditions in which KL mRNA and, in particular, KL-2 transcripts are upregulated in granulosa cells. Both FSH and membrane-permeable analogues of cAMP increased total KL mRNA expression, although their effect on the amount of KL-1 and KL-2 transcripts have not been determined. On the assumption that culturing granulosa cells in the presence of these additives will result in elevated levels of KL-2 mRNA, oocytes could

be cocultured with granulosa cells to determine the biopotency of membrane-associated KL (produced by granulosa cells) on oocyte meiotic maturation. Alternatively a cell line could be made to express membrane-bound KL by the stable transfection with a KL-2 cDNA construct and this membrane-bound KL-producing cell line could then be used for coculture with oocytes. The prediction would be that stimulation of oocytic Kit receptors with membrane-bound KL would prevent spontaneous meiotic maturation or further delay their G_2 to M progression compared with when oocytic Kit receptors are stimulated with soluble KL.

CHAPTER 6 - CHARACTERIZATION OF OVARIAN SURFACE EPITHELIUM

6.1 Introduction

The ovarian surface epithelium is a single continuous layer of epithelial cells that surrounds the ovary. At ovulation, this cell layer is ruptured to allow for the liberation of the oocyte into the oviduct for fertilization. Around the time of ovulation, the ovarian surface epithelial (OSE) cells in the area of the stigma (the site of ovulatory rupture) secrete proteases that aid in follicular rupture (Bjersing and Cajander, 1975) and those directly overlying the stigma undergo apoptotic cell death (Murdoch, 1994). After ovulation, there is a burst in proliferative activity in OSE cells to repair the postovulatory wound and restore the continuity of the epithelium (Murdoch, 1996; Nicosia et al., 1991).

Investigators have shown that OSE cells, in contrast to other adult epithelia, are relatively uncommitted and pluripotent (Dyck et al.., 1996). The OSE cells express both epithelial and mesenchymal markers *in vivo* (Czernobilsky et al., 1985; van Niekerk et al., 1993) and *in vitro* (Auersperg et al., 1984; van Niekerk et al., 1989). In culture, OSE cells exhibit a marked phenotypic plasticity ranging from a typical cobblestone epithelial phenotype when confluent to an almost fibroblast-like phenotype when the monolayer is disrupted (Dyck et al., 1996; Auersperg et al., 1994). *In situ*, less epithelial phenotypes of OSE cells are seen in regions on the ovarian surface at sites of ovulatory rupture and in regions where OSE cells overlie sites of previous postovulatory wounds (Gillet et al., 1991). Epithelial-mesenchymal conversions occur as part of normal wound repair response and are

regulated by intercellular and cell-matrix adhesive interactions (reviewed in Boyer and Thiery, 1993).

Although wound repair in the adult animal is most often associated with a pathological condition, the ovary is an exception in that OSE cells repair postovulatory wounds as part of the normal physiology of the organ. Ovulatory rupture sites are repaired by proliferation of OSE cells at the perimeter of the ruptured follicles (Osterholzer et al., 1985; Gillet et al., 1991; Murdoch, 1994). Transforming-growth factor-beta (TGF-β) has been shown to be a central regulator in epithelial wound repair in other systems (reviewed in Dignass and Podolsky, 1993); however its role in postovulatory wound repair is not known.

There has been a limited number of studies that investigate the biology of OSE cells (reviewed in Godwin et al., 1993 Murdoch, 1996). OSE cells have not been well characterized in terms of their steroidogenic capabilities, nor the factors regulating their proliferation. Although there is no direct evidence of steroid production by OSE cells, human epithelial-derived cancer cells secrete estrogen and/or progesterone both basally and in response to FSH and hCG (Poels et al., 1989; Wimalasena et al., 1991; Abrahamsson et al., 1997).

Direct experimental evidence for the role of growth factors and hormones in the control of OSE cell proliferation is limited. There are conflicting reports about the mitogenic effects of estradiol on cultured OSE cells from rodent and human ovaries (Hamilton et al., 1980; Karlan et al., 1995); however, in some human ovarian cancer lines, estrogen proved to be a powerful mitogen (Langdon et al., 1990). Progesterone stimulation had no significant

effect on rat or human OSE cell growth (Hamilton et al., 1980; Karlan et al., 1995). In addition, there is a single report suggesting a stimulatory effect of testosterone on rat OSE cell proliferation (Hamilton et al., 1980), although androgens had no effect on human OSE cell proliferation (Karlan et al., 1995).

Gonadotropins have been shown to stimulate the growth of normal rabbit OSE cells (Nicosia et al., 1985), as well as ovarian cancer cell lines (Simon et al., 1983). In addition, rat (Godwin et al., 1992) and human (Rodriguez et al., 1991) OSE cells respond to epidermal growth factor (EGF) and transforming growth factor-α (TGF-α) stimulation by proliferating, and receptors for both have been identified in human OSE cells (Berchuck et al., 1990 and 1992; Jindal et al., 1994). Therefore, there is limited data about the responsiveness to growth regulators in the normal OSE cells; however, the involvement of each of these factors in regulating the burst of proliferation in response to the ovulatory wound is not clear.

Changes in cell adhesion, cell migration and proliferation are clearly associated with wound repair mechanisms (Dignass and Podolsky, 1993) and similar cellular activities have been attributed to Kit and KL function in a variety of cell types (see Table 1.3 and reviewed in Broudy, 1997). Given the limited information about the steroidogenic capabilities and the factors that affect the proliferation of OSE cells, this study examined the steroid output and rate of proliferation of OSE under the influence of a variety of hormonal stimuli. Furthermore, the expression of Kit and KL was determined in OSE and ROSE 199 cells as well as its hormonal regulation.

6.2 Materials and Methods

6.2.1 Animals

Prepubertal and adult (3 to 12 month old) female Sprague-Dawley rats were obtained from the animal colony at the University of Ottawa and maintained as previously described (section 2.2.1). Adult animals were sacrificed by asphyxiation with carbon dioxide.

6.2.2 OSE cell isolation

OSE cells were isolated from ovary explants using a method described previously (Adams and Auersperg, 1981). Ovaries were held with forceps and cut across the hilium portion using a razor blade. Ovary pieces were placed cut side down on tissue-culture treated dishes (Falcon) and explants were allowed to dry for 7 min. After this drying time, media (WAY supplemented with 100 IU/ml penicillin G (Sigma) and 100 µg/ml of streptomycin (Sigma) and 25 % FBS) was added to each dish (2 ml/dish) and explants were incubated for 4 days with a media change after 3 days. After this incubation period, a ring of epithelial cells was evident around each explant. To prepare OSE cells for first passaging, explants were removed, any contaminating cells were scraped using a 25-G needle and cells were washed twice with Hanks' Balanced Salt Solution (Gibco BRL). Cells were detached from dishes using 0.25% trypsin in phosphate-buffered saline for 20 min at room temperature. OSE cells were plated at 5000 cells/ml in media and after two passages, the cells were harvested for total RNA. Six individual OSE cultures were isolated and used for RNA extraction and subsequent Northern blot analysis.

6.2.3 Hormone treatment

ROSE 199 cells, a spontaneously immortalized rat OSE cell-derived cell line, a generous gift from Dr. N. Auersperg (Adams and Auersperg, 1985), were used to determine the effects of hormonal stimulation on OSE cell proliferation, steroidogenesis and Kit and KL mRNA expression. These cells were maintained in α-minimum essential medium (α-MEM: Gibco BRL) supplemented with 10% FBS in tissue culture-treated dishes and seeded at $1.5x10^5$ cells in 100 mm tissue culture dishes in the presence or absence of the following additives: E_2, P_4 and T, each at a concentration of 0.5 μM, 150 ng/ml FSH, 5 IU/ml hCG and 1 and 2 mM dbcAMP. In some experiments, ROSE 199 cells were cultured for 48 hr in the presence of 10 ng/ml TGF-β (R & D Systems) diluted from a 20 μg/ml stock dissolved in 4 mM HCl/0.1% BSA .

6.2.4 Steroidogenesis and cell proliferation

To determine effects of hormone treatment on OSE cell steroidogenesis and proliferation, ROSE 199 cells were cultured for 24, 48 and 72 hr with and without hormone treatment. To determine steroidogenic output of these cells, spent media were collected at each time point and stored at -20°C until assayed for E_2 and P_4 by radioimmunoassays (RIAs) that have been described and validated for direct measurements (Leung and Armstrong, 1979; Daniel and Armstrong, 1984). To measure cell proliferation, the remaining media were aspirated, the cells were trypsinized and counted using a hemocytometer. In some cases trypan blue dye exclusion assays were used to estimate cell viablity at the end of the experiment. To do this, cells were resuspended in 0.03 % trypan blue dye (Gibco) in media.

Only those cells that are non-viable take up dye and appear blue in colour. The proportion of viable cells was counted and expressed as a percentage of total cell number. Experiments were done at least 3 times in duplicate. Statistical comparisons involving multiple groups were made by analysis of variance. When significant effects were observed, Fisher's protected least-squared differences test was used for multiple comparisons. Data are the mean ± SEM and statistical significance was inferred at $p<0.05$.

6.2.5 Localization of KL mRNA and protein in rat ovary sections

In situ hybridization and immunohistochemistry were used to localize KL mRNA and protein, respectively. Procedures were performed as described previously (section 2.2.3).

6.2.6 Determination of gene expression in OSE and ROSE 199 cells

To determine levels of mRNA expression of Kit receptor and KL in OSE and ROSE 199 cells, total RNA was isolated using a LiCl precipitation method according to a standard protocol (Sambrook et al., 1989). To determine hormonal regulation of KL and Kit expression, ROSE 199 cells were cultured in the absence or presence of the above described additives for 24 or 48 hr. At each time point, the media was aspirated and total RNA was extracted from the cell monolayers using an RNeasy kit (Qiagen) according to the manufacturer's instructions. Northern blot analysis was done as described in section 2.2.4. When determining Kit receptor mRNA expression, Kit receptor cDNAs (a generous gift from A. Bernstein) were used as probes.

To determine the expression of LH and FSH receptor mRNA in ROSE 199 cells, 16×10^6 ROSE 199 cells were harvested to isolate poly-A+ enriched RNA using Dynabeads

Oligo $(dT)_{25}$ (Dynal, Long Island, NY). Northern blot analysis was done as described in section 2.2.4 using LH and FSH receptor cDNAs (generous gifts from D. Segaloff).

6.2.7 Expression of alternatively spliced KL transcripts in OSE and ROSE 199 cells

Reverse transcription-polymerase chain reaction (RT-PCR) was used to determine the relative proportion of KL-1 and KL-2 transcripts in normal and ROSE 199 cell total RNA preparations using a procedure described previously in section 4.2.4. Some PCR reactions were done using water instead of RT sample to serve as a negative control. There were two positive controls, 1) C13, a cell-line that produces predominantly KL-1 transcripts and, 2) granulosa cells that produce predominantly KL-2 transcripts. This experiment was performed twice with 2 separate RNA preparations and two sets of KL primers.

6.2.8 Effect of dbcAMP on the adhesion of ROSE 199 cells

To determine quantitatively the effect of dbcAMP treatment on the adhesion of ROSE 199 cells to substratum of tissue culture-treated plastic dishes, 1.5×10^5 ROSE 199 cells were seeded in duplicate 60 mm plastic dishes in media alone or with 1.0 mM dbcAMP. After 48 hr, dishes were coded in order to maintain impartiality through the experiment. Cells were rinsed once with PBS and incubated with 1 ml 0.25% trypsin in PBS. After 3 min cells were mildly agitated to dislodge any loosely adherent cells and trypsin/cell suspension supernatants was transferred to a tube containing media to stop the enzyme reaction and to determine the number of cells that had detached (detached cells). Fresh trypsin was added to each dish for 10 min so that the remainder of the cells in each dish would detach (adherent

cells). Percentage of cells that had detached during the first 3 min of trypsinization was calculated as follows:

% detached = [number of detached cells / (number of detached cells + number of adherent cells)] x 100

6.3 Results

6.3.1 Steroidogenesis and cell proliferation of ROSE 199 cells

Steroid assays were performed on spent media to determine the steroidogenic abilities of ROSE 199 cells, a spontaneously immortalized ovarian epithelium-derived cell line. After 96 hr. spent media from untreated cells contained 112 ± 25 pg/ml E_2 and these levels increased significantly ($p<0.01$) to 326 ± 38 pg/ml when T was provided as a substrate (Fig. 6.1). The amount of E_2 was not significantly altered by the addition of gonadotropins either in the presence or absence of T. When cells were stimulated with dbcAMP only 96 ± 46 pg/ml E_2 had accumulated in the media in the presence of T ($p<0.05$) whereas with dbcAMP stimulation alone E_2 levels were similar to control (Fig. 6.1). ROSE 199 cell P_4 accumulation after 48 hr in the absence or presence of steroids, gonadotropins and dbcAMP is shown in Fig 6.2. P_4 was present in spent media at a concentration of 488 ± 63 pg/ml which increased to 2335 ± 156 pg/ml in the presence of the substrate, pregnenolone ($p<0.01$). P_4 accumulation was not influenced by the presence of FSH. However, there was an increase in P_4 output to 3754 ± 92 and 2839 ± 84 pg/ml when ROSE 199 cells were stimulated with hCG and dbcAMP, respectively ($p<0.01$), but this effect was seen only when pregnenolone was present. Addition of T enhanced the basal P_4 output of ROSE 199 cells to 1045 ± 89 pg/ml (Fig. 6.2).

Figure 6.1

Estradiol production by ROSE 199 cells

Estradiol production by ROSE 199 cells cultured for 96 hr in the presence or absence of the substrate, testosterone (0.5 μM). Cells were treated with FSH (150 ng/ml), hCG (5 IU/ml) or dbcAMP (2 mM). Each bar represents means ± S.E.M of 2 experiments with at least 3 independent samples per experiment. *indicates treatments with substrate that are significantly different from the same treatment without substrate and ** indicates significantly different treatments from appropriate control.

* $p<0.01$

** $p<0.05$

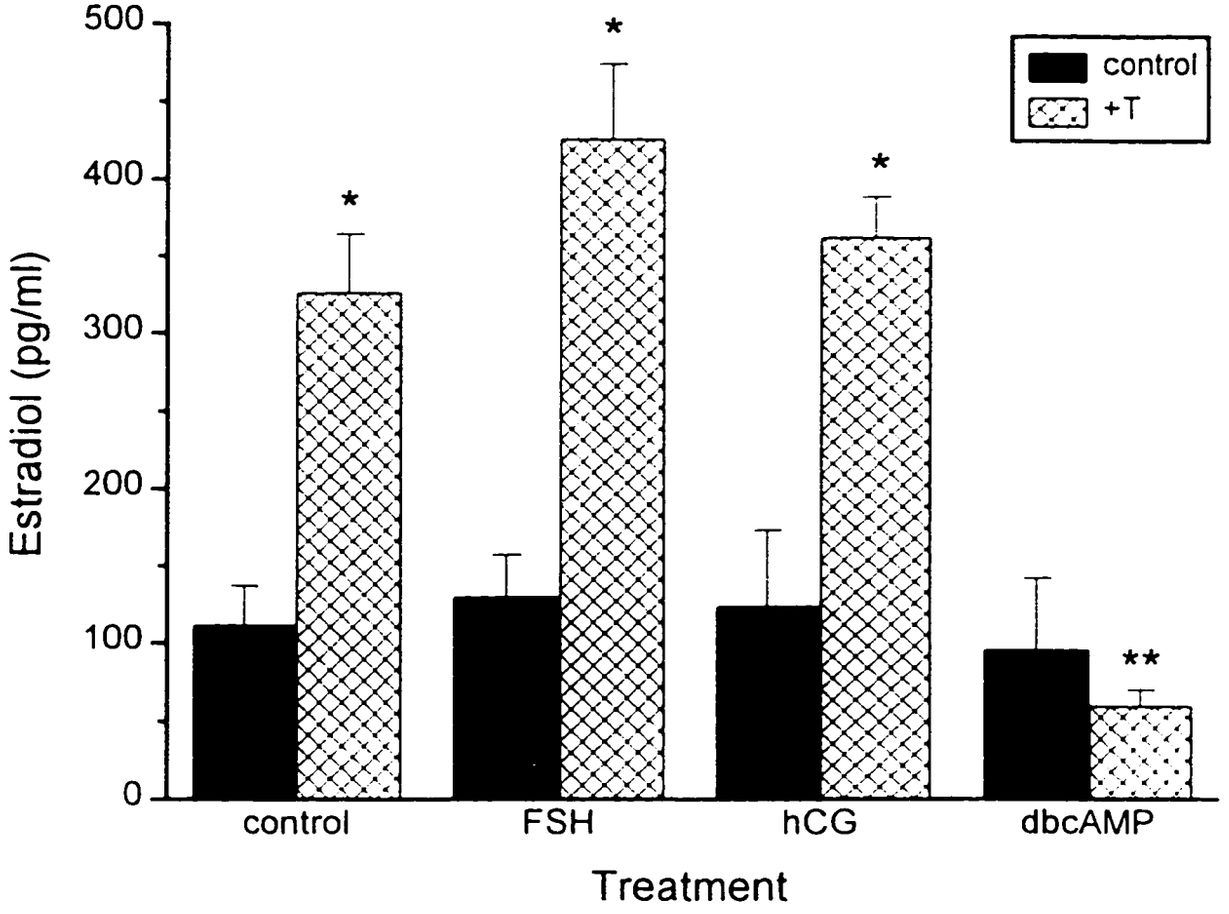
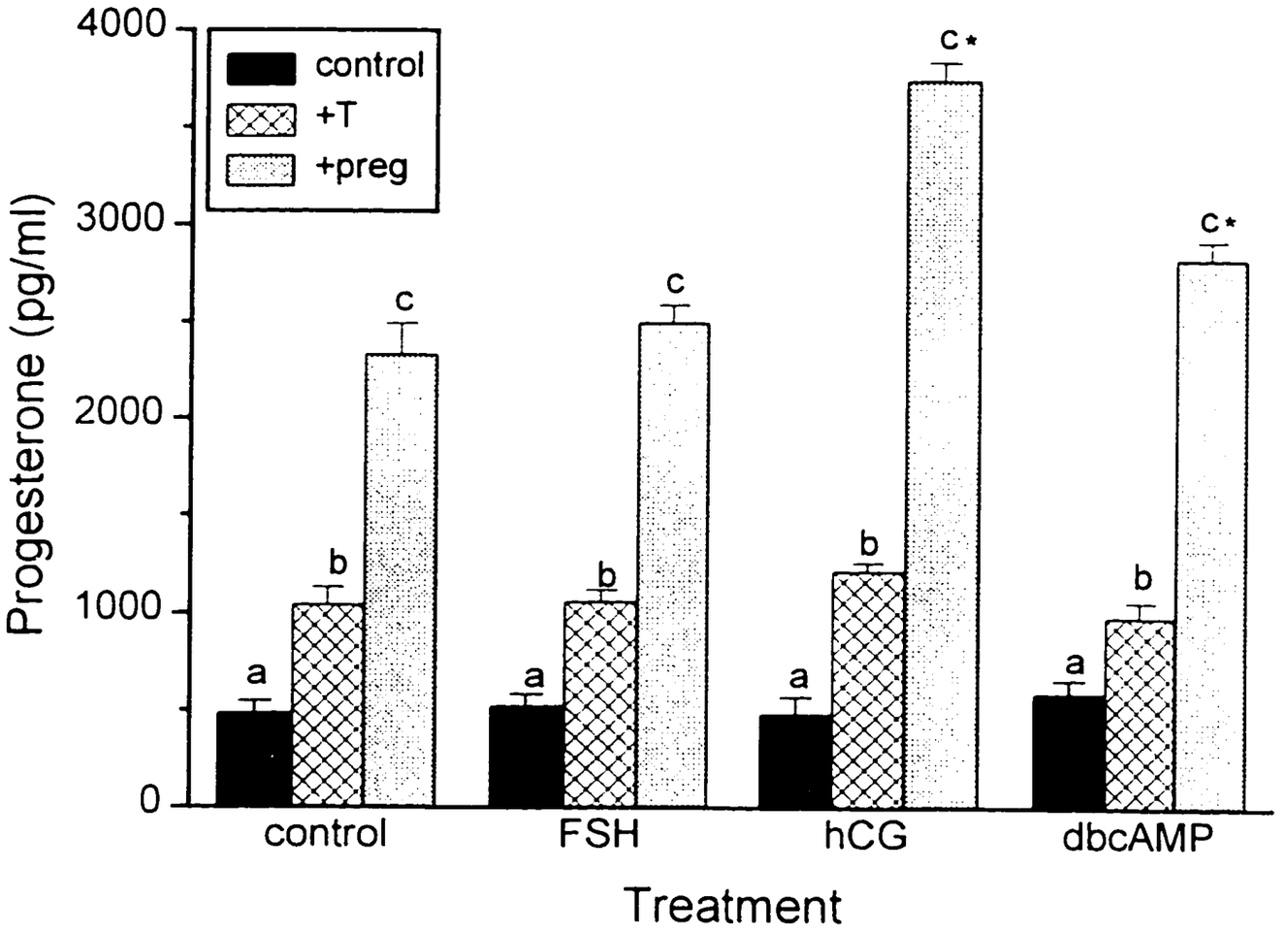

500
400
300
200
100
0
Estradiol (pg/ml)
control
+T
*
*
*
**
control
FSH
hCG
dbcAMP
Treatment

Figure 6.2

Progesterone production by ROSE 199 cells

Progesterone production by ROSE 199 cells cultured for 48 hr in the presence or absence of testosterone (0.5 μM) or the substrate, pregnenolone (0.5 μM). Cells were treated with FSH (150 ng/ml), hCG (5 IU/ml) or dbcAMP (2 mM). Each bar represents the mean ± S.E.M of 2 experiments with 3 independent samples per experiment. Bars with different letters indicate steroid treatments that are significantly different from the respective steroid-free controls and * indicates significantly different treatments from control with pregnenolone ($p<0.01$).


4000
3000
2000
1000
0
Progesterone (pg/ml)
control
+T
+preg
a
b
c
a
b
c
a
b
c*
a
b
c*
control
FSH
hCG
dbcAMP
Treatment

To characterize basal and hormonally stimulated rates of cell proliferation in ROSE 199 cells, 2.5×10^5 cells were cultured alone or in the presence of gonadotropins, steroids or dbcAMP and counted at 24, 48 and 72 hr after seeding (Fig. 6.3). Control ROSE 199 cells (untreated) continued to proliferate over the incubation period so that there were $5.9 \pm 0.5 \times 10^5$, $21.2 \pm 1.9 \times 10^5$, and $59.4 \pm 4.6 \times 10^5$ total cells after 24, 48 and 72 hr respectively. Proliferation of control ROSE 199 cells was set to 100 % at each time point. No significant changes compared to control ROSE 199 cell number were associated with E_2 or P_4 treatment (Fig. 6.3I) or with gonadotropin stimulation (Fig. 6.3II). There was a trend towards an increase in proliferation with testosterone treatment after 48 hr (Fig. 6.3I); however, these results did not attain significance. This trend persisted when ROSE 199 cells were cultured in 10- and 100- fold higher concentrations of testosterone under serum-free conditions but was not statistically significant (data not shown).

Dibutyryl cAMP treatment inhibited cell proliferation in ROSE 199 cells at every time point examined when compared with untreated cells (Fig. 6.3II), $p<0.05$. Cell viability was 97.6 ± 1.1 % in the presence of 1.0 mM dbcAMP at 48 hr and was not significantly different from untreated cells (96.3 ± 2.2 %). However, the average calculated time required for the cells to double in cell number (to 5×10^5 cells) had increased to 44.8 hr with dbcAMP treatment compared to 13.3 hr for untreated cells.

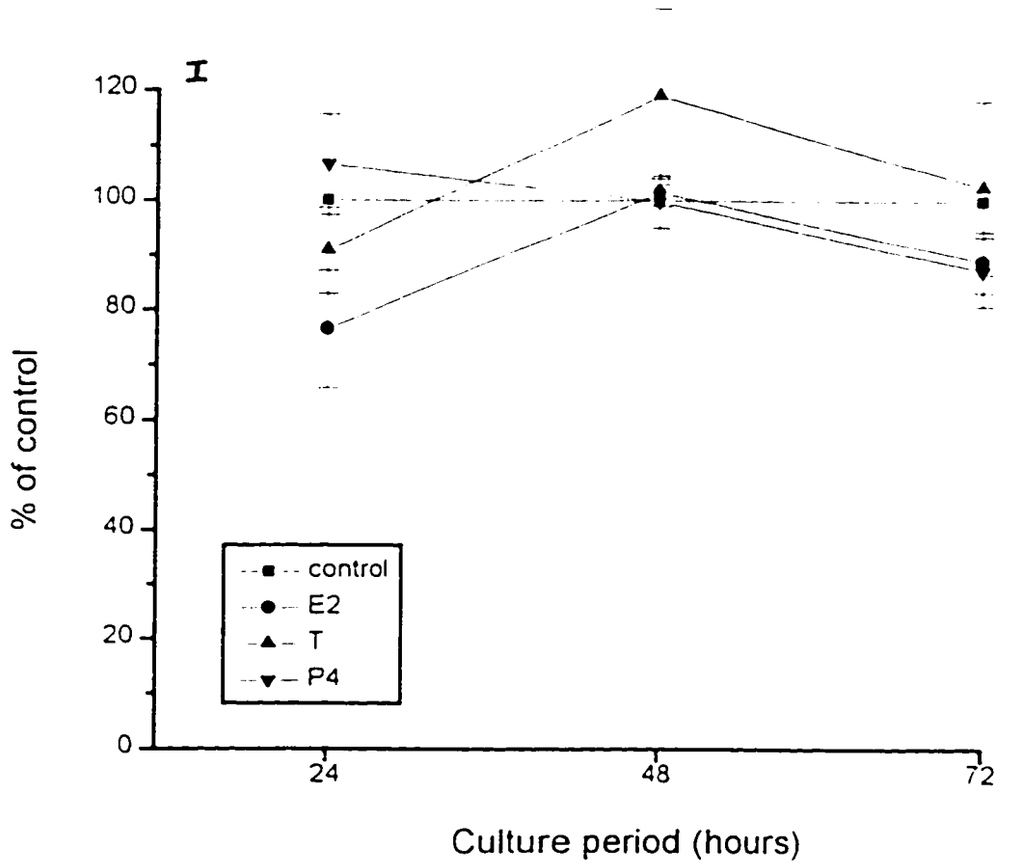
6.3.2 Expression of KL and Kit in OSE cells

In situ hybridization was performed to localize KL mRNA transcripts in sections of 28 day old rat ovaries. Part of an ovary section from a PMSG-primed rat is shown in Fig. 6.4 I.

Figure 6.3

Proliferation of ROSE 199 cells cultured for 72 hr in the presence of gonadotropins, dbcAMP or steroid hormones

Cells were cultured in the presence of (I) 0.5 μM E_2, P_4 or T, or (II) hCG (5 IU/ml), FSH (150 ng/ml) or dbcAMP (2 mM), for up to 72 hr and cell counts in duplicate dishes were done every 24 hr using a hemocytometer. Values are presented as percentage of control and * indicates treatments for which cell numbers are significantly different from control $p<0.05$. Each data point represents the mean ± S.E.M of at least 2 experiments with 2 independent samples per experiment.


I
120
100
80
60
40
20
0
% of control
control
E2
T
P4
24
48
72
Culture period (hours)

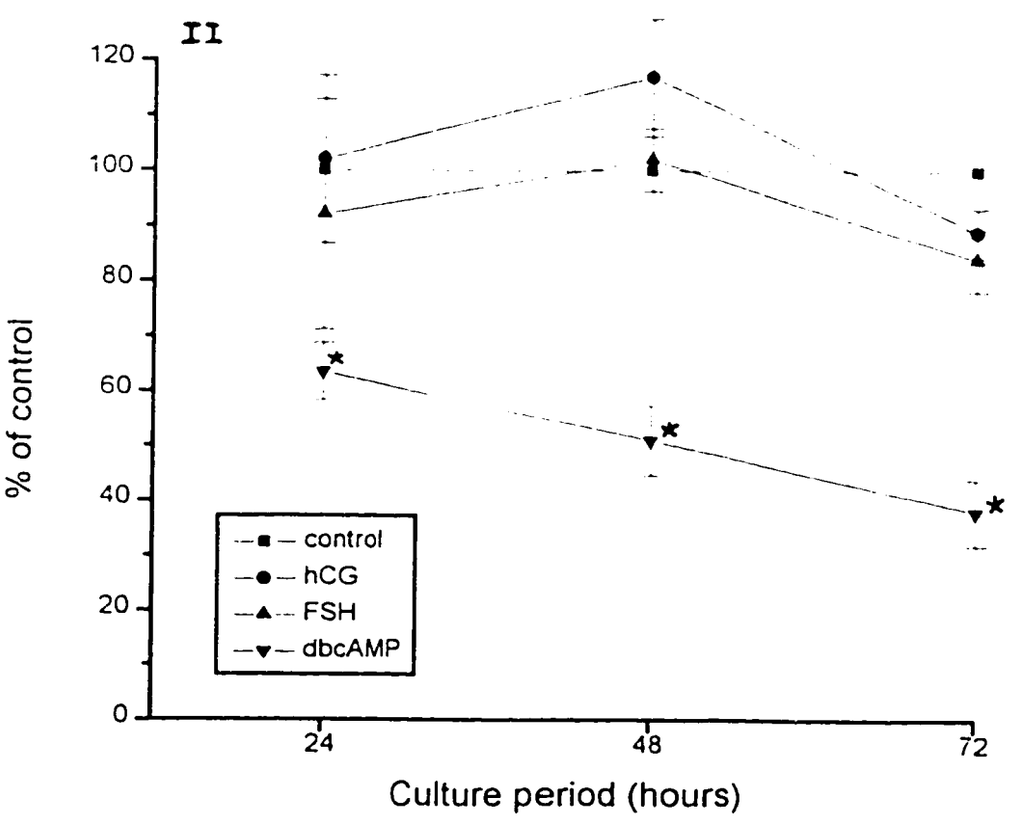
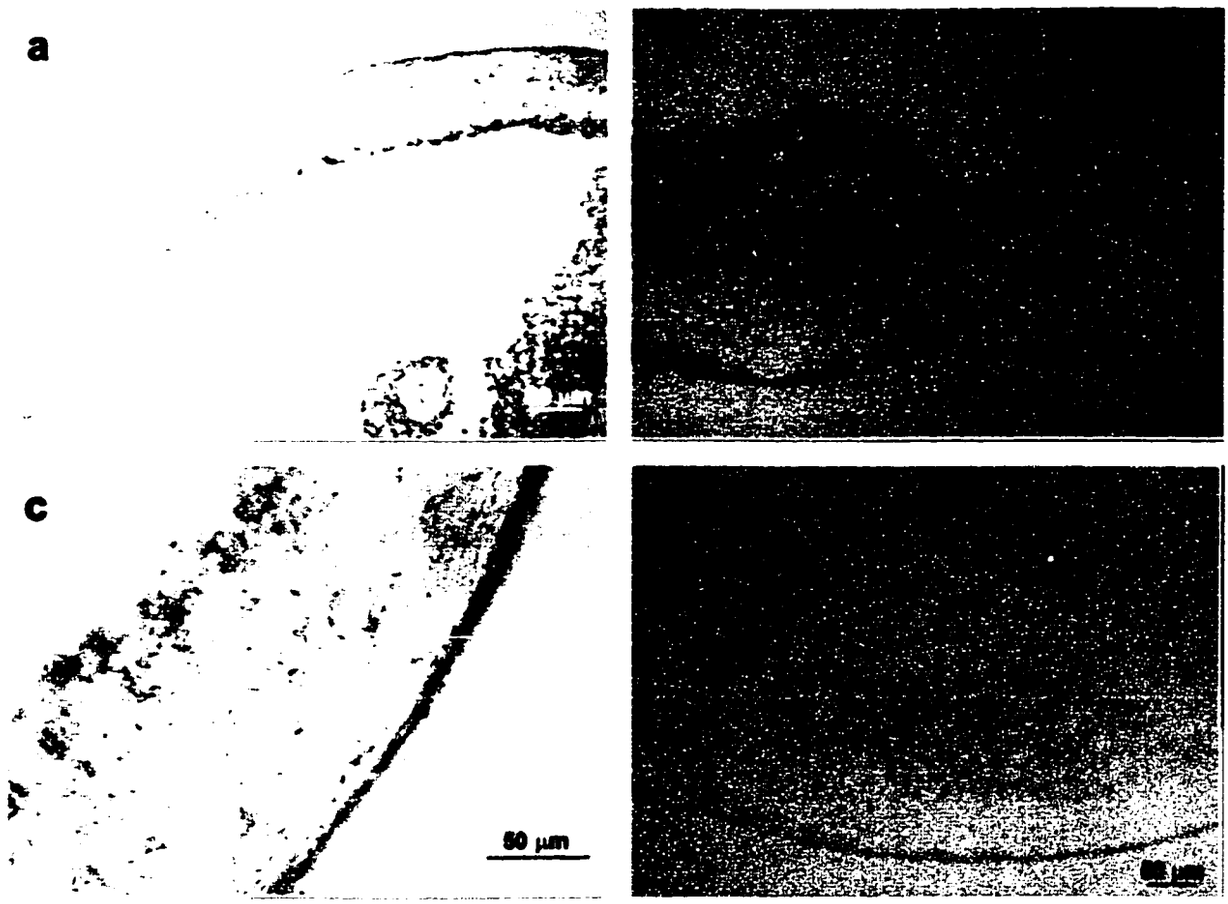

II
120
100
80
60
40
20
0
% of control
control
hCG
FSH
dbcAMP
*
*
*
24
48
72
Culture period (hours)

Figure 6.4

Localization of kit ligand (KL) mRNA (I) and protein (II) in the rat ovarian surface epithelium

In situ hybridization (I) or immunohistochemical detection (II) was utilized to detect either KL mRNA (I) or protein (II) in sections through ovaries of 28-day-old PMSG- rats. In (I), photomicrographs are bright field images of sections of ovary hybridized with a KL probe (a, b and c) and KL mRNA expression is evident in the surface epithelium. Non-specific binding of labelled-probe in the presence of 100-fold excess unlabelled-KL probe (d) is not evident outside of the hilium region (see Fig 2.4c) of the ovary. A region in (b) of the surface epithelium is magnified in (c). In (II), sections were incubated with antibodies against KL and a brown product indicates positive KL staining (a). To identify non-specific staining of the antibody, sections were taken through the procedure in the absence of antibodies against KL (b).

I.



II.

Probe hybridization is evident as a dark blue reaction product. Staining is seen throughout the ovarian epithelium (Fig.6.4 Ia,b) and is more evident at higher magnifications (Fig. 6.4 Ic). When sections of ovaries from PMSG-primed rats were incubated with excess unlabelled probe no non-specific binding was seen in the surface epithelium (Fig. 6.4 Id) except in the hilium region (Fig. 2.4c).

To determine the presence of KL protein in rat OSE cells, sections of PMSG-primed ovaries were exposed to antibodies against KL and positive staining was seen as a dark brown reaction product. KL protein was detected throughout the surface epithelium (Fig. 6.4 IIa). When antibodies were omitted, the dark reaction product was not evident (Fig. 6.4 IIb), indicating that the reaction product is present only under circumstance of specific antibody-antigen reaction.

To further investigate if KL and Kit are present in OSE cells, RNA was isolated from OSE cells of ovary explants and from ROSE 199 cells, and used for northern blot analysis. KL transcripts were present in OSE and ROSE 199 cells (Fig. 6.5A); however, these cells do not express message for Kit receptors as seen by lack of signal in these preparations but an abundance of signal in the positive control (MC/9) lane (Fig. 6.5B). To determine the form of KL transcripts produced by OSE cells, RT-PCR was performed with primers A and C (Fig. 4.1) using total RNA from two preparations of OSE cells and ROSE 199 cells. Although both KL-1 and KL-2 cDNAs were amplified in OSE and ROSE 199 cells, there was a predominance of KL-1 cDNA (Fig. 6.6). Thus OSE cells, both normal and immortalized, produce KL transcripts and the majority of these transcripts are in the form that, when translated, is preferentially cleaved to yield soluble KL.

Figure 6.5

Northern blot analysis of Kit receptor and KL mRNA expression in primary cultures of ovarian surface epithelial cells (OSE cells) and immortalized OSE cells (ROSE 199 cells)

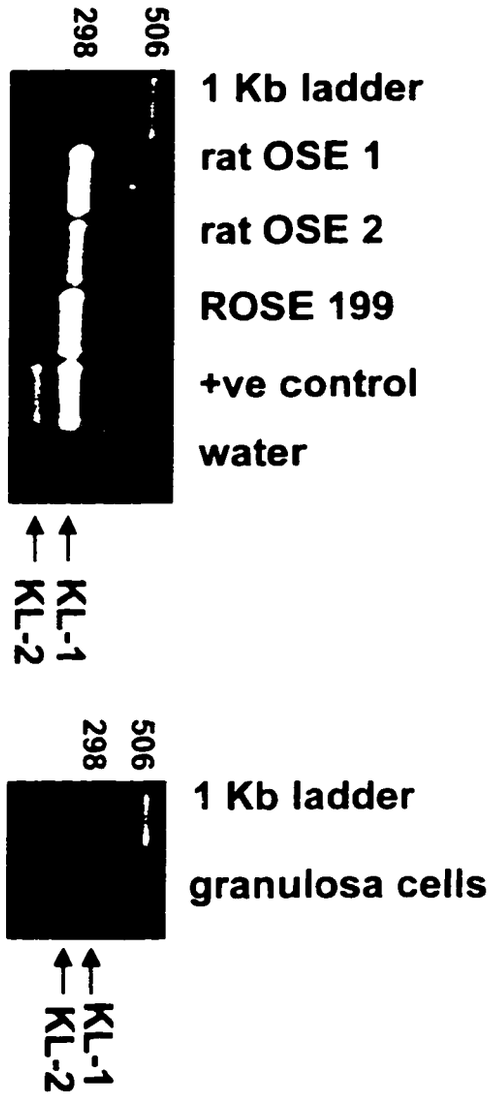
Gel electrophoresis was done with 15 μg of total OSE or ROSE 199 cell RNA in each lane and total RNA samples from NIH 3T3 fibroblasts and MC/9 mast cells were included in the northern blot analysis to serve as KL positive/Kit negative and KL negative/Kit positive mRNA controls. Blots were first probed with ^{32}P-labelled KL cDNAs (A), stripped and reprobed with ^{32}P-labelled Kit receptor cDNAs (B). The blots were reprobed with ^{32}P-labelled α-tubulin cDNA (C) to ensure integrity of RNA.

NIH 3T3
MC/9
rat OSE
ROSE 199
A
KL
28s
B
Kit
28s
C
α-tubulin

Figure 6.6

Reverse transcription-polymerase chain reaction to identify KL total RNA from rat OSE cells and ROSE 199 cells

KL primers spanning exon 6 of KL mRNA were used to amplify KL-1 (287 bp) and KL-2 (203) transcripts. C13, a KL-producing epithelial cell line was used as a positive control. RNA was substituted with water prior to RT-PCR to serve as a negative control (water). At the same time, RT-PCR was also done using granulosa cell RNA, to serve as a positive control for KL-2 cDNA. Similar results were obtained with a second pair of PCR primers (data not shown). Amplified products were separated on 2% nuseive: agarose gels and DNA was visualized by staining the gels with ethidium bromide.


506
298
1 Kb ladder
rat OSE 1
rat OSE 2
ROSE 199
+ve control
water
KL-1
KL-2
506
298
1 Kb ladder
granulosa cells
KL-1
KL-2

6.3.3 Hormonally-regulated expression of KL and Kit mRNA

ROSE 199 cells were cultured with steroids, gonadotropins or dbcAMP, and northern blot analysis was performed to determine the effects of these additives on KL and Kit mRNA expression. Since Kit was not detectable in normal and ROSE 199 cells, the possibility that hormone treatment would induce Kit message in these cells was investigated. However, Kit mRNA continued to be undetectable in the presence of additives (data not shown). No change in KL mRNA expression was seen in response to gonadotropins or steroids compared to control (untreated) ROSE 199 cells (data not shown). Interestingly, when ROSE 199 cells were cultured for 40 hr with 1 mM dbcAMP, KL mRNA expression increased 3.0 ± 0.2 fold compared to untreated (0) ROSE 199 cells (Fig. 6.7). Treatment with 1 and 2 mM dbcAMP elicited similar increases in KL mRNA expression ($p<0.05$).

Except for the moderate increase in P_4 accumulation, gonadotropin stimulation caused very little changes in steroid output, proliferation or gene expression in ROSE 199 cells and this lack of effect could arise due to the lack of receptors. Since the presence of gonadotropin receptors have not been investigated in this cell line, northern blots with total and poly-A+ enriched ROSE 199 cell RNA were prepared to probe for FSH and LH receptor mRNAs. No FSH or LH receptor mRNAs were detected in ROSE 199 cell RNA preparations, whereas multiple transcripts for FSH and LH receptor mRNA were seen in granulosa cell samples (Fig. 6.8) and transcript sizes were consistent with those previously published (Dunkel et al., 1994; Zhang et al., 1994).

Figure 6.7

Northern blot analysis of KL expression in ROSE 199 cells in the presence or absence of 1 and 2 mM dbcAMP

Northern blot analysis of ROSE 199 cells cultured alone or for 40 hr in the presence of 1 or 2 mM dbcAMP, and probed with a ^{32}P-labelled rat KL cDNA probe (A). Gel electrophoresis was done with 15 μg of total RNA in each lane and total RNA samples from NIH 3T3 and Sl^4 fibroblasts were included in the northern blot analysis to serve as positive and negative KL mRNA controls, respectively. Nitrocellulose membranes were used to prepare northern blots and RNA was immobilized by baking membranes at 80°C under vacuum for 3 hr. The blots were reprobed with ^{32}P-labelled 28s ribosomal marker cDNA (B). To quantitate the effect of treatment, the intensity of the bands on the phosphoimager was estimated using ImageQuaNT software, and the ratio of KL/28s ribosomal marker values were standardized to control levels which were arbitrarily set to 1.0. A histogram illustrating the differences in KL/28s ribosomal marker ratios where each bar represents the mean ± S.E.M. of 4 experiments is shown in C. Bars indicated with different letters are significantly different ($p<0.05$).

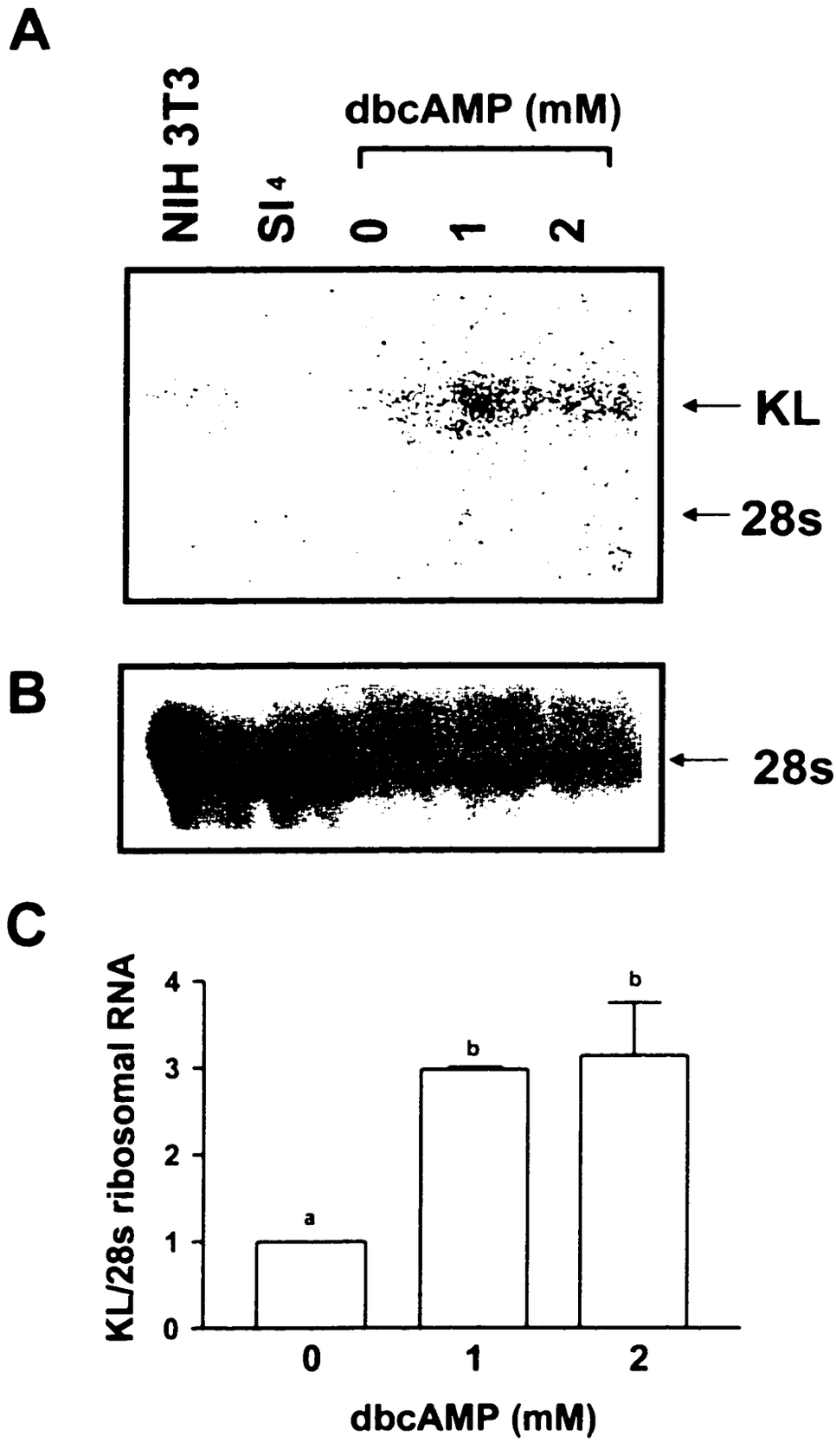
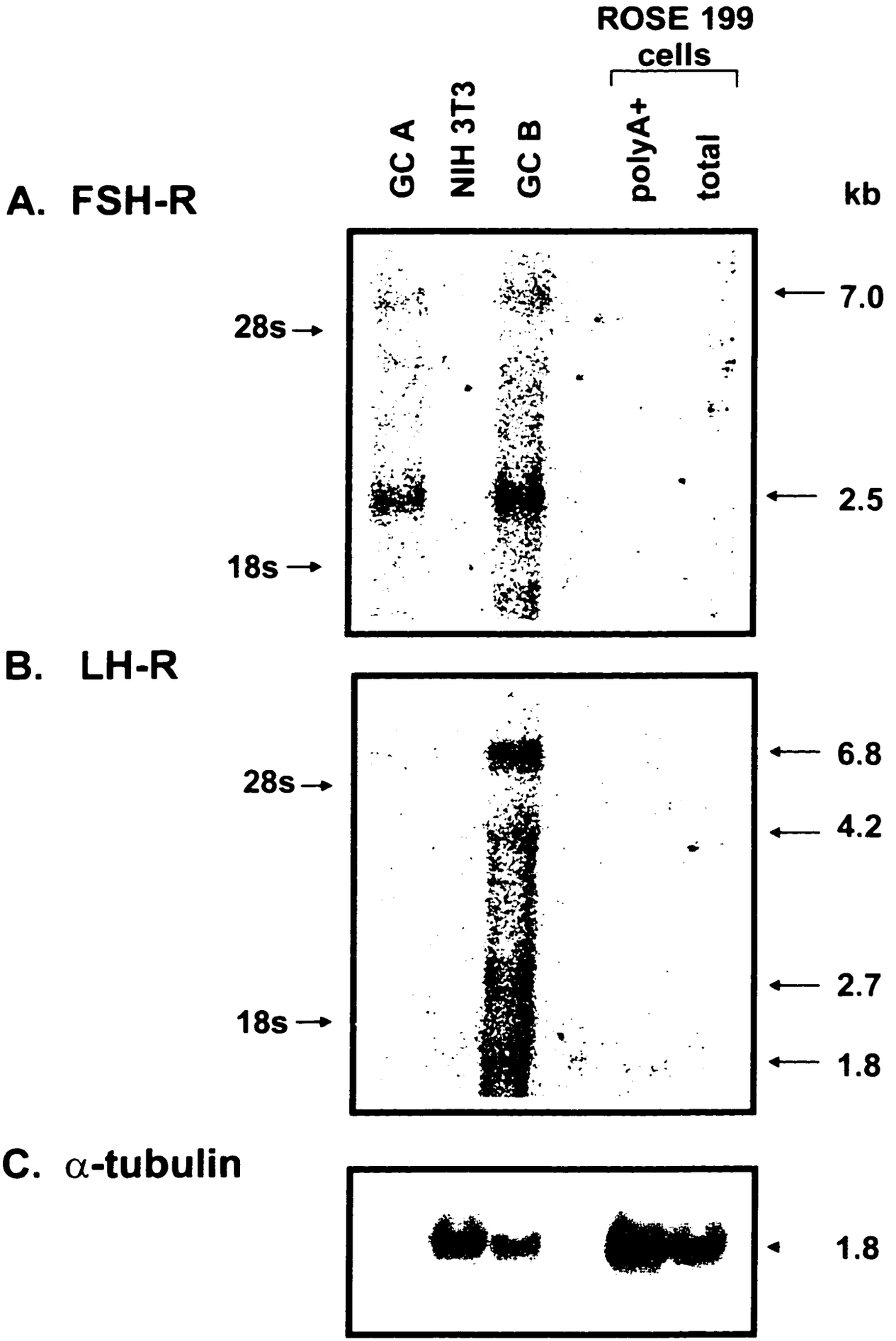

A
NIH 3T3
SI⁴
dbcAMP (mM)
0
1
2
KL
28s
B
28s
C
KL/28s ribosomal RNA
4
3
2
1
0
a
b
b
0
1
2
dbcAMP (mM)

Figure 6.8

Northern blot analysis of FSH and LH receptor mRNA expression in ROSE 199 cells

Gel electrophoresis was done with 15 μg of total ROSE 199 cell RNA or 5 μg of polyA+-enriched ROSE 199 cell RNA. Total RNA samples from 40 hr PMSG-primed granulosa cells (GCA) and 48 hr hCG-primed granulosa/luteal cells (GCB) were included as positive controls for FSH and LH receptor mRNA, respectively and NIH 3T3 fibroblasts was included as a negative mRNA control for both gonadotropin receptor mRNAs. Northern blots were prepared and first probed with ^{32}P-labelled FSH receptor cDNA (A) then stripped and reprobed with ^{32}P-labelled LH receptor cDNA (B). The blots were reprobed with ^{32}P-labelled α-tubulin cDNA (C) to ensure integrity of RNA. Band sizes were estimated by their positions relative to 28s and 18s.


ROSE 199 cells
GC A
NIH 3T3
GC B
polyA+
total
kb
A. FSH-R
28s
18s
7.0
2.5
B. LH-R
28s
18s
6.8
4.2
2.7
1.8
C. α-tubulin
1.8

6.3.4 Effect of TGF-β on dbcAMP-stimulated ROSE 199 cell KL expression

TGF-β has been shown to downregulate KL mRNA expression in stromal cells by repressing gene transcription (Heinrich et al., 1995). To determine if TGF-β could cause similar decreases in KL mRNA levels in ROSE 199 cells, ROSE 199 cells were treated for 48 hr with TGF-β and/or dbcAMP, or cultured alone to serve as a control. TGF-β alone was not able to elicit changes in KL mRNA levels, although there was a trend towards decreased KL mRNA expression. As expected, there was an increase in KL mRNA expression with dbcAMP treatment (Fig. 6.9). However, a combined treatment of dbcAMP and TGF-β dramatically decreased KL mRNA, so that levels were lower than those seen in unstimulated ROSE 199 cells (Fig. 6.9).

6.3.5 Morphological and cell attachment characteristics of ROSE 199 cells treated with dbcAMP

Careful observation of cells exposed to the various treatments revealed that dbcAMP treatment caused a notable change in the morphology of ROSE 199 cells compared to untreated cells (Fig 6.10). The dbcAMP-treated cells appeared flattened, slightly enlarged and formed tight foci. In addition, dbcAMP-treated cells were more difficult to enzymatically detach from the culture dishes by trypsinization compared to untreated cells. To determine quantitatively the difference in ROSE 199 cell attachment with or without dbcAMP treatment, the percentage of ROSE 199 cells detached at 3 min of trypsinization was determined and is illustrated in Fig. 6.11. We found 29.3 ± 5.6 % of cells detached in cultures treated with dbcAMP compared to 63.4 ± 3.4 % of untreated cells ($p<0.05$).

Figure 6.9

Northern blot analysis of KL expression in ROSE 199 cells in the presence or absence of 10 ng/ml TGF-ß and/or 1 mM dbcAMP

ROSE 199 cells were cultured for 48 hr with vehicle alone or in the presence of 10 ng/ ml TGF-ß and/or 1 mM dbcAMP. Northern blots were prepared from isolated RNAs and probed with a ^{32}P-labelled rat KL cDNA probe (A). Gel electrophoresis was done with 15 µg of total RNA in each lane and total RNA samples from NIH 3T3 and Sl^4 fibroblasts were included in the northern blot analysis to serve as positive and negative KL mRNA controls, respectively. The blots were reprobed with ^{32}P-labelled α-tubulin cDNA (B). To quantitate the effect of hormone treatment, intensity of the bands on the phosphoimager was estimated using ImageQuaNT software, and the ratio of KL/α-tubulin values were standardized to control levels which were arbitrarily set to 1.0 . A histogram illustrating the differences in KL/α-tubulin ratios where each bar represents the mean values of 2 experiments is shown in C. Error bars represent maximum and minimum data points. The dbcAMP bar represents the results of a single data point. Bars indicated with * are significantly different from control (untreated) (p<0.05).

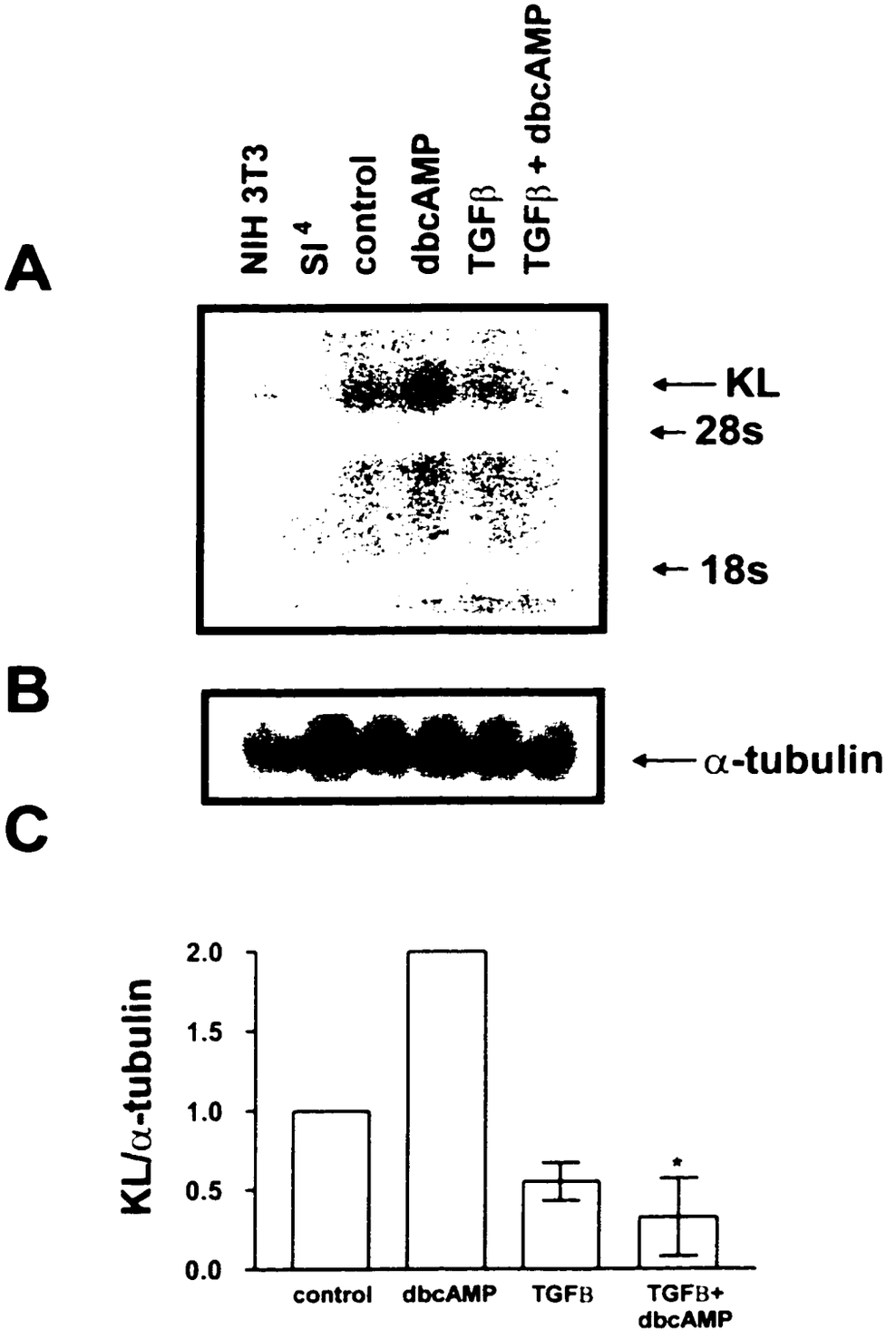

A
NIH 3T3
SI 4
control
dbcAMP
TGFβ
TGFβ + dbcAMP
KL
28s
18s
B
α-tubulin
C
KL/α-tubulin
2.0
1.5
1.0
0.5
0.0
control
dbcAMP
TGFB
TGFB+
dbcAMP
*

Figure 6.10

Effect of dbcAMP treatment on ROSE 199 cell morphology

ROSE 199 cells were cultured in the absence (a) or presence (b) of 1 mM dbcAMP. With dbcAMP treatment, cells formed enclosed foci (open arrows) of flattened cells.

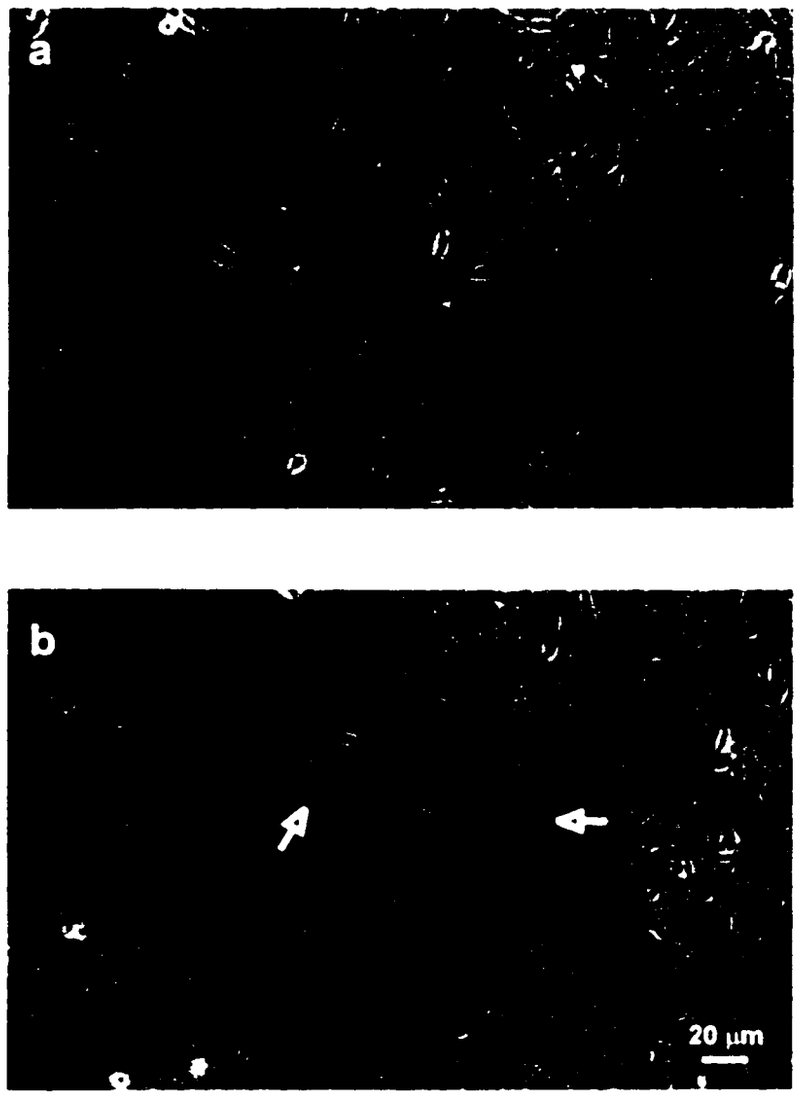

a
b
20 μm

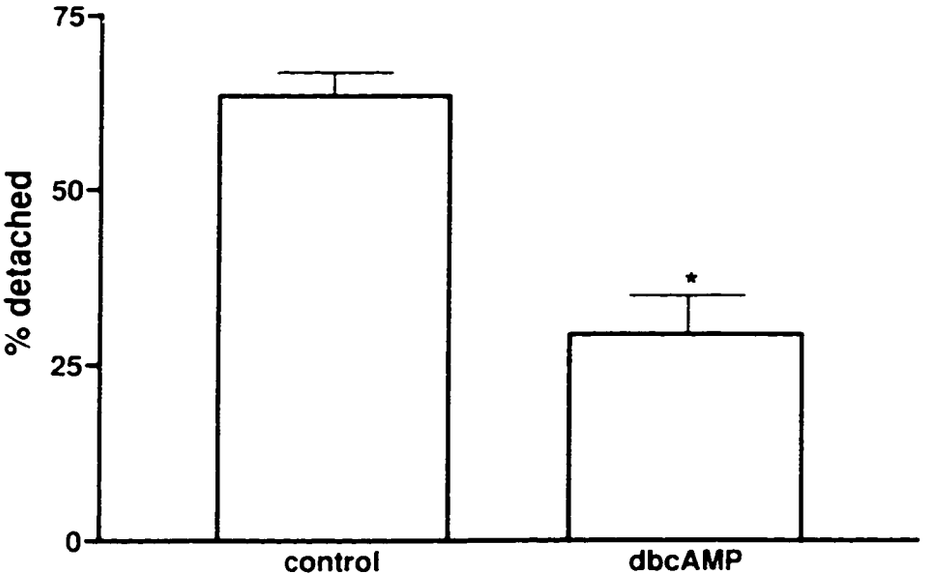
Figure 6.11

Effect of dbcAMP on the adhesion of ROSE 199 cells after 3 min of trypsinization

ROSE 199 cells were cultured alone (control) or in the presence of 1.0 mM dbcAMP . After 48 hr. cell monolayers were exposed to 0.25% trypsin-edta and the percentage of total cells that had detached after 3 min of enzyme treatment was determined using the following equation:

$$\% \text{ detached} = [\text{number of detached cells} / (\text{number of detached cells} + \text{number of adherent cells})] \cdot 100$$

Each bar represents the mean ± S.E.M of 13 data points in a total of 3 experiments and *represents significant difference from control values ($p<0.05$).


75
50
25
0
% detached
*
control
dbcAMP

6.4 Discussion

The experiments reported here demonstrate that immortalized OSE cells were able to produce estradiol and progesterone. Estradiol production was not under gonadotropin control; however, progesterone production was elevated in the presence of hCG and dbcAMP but only when exogenous substrates were included in the culture media. The rate of ROSE 199 cell proliferation was not influenced by steroid hormones or gonadotropins; however, dbcAMP treatment caused a significant decrease in cell proliferation. Furthermore, the expression of KL in primary and immortalized rat OSE cells and its regulation by dbcAMP and TGF-β was demonstrated. In addition to its effects on KL expression, dbcAMP treatment influenced the morphology and adhesive properties of ROSE 199 cells. Under our culture conditions, Kit mRNA was not expressed in either normal or immortalized OSE cells and its expression could not be induced by hormone treatment. Therefore, the role of Kit/KL interactions in OSE cell proliferation after ovulation is still unclear.

The ability of normal OSE cells to produce steroid hormones has not been reported previously. In our experiments, we demonstrated that ROSE 199 cells were able to produce both estradiol and progesterone under resting conditions, as well as when given appropriate substrates, suggesting the presence of the steroidogenic enzymes, aromatase and 3β-hydroxysteroid dehydrogenase (3β-HSD) in these cells. Furthermore, basal progesterone activity was enhanced by the presence of testosterone indicating that 3β-HSD activity can be enhanced by androgens in a manner analagous to that seen in granulosa cells (Gore-Langton and Armstrong, 1988). In addition, in preliminary experiments, when spent media from cultures of primary rat OSE cells were examined for steroids, both estradiol and

progesterone were detected by standard RIA (data not shown), indicating that steroid production in the ROSE 199 cell line probably reflects an activity that is similar to the normal phenotype and not one acquired with cell immortalization. Due to difficulties in obtaining large numbers of primary OSE cells, ROSE 199 cells were used for subsequent experiments to determine the hormonal regulation of steroidogenesis. Adams and Auersperg, (1981) have previously reported the expression of 3β-HSD in transformed rat OSE cells.

Gonadotropins are the key regulators of steroidogenesis in the ovary (reviewed in Gore-Langton and Armstrong, 1988). Since FSH receptors have been demonstrated in human OSE cells (Zheng et al., 1996) and gonadotropin responsiveness has been previously demonstrated (Nicosia et al., 1985), experiments were done to determine the influence of gonadotropins on ROSE 199 cell steroidogenesis. Estradiol production was not under gonadotropic control, although progesterone production moderately increased in hCG-treated OSE cells when they were cultured with substrates. FSH receptor mRNA could not be identified in ROSE 199 cell extracts and the lack of effect of FSH stimulation was due to the lack of FSH receptors in these cells. However, it is quite curious that LH receptor mRNA could not be detected in ROSE 199 cells since an LH-stimulated increase in P_4 production was seen. It is possible that the level of LH receptor mRNA was below the sensitivity of the northern blot analysis and small increases due to hCG stimulation were detected because of the sensitivity of the RIA procedure. Whether the lack of gonadotropin receptors simply reflects a lack of expression in normal OSE cells or if these receptors have been lost due to the immortalization process is not known. DbcAMP treatment resulted in dramatic decreases

in the rate of proliferation of ROSE 199 cells and the apparent decrease in estradiol production is most likely due decreased number of cells per dish. Dibutyryl cAMP resulted in significantly higher progesterone accumulation despite a decrease in cell number and hence, this treatment dramatically stimulated progesterone production.

OSE cells exhibit rapid bursts of proliferation *in vivo*, but very little is known about the factors which influence proliferation or gene expression in these cells. Receptors for both estrogen (Adams and Auersperg, 1983) and progesterone (Karlan et al., 1995) have been identified in primary cultures of OSE cells, as well as in transformed OSE cells (Hamilton et al., 1983; Geisinger et al., 1990), and receptors for testosterone have been identified in some transformed cell lines (Hamilton et al., 1983). The expression of steroid receptors has not been investigated in ROSE 199 cells. Estradiol or progesterone stimulation was unable to stimulate ROSE 199 cell proliferation. Although there was a trend towards increased proliferation after 48 hr of testosterone stimulation, no significant differences were seen. These results are in agreement with findings obtained using normal human OSE cells (Karlan et al., 1995) but in contrast to a previous report of the effects of testosterone on rat OSE cell proliferation (Hamilton et al., 1980) and cell lines derived from human ovarian carcinomas (reviewed in Godwin et al., 1993). These results would indicate that steroid hormones do not significantly alter OSE cell proliferation, although it is possible that testosterone responsiveness is acquired with transformation.

KL mRNA has been demonstrated in the epithelium of fetal sheep ovaries; however this expression is lost after birth (Tisdall et al, 1997). These studies are the first to report KL mRNA and protein in the surface epithelium of postnatal ovaries of any species. To further

demonstrate that OSE cells are a site of KL production in the rat ovary, primary cultures of OSE cells were established from ovary explants and used for subsequent northern blot analysis and RT-PCR. It was of importance to distinguish rat OSE cells from other contaminating ovarian cells. Isolation of rat OSE from ovary explants yielded fairly homogeneous populations and one could readily distinguish OSE cells from other ovarian cell types based on differences in morphology of the cells (Adams and Auersperg, 1981). Rat OSE cells in our cultures had clear cytoplasm, well-defined borders and formed confluent monolayers, similar to those described by Adams and Auersperg (1981). Furthermore, the ovarian cells that underlie the OSE cells are theca cells, which express Kit (Motro and Bernstein, 1993; Manova et al. 1993). The absence of Kit expression in our preparations of rat OSE cells would suggest that there was no theca cell contamination with these cells.

Both normal and immortalized OSE cells were shown to have a predominance of KL-1 and, therefore, would preferentially yield soluble KL when translated. Since the underlying theca cells express Kit receptors *in vivo*, there is a possibility that the soluble ligand could activate the theca cell Kit receptors via paracrine interactions to elicit a response. Although the function of Kit receptors in theca cells has not yet been determined, the importance of Kit/KL interactions in paracrine cell-cell communication is well-established (Keshet et al., 1991). Furthermore limited evidence suggests that theca cells may, in turn, affect OSE cell proliferation. This hypothesis is supported by evidence showing that soluble products produced by theca cells and present in conditioned media of bovine theca-interstitial cells increased OSE cell proliferation (Vigne et al., 1994). In

addition, in mice with *Kit* mutations resulting in the expression of inactive Kit receptors, there was a deregulation of ovarian epithelial cell growth (Murphy, 1972; Murphy and Beamer, 1973) suggesting that the presence of thecal Kit receptors may be important for the production of other theca cell products which act on OSE cells to inhibit their proliferation. Therefore, the role of theca cells in the regulation of OSE cell proliferation remains to be elucidated.

Kit transcripts were absent in immortalized rat OSE cells and, although Kit expression has been shown to be regulated by various factors including cAMP (Nishina et al., 1992) and hCG (Horie et al., 1991), Kit expression could not be induced by dbcAMP or any of the hormones tested. Although this observation suggests that the proliferation of OSE cells after ovulation does not occur through the activation of a Kit/KL autocrine growth regulatory loop, it is equally possible that Kit is important for OSE cell proliferation *in vivo*, but is expressed only under specific conditions which were not duplicated in culture.

Dibutyryl cAMP was the only treatment in this study that consistently produced effects on OSE cell proliferation, morphology, and gene expression. There was a significant decrease in cell proliferation in response to dbcAMP treatment and, although these cells continued to divide, they did so at a much slower rate than untreated ROSE 199 cells. Cells treated with dbcAMP appeared enlarged and flattened, formed tight foci and were more adherent to the tissue culture plate relative to untreated cells. The morphological changes in response to cAMP seen in ROSE 199 cells resembled the flattened morphology typically seen in primary cultures of rat OSE cells (our findings) and similar morphological changes have been reported in bovine OSE cells in response to keratinocyte growth factor (Vigne et

al. 1994). One interpretation for the morphological changes associated with dbcAMP treatment could be that these cells have undergone some differentiative process. Cyclic AMP has been shown to be involved in the cellular differentiation of embryonically-derived cells (Tonary and Carnegie, 1996). Since differentiation is normally associated with a decrease in cell proliferation, the decrease in ROSE 199 cell proliferation may simply be a consequence of differentiation processes. An increase in progesterone production is associated with granulosa cell differentiation and increased progesterone production was also seen with dbcAMP treatment of ROSE 199 cells.

Dibutyryl cAMP treatment of ROSE 199 cells resulted in significant increases in cell adhesion to tissue culture dishes and these results are similar to previous reports of cAMP-mediated increases in cell-cell and cell-substratum interaction of renal proximal tubular epithelial cells (Amsler, 1990). It is quite probable that the dbcAMP-enhanced increase in adhesion may be due to an increase in extracellular matrix protein deposition. Although ROSE 199 cells deposit an abundance of extracellular matrix components including fibronectin, laminin and collagen types I and II (Kruk and Auersperg, 1994), which of these may be involved in increases in dbcAMP-stimulated cell adhesion has not yet been determined. The increase in cell adhesion reported in these experiments is coincident with an increase in KL mRNA expression. In endothelial cells, a 3-fold increase in KL mRNA levels paralleled a marked increase in the levels of intercellular adhesion molecule-1 mRNA (Buzby et al., 1994). Whether the increase in KL expression, directly or indirectly, alters the expression of cell adhesion markers in ROSE 199 cells and the role of KL, if any, in enabling cell matrix interactions remains to be elucidated.

Dibutyryl cAMP treatment resulted in a 3-fold increase in KL mRNA expression and similar increases in KL expression in response to cAMP analogs have been reported previously in other cell types (Rossi et al., 1991, and 1995; Tajima et al., 1993; Manova et al., 1993) including rat granulosa cells (Fig 5.8). Increases in KL mRNA expression could be inhibited if ROSE 199 cells were cultured in the presence of TGF-ß while being stimulated with dbcAMP (Fig. 6.10), suggesting that TGF-ß prevented dbcAMP-stimulated increases in KL expression.

After ovulation, ovarian epithelial cells contribute to postovulatory wound repair by degrading existing wound-associated extracellular matrix and depositing new matrix material in order to allow for the reepithelialization of the stigma region by the migration and proliferation of OSE cells from the perimeter of the ruptured follicle (Murdoch, 1994). One of the key regulators of wound repair is TGF-β, a soluble growth factor that is produced predominantly by theca cells in the ovary. TGF-β secretion is upregulated in large follicles destined to ovulate (Skinner et al., 1987). Since theca cells underlie OSE cells, TGF-β produced in the theca cell layer may be able to diffuse across the basement membrane which separates these two cell types and affect OSE cell function. TGF-β stimulation resulted in a decrease in KL mRNA expression in ROSE 199 cells. We propose that the dbcAMP-treated cells closely resemble OSE cells under resting conditions: having a flat epithelial cell morphology typically seen in the surface epithelium and a low proliferation rate, possibly due to increased OSE cell-to-basement membrane contacts (Fig. 6.12). After ovulation, when OSE cells become activated to repair the post ovulatory wound to restore the continuity of the epithelium, there is decreased OSE cell KL expression in response to TGF-ß which,

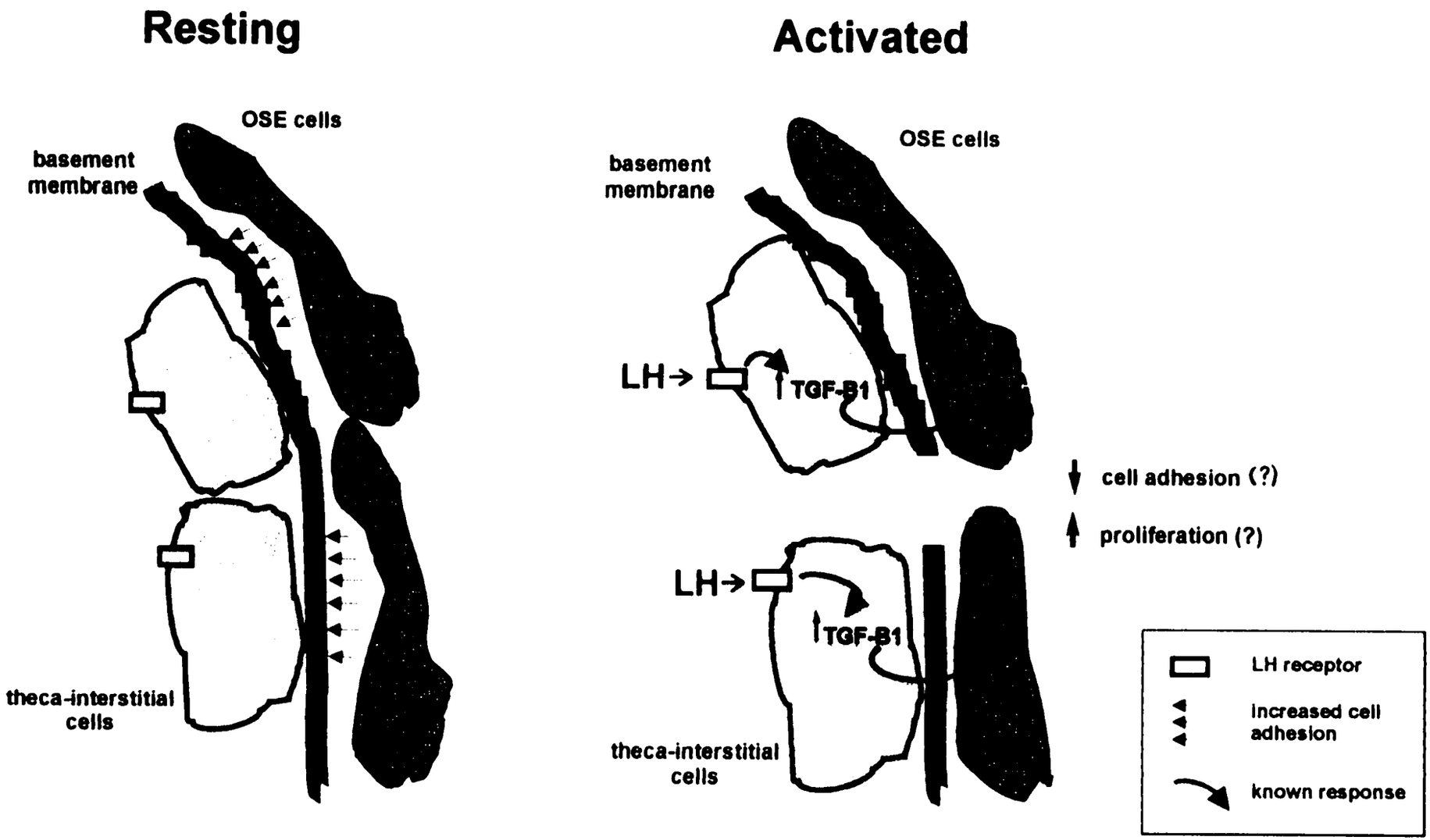
in turn, could result in decreased cell-basement membrane attachment by an as yet unidentified mechanism. This change in cell-matrix attachment may "prime" or enable the cells to respond to factors that enhance the migration or proliferation of OSE cells and allow for ovulatory wound repair. Studies demonstrating an inhibitory action of TGF-ß on KL mRNA expression (Heinrich et al., 1995) or KL activity (Mekori and Metcalfe, 1994; Sott et al., 1994; Ramsfjell et al., 1997) provide support for this hypothesis.

In conclusion, immortalized OSE cells were able to produce both estradiol and progesterone both basally and when given appropriate substrates although steroid hormones and gonadotropins were not able to significantly influence the proliferation rate of ROSE 199 cells. In addition, the OSE cells transcribed and translated KL, and most likely produce predominantly soluble KL. Kit expression was not detected in OSE cells. Dibutyryl cAMP treatment resulted in changes in cell morphology, decreased ROSE 199 cell proliferation, increased KL gene expression and increased cell adhesion. ROSE 199 cell KL expression could be transcriptionally inhibited by the addition of TGF-ß, a molecule well-documented in its modulation of wound repair responses. The inhibition of OSE cell KL mRNA expression may result in decreased OSE cell-basement membrane attachments which, in turn, may be permissive for the burst of proliferation associated with postovulatory wound repair.

Figure 6.12

Model of role of KL in postovulatory wound repair mechanisms

Treatment of ROSE 199 cells with dbcAMP results in cells that resemble OSE cells, under resting conditions, having a flattened-epithelial cell morphology, a low proliferation rate and increased OSE cell-to-basement membrane attachments possibly due to increased KL expression. Around the time of ovulation, gonadotropin stimulation enhances the production of TGF-ß in periovulatory follicles. Soluble TGF-ß diffuses easily across the basement membrane and decreases OSE cell KL mRNA expression. After ovulation, when OSE cells become activated to repair the postovulatory wound and restore the continuity of the epithelium, there may be decreased cell adhesion due to a decrease in KL expression. This change in cell-matrix attachment may "prime" or enable the cells to respond to factors that enhance the migration or proliferation of OSE cells and allow for post ovulatory wound repair.


Resting
OSE cells
basement membrane
theca-interstitial cells
Activated
OSE cells
basement membrane
LH→
TGF-B1
LH→
TGF-B1
theca-interstitial cells
cell adhesion (?)
proliferation (?)
LH receptor
increased cell adhesion
known response

CHAPTER 7 - EXPRESSION OF KL IN THE RAT REPRODUCTIVE TRACT AND ITS FUNCTION IN PREIMPLANTATION EMBRYO DEVELOPMENT

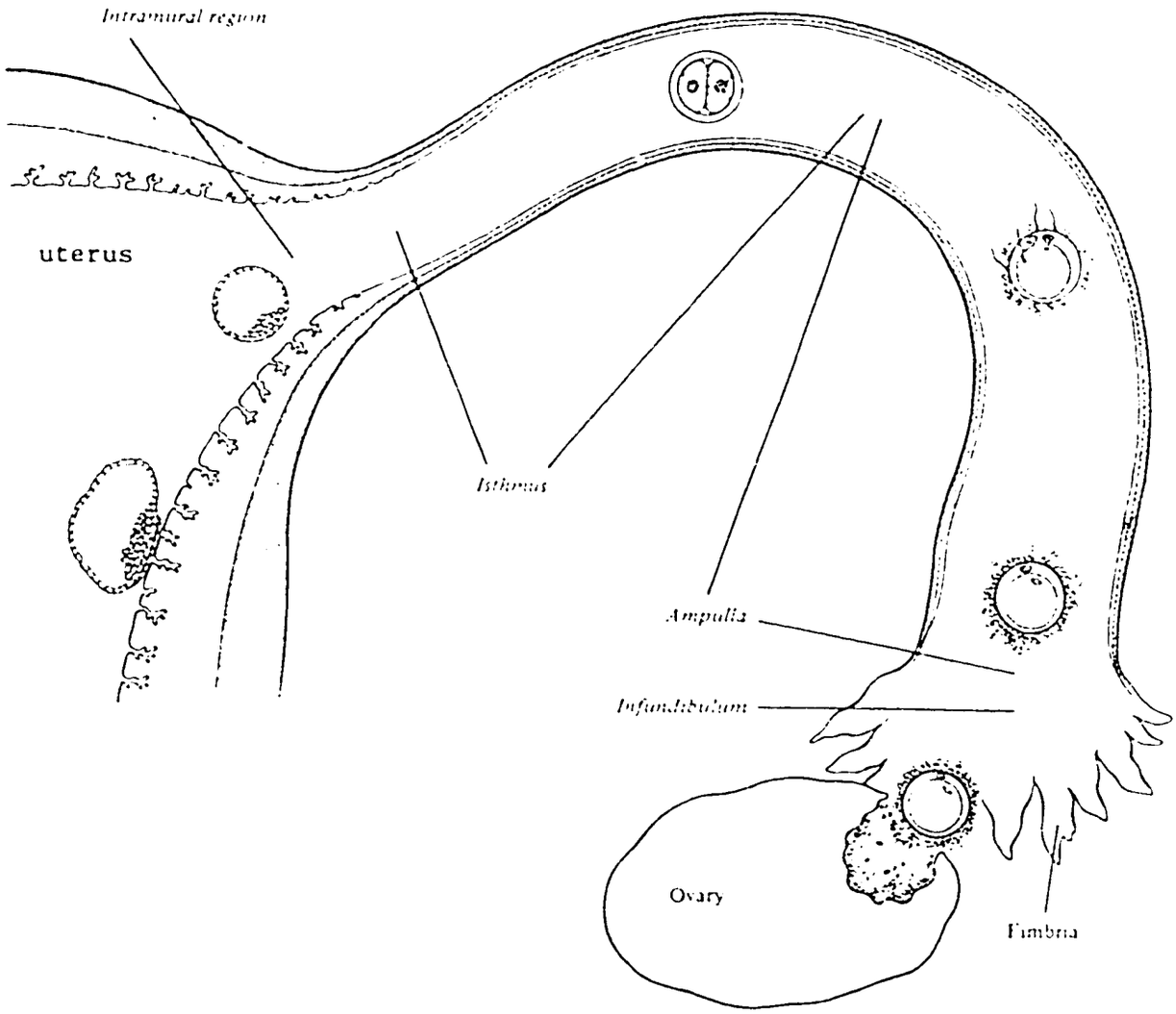
7.1 Introduction

The female reproductive tract in most mammals is comprised of paired oviducts and the uterus and it is in these organs that fertilization and subsequent embryo/fetal development occur until parturition. The oviducts and the uterus are derived from the primitive Müllerian ducts, from which the cranial regions of the bilateral embryonic ducts differentiate to form the oviducts while the caudal segments form the uterine horns in rodents. The ovulated oocyte, once liberated from the ovary, is swept into the oviduct by the rapid movements of the cilia of the *infundibulum*, the region that forms the mouth of the oviduct. In addition to rapid beating of the cilia, the rodent ovary is surrounded by a fluid filled covering called the *bursa* which is continuous with the fimbriae of the infundibulum and enables the transfer of the oocytes into the oviduct. The *ampulla* region of the oviduct, the segment which is adjacent to the infundibulum, is the site for fertilization. Embryo development continues in the lumen of the oviduct in the isthmus segment as ciliary movement transports it through the uterotubal junction and into the uterus for subsequent implantation (Fig 7.1). The rodent preimplantation embryo remains in the oviduct for 95-100 hr prior to entry into the uterus. At this point the embryo has differentiated into a morula. Once implanted in the uterus, the development of the embryo continues until parturition. Successful implantation and sustained pregnancy requires the timely development of preimplantation embryos.

Figure 7.1

Embryo development in the reproductive tract

Illustration of preimplantation embryo development in relation to its location in the oviduct and part of the uterus


Intramural region
uterus
Isthmus
Ampulla
Infundibulum
Ovary
Fimbria

Two distinct features of preimplantation embryo development are the activation and cleavage of the fertilized egg and differentiation of the embryo to the blastocyst stage. During preimplantation development, the mitotic cell cycle of the fertilized egg is activated and 3 cell divisions occur over 95-100 hr (in rodents) yielding an 8-cell embryo, and each cell is capable of differentiating into any cell of the body. At this point, compaction of the 8-cell embryo occurs where gap junctions form between cells, and each cell can no longer be separated from each other. Compaction allows for the development of a fluid filled cavity and is essential for the differentiation of the embryo to a blastocyst.

Mouse embryos can undergo preimplantation development in chemically defined media, indicating that the early stages of embryo development are, at least in part, under autocrine control (reviewed in Kane et al., 1997). However, this observation has been restricted to certain mouse strains and even in these, embryo development *in vitro* occurs at a growth rate that is slower than *in vivo* (Bowman and McLaren, 1970). Furthermore, when *in vitro* developed preimplantation embryos are transferred to a pseudopregnant recipient, there is a significant loss of embryonic viability due to a decreased ability to resume normal development despite the apparent preservation of morphological features during the culture period (Papaioannou and Ebert, 1986).

Embryo development is greatly enhanced when developing embryos are cocultured with monolayers of feeder cells and the best results are obtained using oviductal or uterine cells (reviewed in Pampfer et al., 1991), suggesting that cells of the maternal reproductive tract synthesize growth factors that enhance embryonic development. The growth factors detected in the reproductive tract include colony stimulating factor-1 (CSF-1) (Pampfer et

al., 1991). EGF (Huet-Hudson et al., 1990) , TGF-α and insulin like growth factors (Paria and Dey, 1990). Both CSF-1 receptor (c-fms) (Arceci et al., 1992) and EGF receptor (Paria and Dey, 1990) mRNA are produced in mouse embryos. Furthermore, evidence suggests that EGF produced in the apical border of the epithelium of the reproductive tract may enhance morula to blastocyst differentiation, zona pellucida shedding and blastocyst activation in a paracrine manner (Paria and Dey, 1990 and reviewed in Anderson, 1993).

Kit receptor mRNA is abundantly expressed in ovulated oocytes; however, in early 2-cell embryos Kit mRNA is no longer undetectable. Kit mRNA expression reappears in late 2-cell embryos and is present at all subsequent stages of preimplantation development (4-cell, 6-8 cell, morula, and early, expanded and hatched blastocysts). Using immunofluorescent and immunocytochemical techniques, Kit receptor protein was demonstrated in embryos up to the 8-cell stage (Manova et al., 1990; Horie et al., 1991). The function of Kit receptor in preimplantation embryos remains to be elucidated.

The expression and hormonal regulation of KL has been examined in the rat ovary in previous chapters. Kit receptors continue to be expressed in ovulated oocytes and to a lesser extent in the developing preimplantation embryo (Manova et al., 1990). Since no KL is produced in the rodent embryo (Arceci et al., 1992), the expression of KL in the rodent reproductive tract was examined and the effect of exogenous KL on preimplantation embryo development or timing of cleavage *in vitro* was determined.

7.2 Materials and Methods

7.2.1 Animal treatment and determination of KL expression

Prepubertal rats were injected with 7.5 IU PMSG and then 40 hr later with 7.5 IU hCG. Animals were sacrificed 12, 24, and 40 hr after PMSG injection and 3, 6, 12, 24 and 48 hr after hCG injection. The reproductive tracts were dissected, and oviducts and bisected uteri were immediately submerged in LiCl/urea for RNA extraction and subsequent northern blot analysis (section 2.2.4). In some cases organs were placed into 4% paraformaldehyde to prepare tissue for *in situ* hybridization as described in section 2.2.3.

7.2.2 Embryo development

Female (C57BL/6 × CBA/J) F1 mice were treated with 5 IU PMSG, followed by 5IU hCG 40 hr later and paired with male mice. Zygotes were collected from ampullae of oviducts of mated animals 24 hr after hCG injection, and expanded cumulus cells were dispersed with 1 mg/ml hyaluronidase (Sigma) in order to obtain cumulus cell-free embryos. Isolated embryos were washed 3 times in media and transferred as zygotes to 50 µl drops of CZ-B medium supplemented with 1 mM L-glutamine (Chatot et al., 1986). Embryo development was monitored every 4 hr for 5 days to determine timing of cleavage and stage of development. Cleavage rate was calculated based on results from only those embryos that had developed. Experiments were done 4 times.

7.3 Results

7.3.1 Expression of KL in the rat oviduct

KL transcripts of 6.5 kb were seen in oviduct samples and the levels of KL mRNA remained relatively constant with PMSG treatment (Fig 7.2). At 12 hr after hCG injection there was a 2.0 ± 0.1 fold increase in KL mRNA levels compared to levels in oviducts of untreated (control) rats ($p<0.05$). KL mRNA levels returned to those seen in control oviducts within 24 hr of hCG stimulation.

To localize KL mRNA in the rat oviduct, 6 μm thick sections were sliced and exposed to digoxigenin-labelled oligonucleotide probes for KL. A cross section of the isthmus portion of the oviduct from rats before (a,b) and 12 hr after hCG treatment (c,d) are shown in Fig. 7.3. In untreated animals, KL mRNA expression was restricted to the epithelial lining of the apical regions of the mucosal folds that extend into the lumen of the oviduct. Twelve hours after hCG treatment, KL expression was seen throughout the mucosa which extends into the lumen of the oviduct (Fig. 7.3c,d). To quantitate the differences in KL expression seen before and after hormone treatment, colour densitometry was done using a confocal microscope so that blue, green yellow and red indicate staining from lowest to highest intensity, respectively (Fig. 7.3 b,d). There was an increase in KL mRNA expression 12 hr after hCG treatment based on the abundance of red and yellow colour seen in the epithelial lining and in the mucosa (Fig 7.3d). In control experiments, in which probe was excluded from the hybridization mixture, there was no detectable staining (data not shown).

Figure 7.2

Northern blot analysis of KL expression in rat oviducts.

Total RNA of oviducts from untreated (control) and hormone-primed (PMSG/hCG) rats probed with a ^{32}P-labelled 2.06 kb KL cDNA probe (A). NIH 3T3 and *Sl*^{d} cells served as positive and negative controls, respectively. The blot was then re-probed with ^{32}P-labelled α-tubulin cDNAs (B). To quantitate the effect of hormone treatment, the intensity of the bands on the autoradiographic film was estimated using densitometry, and the ratio of KL/α-tubulin values were standardized to control levels which were arbitrarily set to 1.0. A histogram illustrating the differences in KL/α-tubulin values where each bar represents the mean ± S.E.M. of 3 experiments is shown in C. Bars indicated with different letters are significantly different and bars indicated by * are different from the 40 hr PMSG group ($p<0.05$).

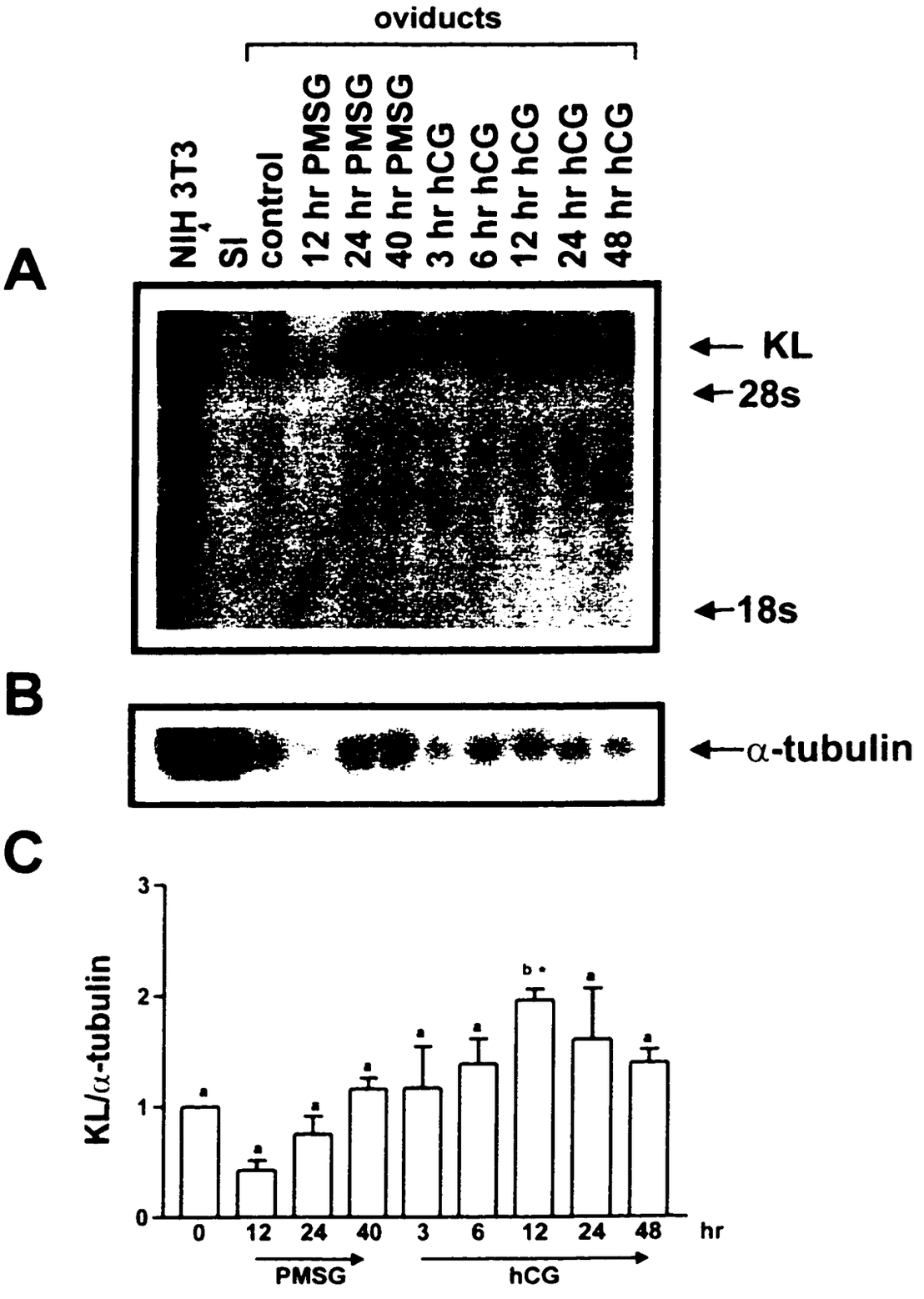
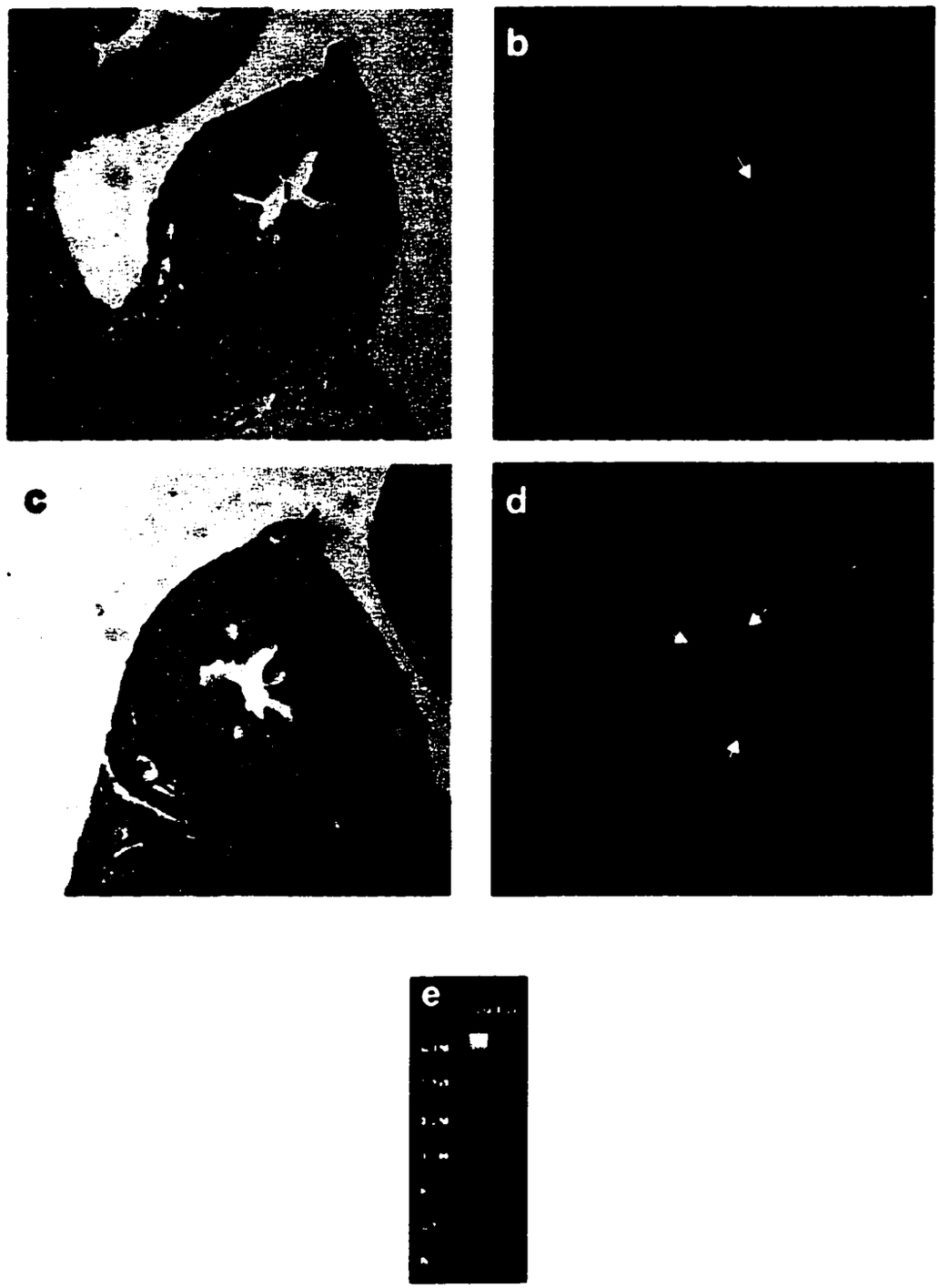

oviducts
NIH 3T3
SI
control
12 hr PMSG
24 hr PMSG
40 hr PMSG
3 hr hCG
6 hr hCG
12 hr hCG
24 hr hCG
48 hr hCG
A
KL
28s
18s
B
α-tubulin
C
KL/α-tubulin
0
1
2
3
0
12
24
40
3
6
12
24
48
hr
PMSG
hCG

Figure 7.3

Localization of KL mRNA in the oviduct by *in situ* hybridization.

Photographs are bright-field (a,c) and confocal (b,d) images of 6 μm sections through whole oviducts from 28-day-old animals from untreated (a,b) or 12 hr hCG-primed (c,d) oviducts hybridized with a KL probe. Colour densitometry was done so that blue, green, yellow and red indicate staining from lowest to highest intensity (e). Hybridizations done excluding labelled-probes showed no KL staining. Photographs are of typical results seen with three independent samples and were done along side of experiment using ovary sections (Fig. 2.3). l=lumen; m=mucosa; arrows represent positive Kit staining Magnification = 26X


b
c
d
e

7.3.2 Expression of KL in rat uterus

Rat uteri were isolated 12, 24 and 40 hr after PSMG and 3, 6, 12, 24 and 48 hr after hCG treatment. Uteri were bisected into anterior and posterior halves, and total RNA was isolated using LiCl precipitation for northern blot analysis. A single KL mRNA transcript of 6.5 kb was evident in all samples tested (Fig 7.4A). There was no significant effect of hormone (PMSG/hCG) treatment in KL mRNA levels in either the anterior or posterior regions of uteri samples, although there was trend towards decreased KL mRNA levels after PMSG and hCG treatment in both sets of samples (Fig 7.4C).

7.3.3 Effect of KL on mouse embryo development

To determine if KL is able to stimulate preimplantation embryo development *in vitro*, embryos were cultured singly or in groups of 10 embryos under conditions described previously to yield suboptimal embryo development (Paria and Dey, 1990). Two parameters were assessed: first, the effect of KL on the rate of cleavage of cells was determined, and second the proportion of embryos progressing to the blastocyst stage. No significant difference in the rate of cleavage of embryos cultured alone or in the presence of 10 or 200 ng/ml KL were seen (Table 7.1). However, in the presence of KL there was a significant increase in the number of embryos that developed to 8-cell embryos when cultured individually (Fig 7.5 I) or the number of embryos that differentiated from morulae to blastocyts when cultured in groups of 10 (Fig 7.5 II).

Figure 7.4

Northern blot analysis of KL expression in bisected rat uteri.

Total RNA of anterior and posterior portions of rat uteri from untreated (control) and hormone-primed (PMSG/hCG) rats probed with a ^{32}P-labelled 2.06 kb KL cDNA probe (A). NIH 3T3 and *Sl*^{d} cells served as positive and negative controls, respectively. The blot was then re-probed with ^{32}P-labelled α-tubulin cDNAs (B). To quantitate the effect of hormone treatment, the intensity of the bands on the autoradiographic film was estimated using densitometry, and the ratio of KL/α-tubulin values were standardized to control levels which were arbitrarily set to 1.0. A histogram illustrating the differences in KL/α-tubulin values where each bar represents the mean ± S.E.M. of 3 experiments is shown in C. No significant effect of hormone treatment was seen.

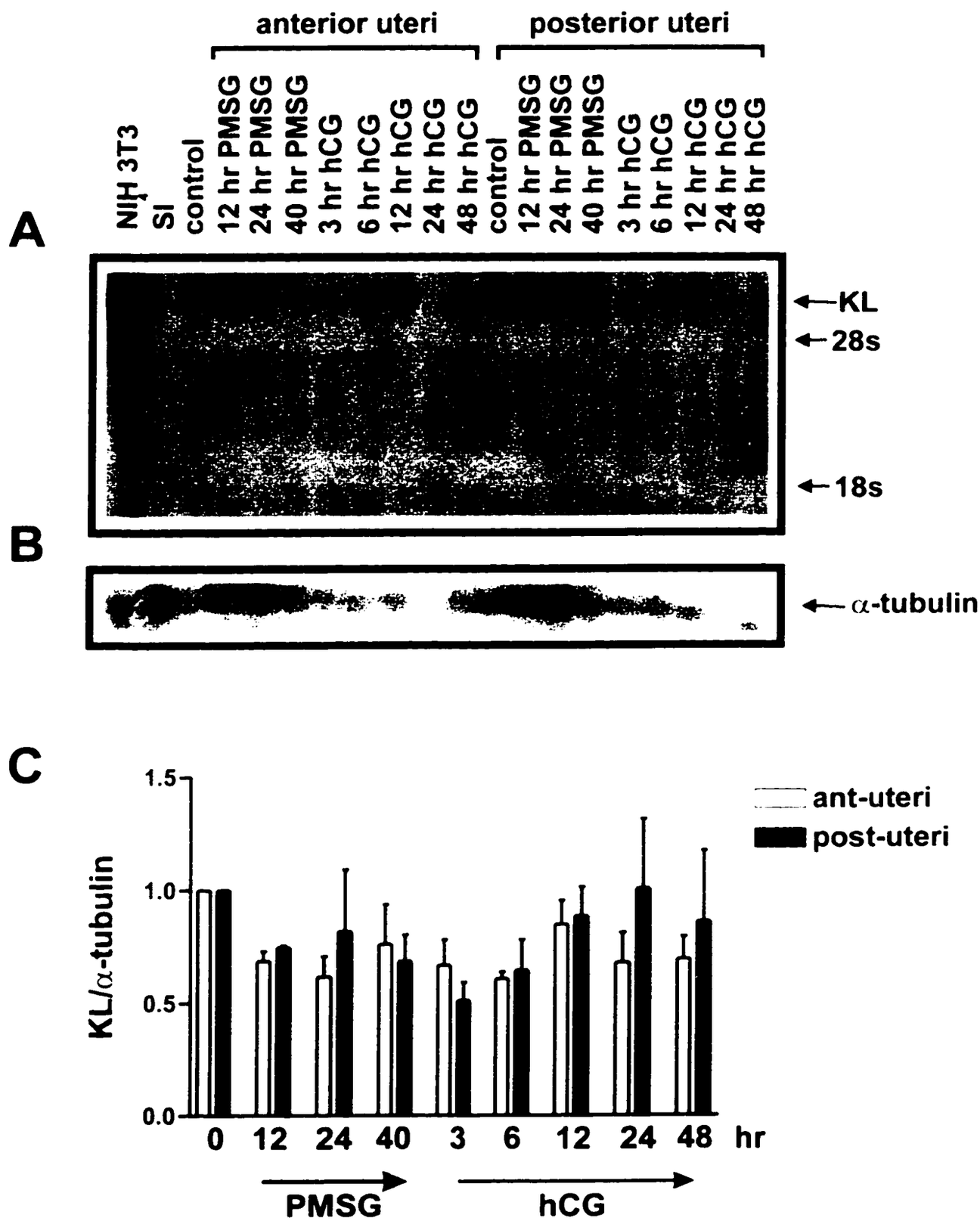

anterior uteri
posterior uteri
NIH 3T3
SI
control
12 hr PMSG
24 hr PMSG
40 hr PMSG
3 hr hCG
6 hr hCG
12 hr hCG
24 hr hCG
48 hr hCG
control
12 hr PMSG
24 hr PMSG
40 hr PMSG
3 hr hCG
6 hr hCG
12 hr hCG
24 hr hCG
48 hr hCG
A
KL
28s
18s
B
α-tubulin
C
KL/α-tubulin
1.5
1.0
0.5
0.0
ant-uteri
post-uteri
0
12
24
40
3
6
12
24
48
hr
PMSG
hCG

Table 7.1

Effect of KL on rate of embryo development.

Zygotes from female (C57BL/6 × CBA/J) F1 mated mice were cultured in the presence or absence of KL (10 or 200 ng/ml) in 50 µl drops of CZ-B embryo culture media. Embryos were assessed every 3 hr to monitor the time required for cleavage from 2- to 4-cells, from 4- to 8- cells, and differentiation from 8-cells to blastocysts. The cleavage times for 20-25 embryos, cultured individually in two independent experiments, were used to calculate average cleavage time.

Rate of Development of Preimplantation Embryos

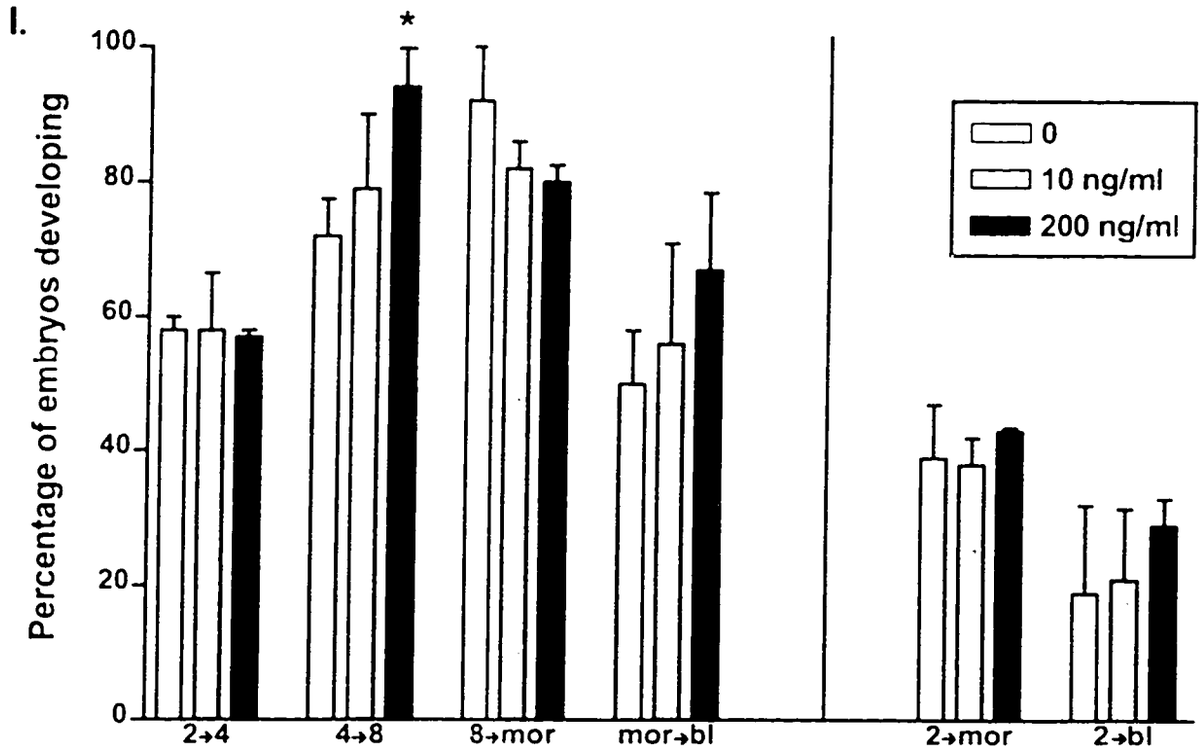
Concentration of KL (ng/ml)	2-cells to 4-cells		4-cells to 8-cells		8-cells to blastocysts	
	Mean Time	Range (h)	Mean Time	Range (h)	Mean Time	Range (h)
0	30 h	27-33	20 h	15-22	54 h	52-56
10	31 h	27-36	21 h	18-32	50 h	44-56
200	32 h	30-36	19 h	18-20	ND	ND

Figure 7.5

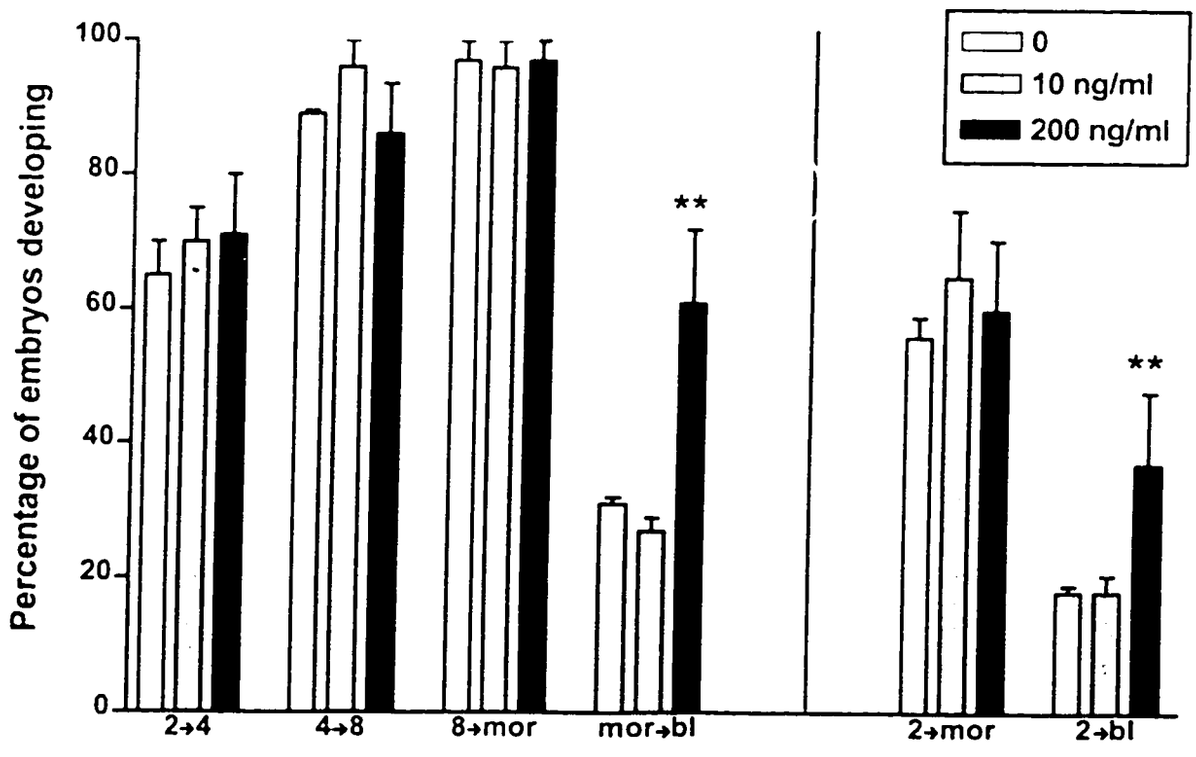
Effect of KL on mouse preimplantation embryo development

Zygotes from female (C57BL/6 × CBA/J) F1 mated mice were cultured individually (I) or in groups of 10 (II), in the presence or absence of KL (10 or 200 ng/ml) in 50 µl drops of CZ-B embryo culture media. Embryos were assessed every 3 hr to determine the number of embryos that develop from 2-cell to blastocyst. Each bar represents assessments of 31-32 (I) or 40-57 (II) embryos, and * or ** represent groups that are significantly different ($p<0.05$).

I.


Percentage of embryos developing
100
80
60
40
20
0
*
0
10 ng/ml
200 ng/ml
2→4
4→8
8→mor
mor→bl
2→mor
2→bl

II.


Percentage of embryos developing
100
80
60
40
20
0
**
**
0
10 ng/ml
200 ng/ml
2→4
4→8
8→mor
mor→bl
2→mor
2→bl

7.4 Discussion

KL mRNA was detected in the rat oviducts and uteri, and oviductal KL mRNA levels were increased with hCG treatment. In addition, preimplantation embryo development and survival was significantly enhanced in the presence of KL although there was no effect on the rate of cleavage of preimplantation embryos.

Oviductal and uterine tissues are responsive to changes in steroid hormones that are produced in a cyclic manner by the ovary in response to gonadotropin stimulation. The influence of steroid hormones on oviduct and uterine function is well documented and this process is essential to maintain a healthy environment to enable embryo development and subsequent implantation. KL mRNA expression was present in the maternal reproductive tract tissues and oviductal KL mRNA expression increased after hCG treatment. In addition, KL mRNA levels were localized to discrete regions of the epithelial lining of the oviductal mucosa. During the periovulatory period (12 hr after hCG), KL mRNA was seen throughout the mucosa and was most likely expressed in fibroblasts typically located in this region of mucosa. KL mRNA expression has been demonstrated in mouse (Arceci et al., 1992; Bedell et al., 1995) oviducts and cells from human Fallopian tubes (Barmat et al., 1997), and porcine uterine tissues (Zhang and Anthony, 1994), although hormonal regulation of KL mRNA transcription has not been previously demonstrated in any of these tissues.

Sun and coworkers (1997) recently demonstrated that bovine oviductal epithelial cells contain LH receptors. Although LH receptor expression has not been investigated in the rat oviduct, LH receptors may also be present in rat oviductal cells. It is most likely the stimulation of these receptors with hCG that caused an increase in oviductal KL mRNA.

When comparing the increase seen in granulosa cell KL mRNA expression before hCG stimulation (at 40 hr after PMSG treatment) with the effects seen after hCG stimulation, there was a 2-fold increase in KL expression 6 hr after hCG treatment (Fig 4.5). It may be that oviduct epithelial cells express considerably fewer LH receptors compared to granulosa-luteal cells and this could account for a slower increase in KL mRNA expression (6 hr in granulosa cells versus 12 hr in oviducts) although a similar degree of response was seen.

To minimize cooperative interactions among preimplantation embryos, embryos were cultured in groups of 1 or 10 per 50 µl of media resulting in suboptimal embryo development. Using this method researchers determined positive influences of EGF, TGF-β and TGF-α on preimplantation embryo development (Paria and Dey, 1990). Using a similar strategy, we demonstrated that the presence of exogenous KL promoted differentiation of the embryos to blastocysts and significantly stimulated 4- to 8- cell cleavage when compared to untreated embryos. The concentration of KL used in these experiments was within the doses used to demonstrate KL effects on mast cell differentiation and cell proliferation (see Table 1.3).

Based on the results presented in this chapter we propose the following hypothesis. KL is produced in the oviduct and acts in a paracrine fashion on fertilized eggs to promote embryo development. The LH surge, which causes the ovulation of oocytes from the ovary, also results in an increase in KL production in the mucosa of the oviduct, presumably in preparation for the arrival of the developing embryo. In human embryos, both Kit and KL mRNA are produced after the 6-cell stage and continue to be coexpressed in morulae and blastocysts (Sharkey et al., 1995). Since KL is produced by human oviductal cells (Barmat

et al., 1997), it may be that paracrine stimulation of the human embryo Kit receptors is not sufficient for optimal embryo development, and autocrine stimulation may also be necessary to attain critical levels of Kit/KL interactions. In addition, studies have demonstrated that c-*fms* receptor and its ligand, CSF-1, may also function via paracrine interactions to improve blastocyst development. Since the pattern of expression between Kit/KL and c-*fms*/CSF-1 is identical in mouse developing embryos, there is a strong possibility that KL and CSF-1 produced in the reproductive tract may act in a redundant manner to promote the differentiation to blastocyst. To lend support to this hypothesis, *in vitro* studies demonstrated that CSF-1/c-*fms* may rescue the proliferation defect of mast cells derived from Kit^{W}/Kit^{W} mutant mice (Dubreuil et al., 1991).

The identification of $Mgf^{Sl\text{-}12H}$ mutant mice, which display a preimplantation homozygous lethality phenotype, provides support for the hypothesis that KL is an important regulator of early embryo development. Examination of 3.5 day embryos isolated from maternal oviducts of $Mgf^{Sl\text{-}12}/+ \times Mgf^{Sl\text{-}12}/+$ breedings revealed that there were significantly fewer embryos that had differentiated to blastocysts and greater numbers of embryos that had degenerated, indicating a delay in embryo development and a decrease in embryo preimplantation survival (Cattanach et al., 1988). Although the maternal oviducts continue to produce KL, paracrine stimulation of embryonic Kit receptors by oviductal KL may not be sufficient for optimal embryo development. The reason for failed or delayed embryo development with this mutation is not completely clear. The homozygous mutants had impaired development, implying a role for KL intrinsic to the development of the embryos. Although Arceci and coworkers (1992) have shown that KL is not produced in mouse

preimplantation embryos, the strain of mice used for KL analysis was different than the strain in which the *Mgf*$^{Sl\text{-}12}$ mutation arose. There is a possibility that the preimplantation embryos produce both Kit and KL in the strain of mice carrying the mutation, similar to what is seen in human embryos (Sharkey et al., 1995). Therefore, one interpretation of the observations reported (Cattanach et al., 1988) is that heterozygous mutant and wild type embryos of this strain produce both Kit and KL, and there is autocrine stimulation of embryonic Kit receptors; however, homozygous mutant embryos do not produce KL and produce only Kit , and autocrine stimulation of embryonic Kit receptor is not possible.

In summary, KL mRNA is produced in the rat uterus and in the mucosa of the oviduct, and oviductal KL mRNA expression increases around the time of ovulation. In addition, we have demonstrated an increase in embryo survival and in the proportion of embryos differentiating to blastocysts in the presence of soluble KL under specific culture conditions. Thus, KL from the oviduct may interact with embryonic Kit receptors to promote embryo development.

CHAPTER 8 - CONCLUDING REMARKS

Kit receptor expression was identified in the oocytes of antral follicles and KL expression was seen in granulosa cells in growing and antral follicles. In antral follicles, KL expression was greatest in cumulus granulosa cells and only those mural granulosa cells closest to the oocyte and antral cavity. Low KL expression was seen in the mural granulosa cells closest to the basement membrane. This pattern of KL expression in antral follicles suggested a role for KL in oocyte function.

There was an inhibition of meiotic maturation when oocytes from antral follicles were cultured in the presence of KL compared to untreated oocytes. In addition, under conditions where 50 % of oocytes were maintained in meiotic arrest for a 24 hr period of time, ablation of oocytic Kit receptor expression resulted in oocyte maturation in a greater proportion of oocytes compared to oocytes in which Kit receptor expression was not disturbed. Thus spontaneous meiotic maturation was inhibited by KL/Kit receptor interactions and KL stimulation of Kit receptors may be one mechanism by which oocytes are maintained in meiotic arrest within the follicle.

LH is the key stimulator of oocyte maturation within the follicle. *In vivo* stimulation with LH resulted in a loss of KL expression in cumulus granulosa cells and a shift in the production of membrane-bound KL to more soluble KL in mural granulosa cells. Thus, LH-stimulated oocyte maturation may be partly mediated by the removal of KL in the cumulus granulosa cells and the production of a potentially less active form of KL by mural granulosa cells. These experiments support the hypothesis that *in vivo* oocyte maturation occurs as a result of the withdrawal of meiosis-inhibiting substances in the follicle.

The expression of KL in granulosa cells was upregulated at the transcript level by treatment with FSH, LH, or both *in vivo* and *in vitro*, as well as with membrane-permeable analogues of cyclic adenosine monophosphate (cAMP), whereas KL mRNA expression decreased with stimulation with steroid hormone stimulation.

KL but not Kit mRNA expression was seen in the ovarian surface epithelium. KL expression in ovarian surface epithelium derived-cells was influenced by both dibutyryl cAMP and transforming growth factor-β stimulation. These studies would indicate that the ovarian surface epithelium is a site of KL production and that this expression can be regulated by ovarian factors.

Preimplantation embryos have been previously shown to express Kit receptors. In this study KL expression was demonstrated in oviducts and uteri and gonadotropin-stimulated increases in oviductal KL mRNA expression were seen. Furthermore, preimplantation embryo survival and development were enhanced with KL suggesting that KL has stimulatory effects on the mitotic cell cycle of the embryo.

Based on our results, it is clear that Kit and KL are important in the reproductive system. The ovary and the reproductive tract is made up of a variety of cell types and Kit and KL expression alternates between adjacent cell types. This complementary expression pattern further reinforces the role of Kit and KL in mediating paracrine communication between cells within the reproductive system.

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1995	*Endocrinology and Reproductive Physiology* (PHS 2300), 4 hr tutorial
1992-94	Laboratory Demonstrations for Undergraduate Medical Students
1994-97	Let's Talk Science (Outreach Program)

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PUBLICATIONS

Research Articles:

Rubina S. Ismail, Yuji Okawara, James N. Fryer and Barbara C. Vanderhyden. *Hormonal regulation of the ligand for c-kit in the ovary and its effects on spontaneous oocyte meiotic maturation.* (Molecular Reproduction and Development 43: 458-469, 1996)

Rubina S. Ismail, Manon Dubé & Barbara C. Vanderhyden. *Hormonally-regulated alternative splicing of Kit ligand may regulate Kit-induced inhibition of meiosis in rat oocytes.* (Developmental Biology 184: 333-342, 1997)

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Rubina S. Ismail and Barbara C. Vanderhyden. *Expression of Kit ligand in the rat reproductive tract and its effects on embryo development* (in preparation)

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Abstracts:

Rubina S. Ismail, Elizabeth A. Macdonald and Barbara C. Vanderhyden. (1997) *Characterization of rat ovarian surface epithelial cells: steroidogenic capabilities and regulation of c-kit expression.* Biol Reprod. 56(1): 128.

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IMAGE EVALUATION
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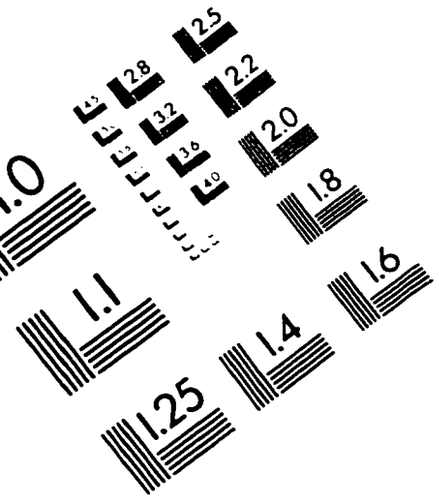

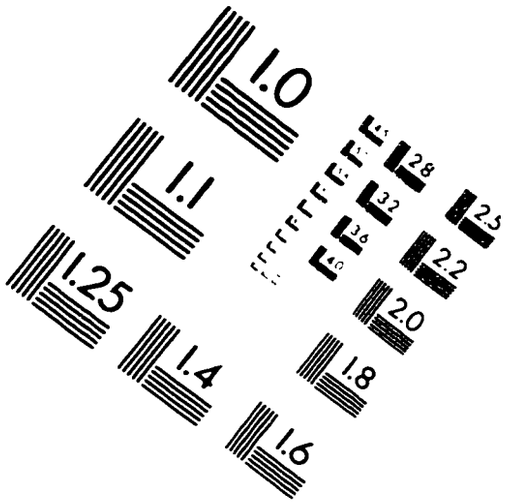

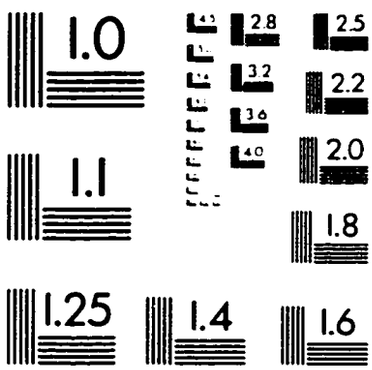

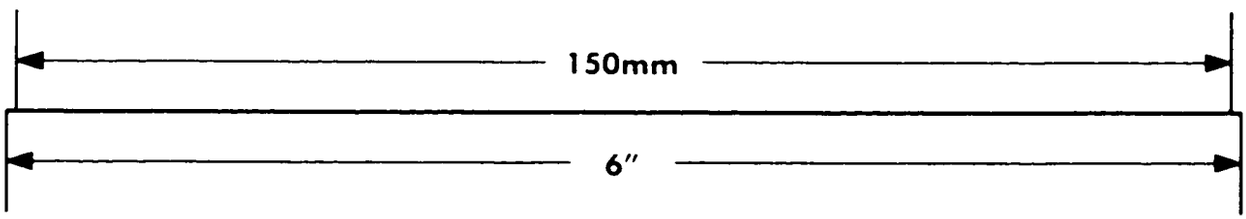

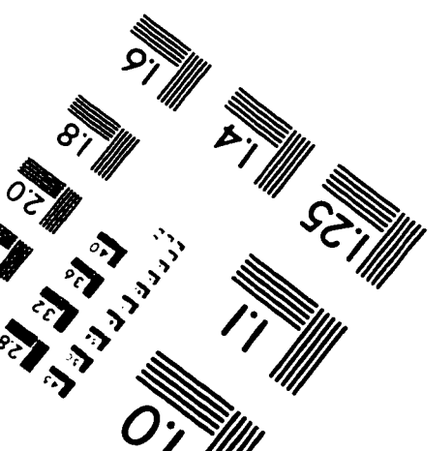

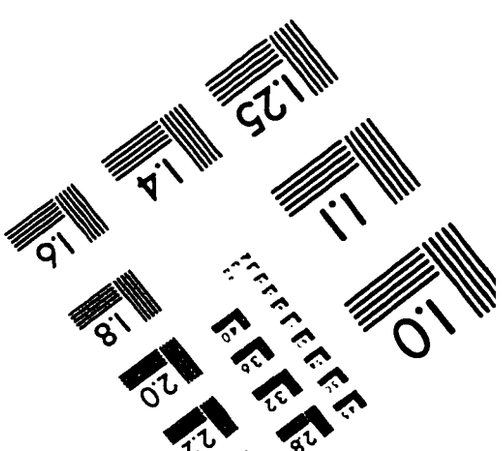

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