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Balwant Singh

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EPIDERMAL GROWTH FACTOR REGULATION OF PORCINE OOCYTE MATURATION AND CUMULUS CELL EXPANSION *IN VITRO*

by

Balwant Singh

Department of Physiology

Submitted in partial fulfilment

of the requirements for the degree of

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ABSTRACT

The overall objective of this study was to investigate the role *in vitro* of epidermal growth factor (EGF) in the regulation of both nuclear and cytoplasmic maturation of the porcine oocyte, and in the expansion of cumulus cells. Investigations were extended to study EGF-interactions with gonadotropins and ovarian steroids which regulate these preovulatory processes, signal transduction mechanisms of EGF-induced oocyte maturation and cumulus expansion, and local EGF production in the ovarian follicle. Additional studies included the elucidation of the role of the porcine oocyte in EGF-stimulated cumulus expansion, and testing of the local follicular production of transforming growth factor-alpha (TGF-α), an additional ligand for EGF receptor-(EGF-R).

Studies aimed at the induction of oocyte maturation and cumulus expansion revealed that EGF is a potent stimulant of the resumption of meiosis in the oocyte and that the addition of FSH, but not LH, to EGF further enhances its effect on germinal vesicle breakdown (GVBD). Both estradiol-17β (E_2) and androstenedione (A_4) inhibited FSH-stimulated GVBD, but had no effect on EGF-stimulated GVBD, suggesting that EGF may be acting through a different mechanism than that of FSH. EGF stimulated cumulus expansion in about 50% of oocyte-cumulus cell complexes (OCC), however FSH was much more effective and induced expansion in $\sim$ 90% of the complexes.

The removal of the oocyte did not impair the ability of porcine cumulus cells to expand in response to EGF, indicating that EGF-induced expansion of porcine cumuli oophori is not dependent on the oocyte. However, the denuded porcine oocytes enabled mouse cumulus cells, which do not undergo expansion in the absence of the oocyte, to expand in response to FSH, indicating that the porcine oocyte is capable of secreting the putative cumulus expansion-enabling factor required for mouse cumulus cell expansion and that this factor is not species specific.

Reverse transcription-polymerase chain reaction (RT-PCR) and immunolocalization studies revealed local follicular production of EGF and the presence of its receptor in the ovarian follicle. From the relative abundance of the mRNAs and immunoreactive

peptides, it appeared that, within the follicle, the oocyte is the primary, although not necessarily the exclusive source of EGF, and the somatic cells that of EGF-R, suggesting that an EGF paracrine system may be in place in the porcine ovarian follicle.

Signal transduction studies indicated that EGF induces cumulus cell expansion by activating its receptor tyrosine kinase (TK) and cAMP-dependent protein kinase A (PKA), and the oocyte maturation by cAMP-PKA phosphorylation, suggesting that the two preovulatory events are not causally related.

Both EGF and FSH influenced cytoplasmic maturation of porcine oocytes as revealed by the success of fertilization and by the changes in the pattern of protein synthesis. Fertilization studies indicated that the effect of EGF is beneficial when added alone to the oocyte maturation media, whereas FSH appeared to influence cytoplasmic maturation adversely. Addition of EGF alone decreased the proportion of polyspermic oocytes and increased the percentage of monospermic oocytes forming male pronuclei, whereas FSH abolished these effects of EGF and increased the percentage of polyspermic oocytes forming more than two pronuclei. Both EGF and FSH altered the synthesis of certain proteins in the oocyte and cumulus cells after different culture periods.

RT-PCR and immunocytochemistry indicated the presence of TGF-α in the cumulus and granulosa cells. Since EGF was also localized in porcine cumulus and granulosa cells, the presence of two ligands which trigger a similar signal transduction cascade after binding to the EGF-R may indicate ligand redundancy.

In conclusion, evidence is provided that EGF of follicular origin may play a vital role in bringing a complete physiological maturation to the porcine oocyte, including expansion of the surrounding cumulus cells.

To my mother

Kartar Kaur Jhajj

and

To my Daughters

Amrit and Jasleen

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LIST OF ABBREVIATIONS

AGP	Aminoglutethimide phosphate
ANOVA	Analysis of variance
A_4	Androstenedione
BSA	Bovine serum albumin
cAMP	Cyclic adenosine 5'-monophosphate
cDNA	Complementary deoxyribonucleic acid
CSF	Cytostatic factor
DAG	Diacylglycerol
dbcAMP	Dibutyryl cyclic adenosine monophosphate
DES	Diethylstilboestrol
DPBS	Dulbecco's phosphate-buffered saline
EGF	Epidermal growth factor
EGF-R	Epidermal growth factor receptor
E_2	Estradiol-17β
FSH	Follicle-stimulating hormone
GAGs	Glycosaminoglycans
GnRH	Gonadotropin-releasing hormone
GV	Germinal vesicle
GVBD	Germinal vesicle breakdown
GVI	Germinal vesicle intact
HBSS	Hanks balanced salt solution

hCG	Human chorionic gonadotropin
IBMX	Isobutylmethylxanthine
IGFs	Insulin-like growth factors
IGFBPs	Insulin-like growth factor binding proteins
IVF	*In vitro* fertilization
IVM	*In vitro* maturation
Iαl	Inter-α-trypsin inhibitor
kDa	Kilo daltons
LH	Luteinizing hormone
M	Molar
MAP	Mitogen activated protein
M-CCC	Mouse cumulus cell clumps
MEM	Minimal essential medium
MIS	Mullerian inhibiting substance
mm	Millimeter
mM	Millimolar
MPF	Maturation-promoting factor
MPGF	Male pronucleus growth factor
mRNA	Messenger ribonucleic acid
ng	Nanogram
OCC	Oocyte-cumulus cell complexes
OMI	Oocyte maturation inhibiting factor

PAGE	Polyacrylamide gel electrophoresis
P-CCC	Porcine cumulus cell clumps
PDE	Phosphodiesterase
PDGF	Platelet-derived growth factor
P-DO	Porcine denuded oocytes
PGE_2	Prostaglandin E_2
PKA	Protein kinase A
PKC	Protein kinase C
PMSG	Pregnant mare serum gonadotropin
P-OOX	Porcine oocytectomized oocyte-cumulus cell complexes
RIA	Radioimmunoassay
RT-PCR	Reverse transcription-polymerase chain reaction
SDS	Sodium-dodecyl sulphate
SEM	Standard error of the mean
TGF-β	Transforming growth factor-beta
TGF-α	Transforming growth factor-alpha
TK	Tyrosine kinase
μg	Microgram
μl	Microliter
μm	Micrometer
~	Approximately
<	Less than

CHAPTER 1 INTRODUCTION

The ability of any mammalian species to reproduce itself is dependent upon the production of germ cells containing sufficient genetic information for the subsequent development of each new individual. The contribution of the oocyte in this regard is exemplified by the statement "Omne vivum ex ovo" ("All living things come from eggs"), which is attributed to William Harvey. Overwhelming experimental evidence has proven today that the zygote inherits from the oocyte an extensive reserve of macromolecules and organelles that support, to varying degrees, the nutritional, synthetic, energetic, and regulatory requirements of the early embryo.

The mammalian oocyte acquires the capacity for fertilization and subsequent embryonic development after a prolonged period of growth and differentiation within the ovarian follicle. Oogenesis commences in early embryonic life and culminates in a complex sequence of maturational changes prior to ovulation in the sexually mature female. Around the time of birth, the ovary possesses a finite stock of nongrowing oocytes that have progressed up to the diplotene stage of meiotic prophase. At this stage meiosis is arrested, chromosomes decondense, the nuclear membrane reappears and a prominent nucleus, called the germinal vesicle, is formed. The oocytes remain arrested at this stage of meiosis until puberty when in each oestrus or menstrual cycle a preovulatory surge of gonadotropins triggers the resumption of meiosis in a selected oocyte or group of oocytes (depending upon species: monotocous or polytocous). The mechanism by which selection of oocyte(s) is made in each cycle for induction of meiosis and later ovulation at metaphase II (MII) stage, is unknown. The resumption of meiosis is morphologically identified by breakdown of the germinal vesicle (GVBD) and is accompanied by the expansion of the compact layers of cumulus cells surrounding the oocyte, due to deposition of mucoelastic material in the extracellular matrix. At ovulation, the oocyte enclosed by expanded cumulus is released from the follicle into the fallopian tube where the mucified matrix and other cumulus cell secretory products appear to provide an appropriate environment for sperm penetration and fertilization (Gwatkin *et al.*, 1972; Schuetz & Dubin, 1981; De Felici & Siracusa, 1982; Meizel,

1985).

The germinal vesicle-stage oocytes, once liberated from their follicles and maintained under suitable conditions in hormone-free media *in vitro*, resume meiosis spontaneously and yield morphologically normal secondary oocytes (Pincus & Enzmann, 1935). Cumulus expansion can also be induced in isolated oocyte-cumulus cell complexes (OCC) *in vitro* by supplementing the maturation media with hormones such as gonadotropins (Eppig, 1979a; Hillensjo & Channing, 1980) or prostaglandin E_2 (Eppig, 1981a). Although spontaneous maturation *in vitro* can yield visibly normal metaphase II oocytes with expanded cumuli oophori, the functional normality of such oocytes is not assured. Such oocytes often fail to transform sperm into male pronuclei and their ability to support subsequent embryonic development is very low compared to oocytes matured *in vivo* (Cross & Brinster, 1970; Niwa *et al.*, 1976; Moor & Trounson, 1977; Thibault, 1977; Shalgi *et al.*, 1979; Leibfried & Bavister, 1983; Nagai *et al.*, 1984; Leibfried-Rutledge *et al.*, 1987). These deficiencies are attributed to inadequate cytoplasmic maturation of oocytes undergoing spontaneous maturation *in vitro*, even though apparently normal nuclear maturation is observed (Thibault, 1977; Leibfried-Rutledge *et al.*, 1987).

The pig is an excellent model for the production of embryos *in vitro* both for research and commercial purposes. The large size of the ovaries and the fact that multiple ovulations are physiological in this species make it feasible to obtain relatively large numbers of OCC. Suitable tissue is readily available from abattoirs: gilts (nulliparous females) are normally slaughtered shortly before their first ov ation, providing a relatively synchronized population of follicles without the complication of variations in stages of the oestrus cycle. However, despite continuous efforts, *in vitro* maturation (IVM) and fertilization (IVF) in the pig has met with a very limited success. Polyspermy is a major problem encountered with pig oocytes matured and fertilized *in vitro*, and as many as 80% of the oocytes have been reported to be polyspermic in some studies (Cran and Cheng, 1986; Mattioli *et al.*, 1988b, 1989). Furthermore, only a very low proportion of these *in vitro* matured and fertilized oocytes is capable of transforming

sperm into the male pronucleus (Nagai *et al.*, 1984; Mattioli *et al.*, 1988b; Yoshida *et al.*, 1990; Nagai, 1994). On the contrary, *in vivo* matured porcine oocytes exhibit lower incidences of chromosomal anomalies and polyspermy, and higher rates of development following IVF than those matured *in vitro* (Yoshida *et al.*, 1990). This inadequate maturation of *in vitro* matured oocytes may result from deficiencies in the culture media used for IVM. Indeed reports using media with different compositions indicate that the selection of IVM media influences the outcome of porcine IVF (Wang *et al.*, 1992; Yoshida *et al.*, 1992a; Nagai, 1994).

In vivo oocyte maturation takes place in an intrafollicular milieu that is determined by the surrounding somatic cells of the follicle which, in turn, are under the regulatory influences of a variety of hormones, in particular the gonadotropins. It is perhaps not surprising, therefore, that the coculture of somatic follicular cells during IVM was found to increase fertilizability and subsequent developmental potential of oocytes following IVF (Staigmiller & Moor, 1984; Critser *et al.*, 1986; Lu *et al.*, 1987; Lutterbach *et al.*, 1987; Xu *et al.*, 1987; Mattioli *et al.*, 1988b, 1989). Thus it would appear that the somatic cells secrete undefined factor(s) into the culture medium which facilitate oocyte maturation and subsequent embryonic development.

The involvement of locally produced growth factors in ovarian physiology has been an active area of research in recent years, and studies from several laboratories suggest that such peptides may play a vital role in the regulation of oocyte and follicular development. Increasing evidence has appeared which suggests that the mammalian ovary is the site of production, secretion and action of several different growth factors. Selection of follicles into dominant or atretic pools further implies that the follicular selection may be mediated through locally produced factor(s). Although several growth factors have been implicated in ovarian physiology, epidermal growth factor (EGF) was the only one of ten known growth factors tested, which stimulated oocyte maturation and cumulus cell expansion in the mouse *in vitro* (Downs, 1989), suggesting a possible role for this peptide in preovulatory processes.

The present study was aimed to investigate the role of EGF in the regulation of

porcine oocyte maturation (both nuclear and cytoplasmic) and cumulus cell expansion, using an *in vitro* model system. Studies were extended to investigate interactions of EGF with gonadotropins and ovarian steroids that regulate these preovulatory processes, signal transduction pathways of EGF-induced GVBD and cumulus expansion, and local EGF production in the porcine ovarian follicle. Additional studies included the elucidation of the role of the porcine oocyte in EGF-stimulated cumulus cell expansion, and the local follicular production of transforming growth factor-α (TGF-α), an additional ligand for EGF receptor, extensively studied for its roles in follicular functions.

CHAPTER 2 REVIEW OF THE LITERATURE

2.1 INTRODUCTION

2.1.1 Follicular Morphology

Depending upon the mammalian species, the number of oocytes within each ovary reaches a zenith prior to or at about the time of birth, with no proliferation of germ cells occurring in the remainder of animal's life (Challoner, 1974; Mauleon & Mariana, 1977). Oogenesis, which refers to the process of growth and development of an oocyte, commences during embryogenesis and is arrested in the dictyate stage of meiotic prophase during fetal life. These oocytes in meiotic arrest are surrounded by a squamous layer of follicular cells and constitute the resting stockpile of nongrowing follicles which are progressively depleted during the reproductive life span. Folliculogenesis, which refers to the growth and development of an ovarian follicle, is also initiated before birth. But unlike oogenesis, it is a continuous process with groups of follicles undergoing cyclical growth and development throughout life in most mammals. The ovaries of prepubertal and adult females therefore contain a large pool of nonproliferative "primordial" follicles from which follicles are periodically recruited for growth and development culminating in either ovulation or atresia at some stage of their subsequent development (Peters *et al.*, 1975).

After birth, selected pools of oocytes and their surrounding follicular cells begin a coordinated growth and progress through a series of definable morphological changes (Peters, 1978). Commencement of oocyte growth is apparently regulated within the ovary and the number of oocytes entering the growth phase is a function of the size of the pool of nongrowing oocytes (Krarup *et al.*, 1969). In adult mice, the oocyte completes growth in 2-3 weeks, before the formation of the follicular antrum, and grows in size from a diameter of $\sim 10\ \mu m$ to $\sim 80\ \mu m$ (Wassarman & Albertini, 1994). During this time, the oocyte acquires a proteinaceous extracellular coat called the zona pellucida and becomes one of the largest cells of the body. For many years, the question of whether the zona material is synthesized by the oocyte or surrounding follicle cells was controversial (Chiquoine, 1960; Guraya, 1974). However, studies in the mouse

have

clearly indicated that in this species, and perhaps in many others, the zona is synthesized exclusively by the oocyte. This view is supported by the evidence that growing oocytes freed of zonae are able to resynthesize all the zona glycoproteins and that the messages for ZP2 and ZP3 glycoproteins, as revealed by *in situ* hybridization in the ovary using cDNA and oligonucleotide probes, were detected only in the oocyte, but not in follicular or in any other cell types (Bleil & Wassarman, 1980; Philpott *et al.*, 1987; Roller *et al.*, 1989; Liang *et al.*, 1990; Wassarman, 1990). The synthesis and secretion of zona glycoproteins begin as the oocyte starts to grow and continue until close to the time of ovulation, with a peak activity at about the time when the oocyte attains full size. Throughout its period of growth, the oocyte remains arrested in the dictyate stage of the first meiotic prophase.

Although the oocyte completes its growth relatively quickly, the follicle continues to grow. By a series of mitotic divisions, the monolaminar follicle is converted into a multilaminar preantral follicle. Thecal investment in the pig is a late event, not usually observed until the preantral follicle is 200-300 μm in diameter and has an average of 11 layers of granulosa cells (Morbeck *et al.*, 1992). Subsequently a fluid filled antrum appears and the follicle grows further resulting in a mature, Graafian follicle with a diameter of greater than 8 mm in the pig. In this species, the antrum is fully formed in follicles 0.4-0.8 mm in diameter (Crozet *et al.*, 1981). The follicular fluid is comprised of serum transudate, glycosaminoglycans and other substances secreted by the granulosa and theca cells. As the antrum expands, the oocyte takes up an eccentric position and is surrounded by a dense mass of granulosa cells called the cumulus oophorus. The cells of the cumulus oophorus (cumulus cells) and their enclosed oocyte, the oocyte-cumulus cell complex, is attached by a thin stalk of cells to the mural granulosa cells which line the periphery of the antral cavity. The innermost layer of cumulus cells becomes columnar in shape and constitutes the corona radiata, the cells of which form specialized gap junctions with the oolemma.

In prepubertal females (gilts), it is estimated that a primary follicle with one to

two layers of granulosa cells takes about 84 days to grow to a small antral follicle (~400 μm), and that an additional 14 day period is required for the follicle to reach a size of 3 mm (Morbeck *et al.*, 1992). To acquire a preovulatory size of about 8 mm, the follicle requires another 5 days. The domestic pig (*Sus scrofa*) has an oestrous cycle of 21±1 days, the first day of behavioral estrus being 'day 0'. Estrus lasts about 1-3 days, and ovulation occurs during the latter part of this period. The selection of follicles for ovulation occurs some time between days 14 and 16 of the cycle, as revealed by the studies involving exogenous gonadotropins (Hunter *et al.*, 1976), electrocauterization of follicles (Clark *et al.*, 1975; Dailey *et al.*, 1976), and unilateral ovariectomy (Clark *et al.*, 1982; Coleman *et al.*, 1984).

2.1.2 Evidence for Local Follicular Regulators

A Growth factors

Although the traditional roles of gonadotropins and ovarian steroids in follicular functions remain undisputed, recent studies from several laboratories suggest that locally produced growth factors may also play a vital role in the regulation of oocyte and follicular development. Selection of follicles into dominant or atretic pools further implies that the follicular selection may be mediated through locally produced factor(s). In addition, evidence suggests that the earliest stages of follicular growth are independent of circulating gonadotropins (Richards, 1980). Even after the formation of the antrum, when follicular development seems to depend on gonadotropins and ovarian steroids, it is difficult to assign all aspects of follicular growth to these hormones. This notion is supported by the results of several *in vitro* investigations, including the observations that granulosa and theca cells secrete substances that enhance proliferation of theca and granulosa cells respectively (McNatty *et al.*, 1980; Makris *et al.*, 1982, 1983). During the past few years increasing evidence has appeared which suggests that the mammalian ovary is the site of production, secretion and action of several different growth factors. Among these peptides, the following growth factors or families of growth factors have largely been considered for their putative role in follicular physiology.

Insulin-like growth factors (IGFs) and their binding proteins (IGFBPs)

This family of growth factors constitute homologous, low-molecular weight, single chain polypeptides named for their remarkable structural and functional similarity to insulin (Adashi *et al.*, 1985a). Based on physicochemical criteria, they are classified into two distinct groups, IGF-I and IGF-II. Among the various growth factors now under study for their potential roles in ovarian physiology, IGF-I perhaps is the most thoroughly evaluated.

A large body of evidence suggests that an IGF system complete with its ligands, receptors and binding proteins exist in the ovary (Adashi *et al.*, 1985a). The mRNA and/or immunoreactive peptide for IGF-I has been detected in the ovarian follicle of several species including the rat (Murphy *et al.*, 1987; Hansson *et al.*, 198°; Hernandez *et al.*, 1989), pig (Hammond, 1981; Hammond *et al.*, 1988), and human (Eden *et al.*, 1988; Geisthoevel *et al.*, 1989; Rabinovici *et al.*, 1990). In the rat, the ovary displayed the third highest levels of IGF-I mRNA of all adult tissues tested, second only to the uterus and the liver (Murphy *et al.*, 1987). In contrast, IGF-II gene expression in the murine ovary, although detectable, was much less pronouced (Hernandez *et al.*, 1990).

In terms of cellular source, IGF-I and IGF-II gene ex, ..ion is highly compartmentalized within the ovary, with two IGF species being expressed in two distinct somatic cell types. While granulosa cells appear to be the primary site of IGF-I production in the murine (Hansson *et al.*, 1988; Hernandez *et al.*, 1989; Oliver *et al.*, 1989) and porcine (Hammond *et al.*, 1985; Mondschein *et al.*, 1988) ovary, IGF-II mRNA was localized exclusively in theca-interstitial compartment of the murine ovary (Hernandez *et al.*, 1990). On the other hand, in the human ovary, granulosa cells seems to be a site for IGF-II rather than IGF-I production (Geisthovel *et al.*, 1989). Gonadotropins, steroids and other growth factors seem to regulate the ovarian production of IGF-I and IGF-II. Estradiol-17β and FSH stimulate the production of IGF-I by porcine granulosa cells *in vitro* (Hsu & Hammond, 1987). Treatment of hypophysectomized immature female rats with a potent oestrogen such as diethylstilboestrol (DES) implants increased the abundance of ovarian IGF-I mRNA by

2-fold, but resulted in a 2.6-fold decrease at the level of the liver (Hernandez *et al.*, 1989). In contrast, the ovarian expression of IGF-II mRNA was decreased in rats treated with DES *in vivo* (Hernandez *et al.*, 1990). Production of immunoreactive IGF-I by porcine granulosa cells from small antral follicles was stimulated by EGF (Mondschein & Hammond, 1988).

While the insulin receptor and type-I IGF receptor share a similar heterotetrameric structure and mediate effects via respective tyrosine kinases, the type-II IGF receptor or mannose-6-phosphate receptor is a monomer and less well understood. The insulin receptor has a high binding affinity for insulin and a lower affinity for IGF-I and IGF-II, whereas the IGF-I receptor has a high affinity for IGF-I and a lower affinity for IGF-II and insulin (reviewed by dePablo *et al.*, 1990). The type-II receptor binds IGF-II and IGF-I, but not insulin, on one site, and proteins containing mannose-6-phosphate residues at another site. It is now well established that high-affinity, low-capacity IGF-I binding sites are present in the granulosa cells of a number of species, including the rat (Adashi *et al.*, 1988a,b), pig (Baranao & Hammond, 1984; Veldhuis & Furlanetto, 1985), sheep (Monget *et al.*, 1989), and human (Gates *et al.*, 1987). Coupled to the promotion of ovarian androgen biosynthesis, functional IGF-I receptors also appear to be present in the murine (Hernandez, *et al.*, 1988; Cara *et al.*, 1990) and human (Poretsky *et al.*, 1985, 1990) theca-interstitial cells. Observations both *in vivo* and *in vitro* have clearly documented the ability of FSH to effect dose-dependent increments in granulosa cell IGF-I binding (reviewed by Adashi, 1992). The murine granulosa cells have also been shown to contain IGF-II receptors (Davoren *et al.*, 1986; Adashi *et al.*, 1990).

In addition to its effects on differentiation (Davoren *et al.*, 1985) and proliferation (May *et al.*, 1988; Xia *et al.*, 1994) of somatic follicular cells, IGF-I has also been shown to modulate steroidogenesis in these cells. Addition of IGF-I to cultured rat granulosa cells under serum free condition augmented the FSH-stimulated production of progesterone and 20-α-dihydroprogesterone (Adashi *et al.*, 1984, 1985b) and aromatase activity (Adashi *et al.*, 1985c). In porcine granulosa cells, IGF-I alone or synergistically with FSH increased progesterone production (Baranao & Hammond, 1984) and also

appeared to stimulate pregnenolone, progesterone and 20-α-dihydroprogesterone independently, without synergism with FSH (Veldhuis & Furlanetto, 1985).

In addition to ligands and receptors, the ovary is a source of IGF binding proteins (IGFBPs), which constitute a heterogenous group of at least six distinct proteins in the rat ranging in mass from 21.5 to 29.6 kDa (non-glycosylated mature proteins). These proteins are capable of binding IGFs, but not insulin, with affinities in the range of 10^{-10} to 10^{-9} M (reviewed by Adashi, 1992). Treatment of gilts with PMSG increases follicular fluid concentrations of IGFBP-3 and decreases those of IGFBP-2 (Samaras *et al.*, 1990). Since the profile of IGFBPs secretion by cultured porcine granulosa cells is very similar to that found in porcine follicular fluid (Mondschein *et al.*, 1991), it appears that IGFBPs present in follicular fluid are primarily of granulosa cell origin. In cultured pig granulosa cells, production of IGFBPs is stimulated by EGF, estradiol-17β, insulin and IGF-I, and inhibited by TGF-β, FSH, and cAMP agonists (Mondchein *et al.*, 1990; Grimes *et al.*, 1991). Although the exact role of the IGFBPs in follicular functions remains a matter for further study, general consensus seems to support their role in the transport and in the regulation of bioavailbility of IGFs (reviewed by Adashi, 1992; Tsafriri & Adashi, 1994).

Transforming growth factor-beta (TGF-β)

The importance of TGF-β as an essential component of cellular regulatory mechanisms is indicated by the fact that it is produced *in vitro* by cells of nearly every origin, whether they be neoplastic or nonneoplastic, embryonic or adult (Sporn *et al.*, 1987). This growth factor possesses a unique capacity to exert cell-specific stimulatory, biphasic, or inhibitory effects on a variety of cellular activities *in vitro,* from cell growth and differentiation to specific cell functions (Pfeilschifter, 1990). Three different isoforms of TGF-β, namely TGF-β1, TGF-β2, and TGF-β3, have been identified. The mature isoform peptides share a 70-80% sequence homology and similar, although not entirely identical, biological activities (Pfeilschifter, 1990). TGF-β1 is a 25 kDa dimer composed of two identical disulfide-linked chains of 112 amino acids each, which are

derived from the carboxy-terminal of a large precursor (Derynck *et al.*, 1985).

Interest in TGF-β as a regulator of gonadal physiology was particularly heightened by the realization that TGF-β is a member of a growing gene family of structurally related regulatory peptides which include inhibin, activin, and mullerian inhibiting substance (MIS) (Roberts *et al.*, 1988). To implicate TGF-β in follicular physiology, it was essential to demonstrate its local availability in the follicle. Indeed, reports indicate that TGF-β is produced in the follicle by granulosa cells (Kim & Schomberg, 1989; Mulheron & Schomberg, 1990), theca-interstitial cells (Skinner *et al.*, 1987; Bendell & Dorrington, 1988; Gangrade & May, 1990), and the oocyte (Chegini & Flanders, 1992). In the rat, theca cells expressed both TGF-β1 and TGF-β2; however only TGF-β2 expression was regulated by the gonadotropins (Mulheron *et al.*, 1991). In the pig, granulosa cells appeared to secrete low levels of TGF-β1 and possibly TGF-β3, but did not produce translatable TGF-β2 mRNA (Mulheron *et al.*, 1992).

As in other systems, TGF-β in the ovarian follicle appears to be a multifunctional molecule with diverse effects. It stimulated the proliferation of murine (Skinner *et al.*, 1987; Bendell & Dorrington, 1988) and porcine (May *et al.*, 1988) granulosa cells, but suppressed the proliferative action of TGF-α on bovine thecal cells (Roberts & Skinner, 1991). In terms of steroidogenesis, TGF-β inhibited gonadotropin-stimulated progesterone production by granulosa (Mondschein *et al.*, 1988) and theca (Caubo *et al.*, 1989) cells, but stimulated estradiol-17β production by theca cells (Caubo *et al.*, 1989) in the pig. On the contrary, in the rat, TGF-β stimulated progesterone production in granulosa (Dodson & Schomberg, 1987; Knecht *et al.*, 1987) and theca-interstitial cells (Magoffin *et al.*, 1989), and stimulated FSH-induced LH receptor binding in granulosa cells (Knecht *et al.*, 1986; Dodson & Schomberg, 1987). Clearly, further studies are needed to understand fully the role of TGF-β in follicular physiology, including its regulation by circulating gonadotropins and ovarian steroids.

Epidermal growth factor/transforming growth factor-alpha

The epidermal growth factor (EGF)-related family of growth factors includes a

number of ligands including EGF, transforming growth factor-alpha (TGF-α), heparin-binding EGF, amphiregulin, cripto and heregulin for a common receptor, the EGF receptor (EGF-R) (Prigent & Lemoine, 1992). However, only EGF and TGF-α have been studied for their potential roles in ovarian functions. EGF is a single-chain polypeptide of 53 amino acids with a relative molecular weight of ~6 kDa. This peptide was originally discovered in submaxillary glands of mice by Stanley Cohen (1962) and has been shown to stimulate the proliferation of epidermal and epithelial cells in whole animals and a variety of cell types in culture (Carpenter & Cohen, 1979). The EGF receptor is a 170 kDa transmembrane glycoprotein consisting of an extracellular ligand binding domain, a hydrophobic short transmembrane domain and a cytoplasmic region containing a highly conserved tyrosine kinase domain (Prigent & Lemoine, 1992). EGF binding results in a stimulation of phosphorylation of its receptor as well as numerous other target proteins in their tyrosyl residues (Carpenter, 1987; Carpenter & Cohen, 1990).

In addition to its effects on oocyte maturation which will be discussed later in Section 2.3.2 D, EGF has also been implicated in the regulation of follicular somatic cell functions including differentiation, proliferation and steroidogenesis. EGF was shown to modulate FSH-mediated granulosa cell differentiation including suppression of FSH-induced LH receptor expression in rat granulosa cells (Mondschein & Schomberg, 1981). EGF also inhibited FSH-stimulated estradiol-17β (Hsueh *et al.*, 1981) and cAMP production (Knecht & Catt, 1983) in rat granulosa cells, and stimulated progesterone and 20α-dihydroprogesterone formation in hamster and rat granulosa cells (Jones *et al.*, 1982; Roy & Greenwald, 1991). Infusion of mouse EGF into the systemic circulation or ovarian artery of the ewe inhibits secretion rates of ovarian estradiol and inhibin, and increases plasma FSH concentration and LH pulse amplitude throughout the follicular phase (Shaw *et al.*, 1985; Scaramuzzi *et al.*, 1988; Murray *et al.*, 1989).

A mitogenic activity for EGF has been demonstrated in bovine, porcine, rabbit, guinea pig, and human granulosa cells (Gospodarowicz *et al.*, 1977a,b; Gospodarowicz & Bialecki, 1979). The mitogenic effect of EGF on granulosa cells was enhanced by several other growth factors, such as TGF-β (Bendell & Dorrington, 1990), platelet-

derived growth factor (PDGF), and α-fetoprotein (May *et al.*, 1990; Keel *et al.*, 1991a,b). Although PDGF was a more potent mitogen than EGF towards *in vitro* cultured porcine theca cells, the addition of EGF further enhanced the mitogenic effect of PDGF in these cells (May *et al.*, 1992). These findings suggest that more than one growth factor may act synergistically to promote somatic cell proliferation associated with rapid follicular growth.

The presence of EGF receptors and their up-regulation by FSH have been reported in the rat ovary (St-Arnaud *et al.*, 1983) and in cultured granulosa cells of the pig (Fujinaga *et al.*, 1992). Despite implication of EGF in ovarian functions, reports concerning its presence in the ovary are controversial and will be discussed in detail in Chapter 7.

Transforming growth factor-alpha is a 50 amino acid protein that has 30-40 % structural homology with EGF. It binds to the EGF-R (Derynck, 1990) and has similar, although not identical, biological activities as EGF (Derynck, 1986). TGF-α is a unique gene product that is produced as a large transmembrane precursor which is processed into a soluble extracellular protein (Bringman *et al.*, 1987). It was first isolated from the conditioned medium of virally transfected fibroblasts (DeLarco & Todaro, 1978) and has subsequently been shown to be produced by a large number of neoplastic cells (Derynck *et al.*, 1987). In the last few years, TGF-α production has been demonstrated in nontransformed cells from postnatal tissues, including cultured bovine anterior pituitary cells (Kobrin *et al.*, 1986), neonatal human foreskin keratinocytes (Coffey *et al.*, 1987), activated macrophages (Madtes *et al.*, 1988) and mouse brain (Wilcox & Derynck, 1988), indicating its role as a regulator in normal adult tissues.

TGF-α has been studied for its potential roles in follicular physiology and has been shown to influence a variety of somatic cell functions. It stimulated the proliferation of isolated theca and granulosa cells from bovine ovarian follicles (Skinner & Coffey, 1988). In the rat, TGF-α attenuated FSH-stimulated basal aromatase activity and inhibited estradiol-17β production by cultured granulosa cells in a dose-dependent manner (Adashi & Resnik, 1986; Mason *et al.*, 1986). Infusion of TGF-α into the

ovarian artery of the sheep inhibited estradiol secretion, increased peripheral LH pulse amplitude and FSH concentration, and delayed the preovulatory surge of LH (Murray *et al.*, 1990). Such observations lead to the formulation of a consensualized view that TGF-α/EGF are negative regulators of FSH-induced differentiated functions in granulosa cells. However, more recently, in a contrasting observation, TGF-α was found to induce aromatase activity in granulosa and theca cells obtained from prepubertal gilts (Gangrade *et al.*, 1991). This may suggest a more complicated nature of actions and interactions of these peptides in follicular functions. Although originally TGF-α was considered predominantly to be a theca cell product (Kudlow *et al.*, 1987; Skinner & Coffey, 1988; Lobb *et al.*, 1989), more recent studies have provided evidence to the contrary suggesting the granulosa cells to be a more likely follicular source of TGF-α (Yeh *et al.*, 1991, 1993). This aspect will be discussed in the light of my own studies included in Chapter 10, which also indicate TGF-α to be a granulosa cell product in the pig.

B Others

In addition to peptide growth factors, a whole host of other factors have been proposed and are being investigated as possible intragonadal regulators of folliculogensis. These include, in addition to many others, activin, inhibin, follistatin, follicle regulatory protein and gonadotropin-releasing hormone (GnRH)-like protein (Montz *et al.*, 1984; Battin & diZerega, 1985; Miyamoto *et al.*, 1985; Ying *et al.*, 1986; Ono *et al.*, 1986; Aten *et al.*, 1986, 1987a,b; Hsueh *et al.*, 1987; Hutchinson *et al.*, 1987; Ying, 1988; Tonetta *et al.*, 1988; LaPolt *et al.*, 1989; Xiao *et al.*, 1989; Klein *et al.*, 1990; LaPolt *et al.*, 1990; Schwall *et al.*, 1990).

In summary, although the traditional roles of gonadotropins and ovarian steroids in the regulation of ovarian follicular growth and development are undisputed, it is becoming increasingly evident that the follicle itself may control its own destiny. Identification in the follicular fluid of a number of these nonsteroidal factors, which modulate follicular cell responses to gonadotropins and ovarian steroids, further supports

such concept. However, the bulk of the information implicating such factors as vital autocrine and paracrine regulators of normal folliculogensis *in vivo* came from their *in vitro* model systems. Thus in the absence of further evidence *in vivo*, these inferences should be guarded with caution.

2.2 OOCYTE MATURATION

Oocyte maturation represents that period during which the female gamete changes from a developmentally incompetent cell to one with the capacity to direct and support the events of fertilization and early embryonic development. Elucidation of the intricate mechanisms which regulate mammalian oocyte maturation is one of the major problems encountered in developmental and reproductive biology today. Although several factors have been identified and implicated in the maturation processes both *in vivo* and *in vitro*, there is no universally accepted model for the coordination of these factors in the regulation of oocyte maturation. *In vivo*, oocyte maturation occurs in an intrafollicular milieu which is determined by the surrounding somatic cells of the follicle. These cells include the cells of the cumulus oophorus and corona radiata with which the oocyte is in most intimate contact, plus the more peripheral cells of the mural granulosa and theca layers. These somatic cells, in turn, are under regulatory influences of a variety of hormones and blood-borne substances that contribute to the intrafollicular environment. Thus, during the period of oocyte maturation *in vivo*, dynamic changes in pattern of gonadotropins and other hormones, through their actions on the follicular somatic cells, cause profound alterations in the micro environment to which the oocyte is exposed. It is now well documented that the endogenous surge of gonadotrophic hormones trigger oocyte maturation during the final stages of preovulatory development in mammals, which may suggest that oocyte maturation is under positive control of these hormones. However, gonadotropins are not necessary for inducing maturation of competent oocytes; once removed from follicles, such oocytes resume meiotic maturation spontaneously in the absence of hormones or follicular factors (Pincus and Enzmann, 1935; Edwards, 1965a,b). This ability of oocytes to resume meiosis after removal from the follicles has

led researchers to pursue a putative oocyte maturation inhibiting factor (OMI) in the follicular environment (Tsafriri and Channing, 1975b). Although such a factor may have some role in the maintenance of meiotic arrest in intrafollicular oocytes, much evidence suggests that oocyte maturation is not accomplished simply by removal of inhibition. This section will provide a detailed description of various aspects of oocyte maturation including the regulatory factors both *in vivo* and *in vitro*.

2.2.1 Acquisition of Developmental Competence by the Oocyte

Critical developmental changes occur in dictyate-stage oocytes near the completion of their growth phase. These changes pertain to the acquisition of competence to complete both nuclear and cytoplasmic maturation and are essential for the formation of an egg having the capacity for fertilization and development into a live offspring. Nuclear maturation refers to the events which reverse meiotic arrest at prophase I and allow the progression of meiosis to metaphase II, with extrusion of the first polar body. Studies in the pig (Tsafriri & Channing, 1975a; Motlik *et al.*, 1984) and in the mouse (Sorensen & Wassarman, 1976) have indicated that competence to resume meiosis and to reach metaphase II is acquired in a stepwise manner during oocyte growth. The ability to undergo germinal vesical breakdown and continue to metaphase I is acquired earlier during folliculogenesis than the ability to reach metaphase II. However, an oocyte's attainment of meiotic competence does not ensure the ability to be fertilized and complete embryonic development (reviewed by Thibault *et al.*, 1987; Downs, 1993). Cytoplasmic maturation encompasses the processes that prepare the egg for activation, formation of pronuclei and subsequent embryonic development. In the mouse oocyte, it appears that the competence to undergo nuclear and cytoplasmic maturation are acquired independently by the developing oocyte (Eppig *et al.*, 1994). The competence to undergo cytoplasmic maturation is also acquired in a stepwise manner. For instance, the mouse oocyte first acquires competence to undergo fertilization and development to the 2-cell stage, but further development at the germinal vesicle (GV) stage is required before the oocyte becomes competent to develop from the 2-cell stage to the blastocyst stage (Eppig & Schroeder, 1989). The developmental changes occurring in the growing

oocyte, therefore, relate not only to the appropriate segregation of chromosomes and the maintenance of diploidy, but also to its ability to sustain embryonic development even beyond the time of zygotic gene activation, which occurs at the 2-cell stage in the mouse (Flach *et al.*, 1982; Tayler & Piko, 1987).

The acquisition of competence to resume meiosis in oocytes is often correlated with the stage of follicular development and oocyte size. Meiotic competence is acquired at the age of 23 days postpartum in hamsters (Iwamatsu & Yanagimachi, 1975), between days 15-21 in mice (Szybek, 1972; Sorensen & Wassarman, 1976) and days 20-26 in rats (Bar-Ami & Tsafriri, 1981). In rodents, it has been demonstrated that acquisition of meiotic competence by the oocyte coincides with formation of an antrum in the enclosing follicle (Erickson & Sorensen, 1974; Iwamatsu & Yanagimachi, 1975; Tsafriri & Channing, 1975a; Bar-Ami & Tsafriri, 1981). The observation that gonadotropin stimulation is required for the development of the antrum (Richards, 1980) led to further investigations of the role of these hormones in the acquisition of developmental competence. Bar-Ami and Tsafriri (1981) demonstrated that hypophysectomy of immature rats on day 15 postpartum prior to acquisition of meiotic competence reduced the proportion of competent oocytes by 58% on day 26. Treatment of these hypophysectomized rats with FSH or pregnant mare serum gonadotropin (PMSG) reversed this effect suggesting a role for gonadotropins in acquisition of meiotic competence. In further studies the authors concluded that both FSH and estrogen are required for the acquisition of meiotic competence by rat oocytes and that the effect of FSH is, at least partly, mediated by estrogen produced by somatic follicular cells (Bar-Ami *et al.*, 1983; Bar-Ami & Tsafriri, 1986). However, formation of the antrum is not closely correlated with meiotic competence in all species; for instance, pig oocytes obtained from early antral follicles resume meiosis in culture only at a very low frequency (Motlik *et al.*, 1984). The ability of oocytes to resume meiotic maturation *in vitro* also appears to be related to their size. Hamster and mouse oocytes successfully mature *in vitro* only when they have reached full size (Iwamatsu & Yanagimachi, 1975; Sorensen & Wassarman, 1976). Similarly, the increase in the size of porcine oocytes

from 100 to 120 μm in small antral follicles appears to be related to their ability to resume meiosis *in vitro* (McGaughey *et al.*, 1979). Only mouse oocytes larger than 60 μm in diameter mature during *in vitro* culture; smaller oocytes remain arrested at the dictyate stage. Furthermore, the fusion of small, meiotically incompetent oocytes with large, meiotically competent oocyte enables the small incompetent oocytes to mature *in vitro* (Balakier & Czolowska, 1977; Balakier, 1978; Fulka *et al.*, 1985). Also in the rat, there seems to be a correlation between the oocyte diameter and the ability to undergo spontaneous maturation (Bar-Ami & Tsafriri, 1981).

Several studies have been undertaken to investigate the details of RNA synthesis and accumulation, and to determine qualitative changes in the pattern of protein synthesis during oogenesis (reviewed by Schultz, 1986). Mammalian oocytes like those of amphibians, are actively synthesizing RNA from early in follicular growth to within an hour or so of GVBD and some of the newly synthesized RNA is released into the cytoplasm before breakdown of germinal vesicle (Rodman & Bachvarova, 1976; Wassarman & Letourneau, 1976; Wolgemuth & Jagiello, 1976). With the use of α-amanitin to inhibit mRNA synthesis, Osborn and Moor (1983) concluded that transcription is essential for the initiation of those changes in protein synthesis required for the resumption of nuclear maturation. Furthermore, it has been shown in the rat, pig, cow and sheep that protein synthesis in the oocyte precedes acquisition of meiotic competence and that new protein synthesis is required for the breakdown of nuclear membrane (McGaughey & Van Blerkom, 1977; Ekholm & Magnusson, 1979; Moor & Crosby, 1986). Although transcription-dependent protein synthesis appears to be essential for GVBD, it is not clear whether the site of transcription for these proteins is the oocyte itself or the cumulus cells (Osborn & Moor, 1983a). Sun and Moor (1991) demonstrated that both total protein synthesis and the extensive reprogramming of protein synthesis observed in the oocyte after GVBD *in vitro*, did not differ significantly between enucleated and control sheep oocytes. Furthermore, the nucleus was not required by the oocyte to acquire the ability to condense chromatin, suggesting that both nuclear and cytoplasmic maturation are not dependent on transcription in the oocyte or on factors

released from the oocyte nucleus. These observations suggest that transcription for protein synthesis during oocyte maturation occurs in the cumulus cells rather than the oocyte. Experiments in the mouse, however, have produced different results and have consistently shown that neither new transcription nor new protein synthesis is required for GVBD (Stern *et al.*, 1972; Golbus and Stein, 1976). Protein synthesis, however, is required for the progression of meiosis after GVBD since continuous inhibition of protein synthesis during maturation of mouse oocytes blocked the chromosomes at the circular bivalent stage (Wassarman *et al.*, 1976).

In the mouse oocyte, additional morphological characteristics of meiotic competence have been identified. Incompetent oocytes exhibit a dispersed, interphase-like microtubule pattern while fewer microtubules and multiple centrosomes (microtubule-organizing centers) are typical of competent oocytes (Mattson & Albertini, 1990; Wickramasinghe *et al.*, 1991). Furthermore, the use of a monoclonal antibody for M-phase phosphoprotein has shown that the phosphorylation state of centrosomes is linked to meiotic competence (Wickramasinghe & Albertini, 1992).

2.2.2 Aspects of Oocyte Maturation

Historically, the term 'oocyte maturation' was used to describe the morphological changes in the oocyte nucleus observed in response to the preovulatory gonadotropin surge. Dissolution of the nuclear envelope was considered as indicative of initiation of maturation whereas the emission of first polar body indicated completion. Oocytes cultured outside the follicle undergo nuclear morphological changes similar to those in preovulatory follicles, but upon fertilization often fail to transform sperm into male pronucleus and lack capacity for further development (Thibault & Gerard, 1973; Moor & Trounson, 1977). As a consequence, a concept of "cytoplasmic maturation" has emerged (Thibault, 1977; Moor *et al.*, 1980a) to explain the lack of satisfactory development when mammalian oocytes undergo spontaneous nuclear maturation in the absence of a suitable follicular environment. It is essential that both these aspects of maturation are fully completed to confer upon the oocyte the ability for normal fertilization and development.

A Nuclear maturation

In mammals, the oocyte destined to ovulate remains arrested in the dictyate stage of meiotic prophase until shortly before ovulation during puberty when the preovulatory surge of gonadotropins triggers the resumption of the meiotic process. Resumption of meiosis can be induced in meiotically competent oocytes by a hormonal stimulus *in vivo* or simply by the release of the oocyte from the enclosing follicle into a suitable culture medium *in vitro* (Tsafriri, 1978; Baker, 1982). During the period of meiotic arrest, the nucleus of the oocyte is prominent and the chromosomes are diffused and transcriptionally active. The onset of nuclear maturation is equated with GVBD, which is morphologically identified by breakdown of the nuclear membrane (Masui & Clarke, 1979). This is followed by condensation of chromatin into distinct bivalents, separation into homologous chromosomes, extrusion of the first polar body and arrest of meiosis at metaphase II with chromosomes aligned on a spindle. However, condensation of chromatin precedes GVBD in the porcine oocyte (Kubelka *et al.*, 1988) suggesting that chromatin condensation may be a more sensitive index of the resumption of meiosis than GVBD in this species. In a few mammals, like the dog, fox and horse, oocytes are ovulated at a dictyate stage and nuclear maturation takes place in the oviduct (Evans & Cole, 1931; Pearson & Enders, 1943; Hamilton & Day, 1945).

B Cytoplasmic maturation

In addition to morphological changes in the nucleus, oocyte maturation is also accompanied by alterations in protein synthesis, cellular metabolism, membrane transport and organization of organelles within the ooplasm (Masui & Clarke, 1979). Ultrastructural studies revealed major reorganizational changes in mitochondria, endoplasmic reticulum, membrane-bound vesicles and cortical granules during oocyte maturation *in vivo* (Cran *et al.*, 1980; Kruip *et al.*, 1983). The biological significance of such remodelling is revealed in the oocytes where the disturbance of cortical granule alignment beneath the plasma membrane and their exocytosis results in failure to prevent polyspermy at fertilization (Sathananthan & Trounson, 1982).

There is an increase in amino acid transport across the sheep oocyte plasma

membrane during oocyte maturation (Moor & Smith, 1978, 1979; Crosby *et al.*, 1981; Osborn *et al.*, 1986). Changes in the pattern of protein synthesis has also been observed in the maturing oocyte of a number of species including the mouse (Schultz & Wassarman, 1977a), pig (McGaughey & Van Blerkom, 1977), sheep (Warnes *et al.*, 1977), rabbit (Van Blerkom & McGaughey, 1978) and cow (Kastrop *et al.*, 1990). However, there is a considerable disagreement over the association of the changes in protein synthesis and the progression of nuclear maturation. One view holds that the changes in protein synthesis occur after GVBD and that the resumption of meiosis is not dependent upon new protein synthesis (Stern *et al.*, 1972; Golbus and Stein, 1976; Schultz & Wassarman, 1977a,b; Warnes *et al.*, 1977; Kastrop *et al.*, 1990). Other investigators have shown that changes in protein synthesis precedes GVBD and that new protein synthesis is required for the resumption of meiosis (McGaughey & Van Blerkom, 1977; Ekholm & Magnusson, 1979; Moor & Crosby, 1986). In any event, there is complete agreement that GVBD is followed by marked changes in protein synthesis during oocyte maturation both *in vivo* and *in vitro*. This reprogramming process involves not only the synthesis of new proteins but also the post-translational modifications of existing proteins by processes such as phosphorylation or glycosylation. In fact, phosphorylation is considered to be one of the major general mechanisms by which intracellular events respond to external physiological stimuli (Cohen, 1982). Kastrop *et al* (1990) have shown extensive phosphorylation of certain oocyte proteins, particularly before GVBD, during bovine oocyte maturation *in vitro*. A change in the phosphorylation of a protein associated with the nucleus during GVBD was also observed in the mouse oocyte (Wassarman *et al.*, 1979).

Attempts have also been made to elucidate the role of nucleoplasm in initiating the remodelling changes in the cytoplasm during oocyte maturation. Prior removal of the nucleus does not alter the reprogramming of protein synthesis during progesterone-induced maturation of amphibian oocytes (Smith & Ecker, 1969; Ecker & Smith, 1971). However, reports concerning the role of the nucleus in cytoplasmic maturation of mammalian oocytes are controversial. Earlier reports indicated that protein

reprogramming in mammalian oocytes depends upon the release of certain nuclear factor(s) into the ooplasm. Using hamster oocytes, Usui and Yanagimachi (1976) observed that, although sperm can penetrate zona-free oocytes at any stage of maturation, decondensation of sperm head is observed only after GVBD. Similar results were observed in the mouse (Iwamatsu & Chang, 1972) and in the rat (Niwa & Chang, 1975). This concept was further supported by the observations in the starfish where mechanical breakdown of the germinal vesicle, in the absence of any hormonal supplementation, was able to induce complete cytoplasmic maturation (Guerrier *et al.*, 1983). However, recent reports in the rat (Meng *et al.*, 1993; Meng & Armstrong, 1994) and in the cow (Yang & Smith, 1994) provide evidence to the contrary and indicate that the cytoplasmic maturation of the oocyte is independent of the intrinsic nuclear factors. Rat oocytes enucleated at GV stage and matured *in vitro* exhibited similar patterns of protein synthesis as the intact oocytes (Meng *et al.*, 1993), and were capable of transforming sperm into the male pronucleus, although the presence of GV during maturation enhanced the rate of male pronuclear formation (Meng & Armstrong, 1994). Cytoplasmic maturation *in vitro* has been shown to be influenced by a variety of factors, the details to which will be provided in subsequent sections.

2.2.3 Systems for Oocyte Maturation

A Oocyte maturation *in vivo*

In vivo, a preovulatory surge of pituitary gonadotropins, LH and FSH, triggers the resumption of meiosis. Following gonadotropin injection (usually human chorionic gonadotropin, hCG), GVBD occurs after 2 hours in the mouse (Edwards & Gates, 1959) and rabbit (Moricard & Henry, 1967), 2-3 hours in the rat (Vermeiden & Zeilmaker, 1974) and hamster (Iwamatsu & Yanagimachi, 1975), 18 hours in the pig (Hunter & Polge 1966), and about 25 hours in the human (Edwards, 1965b; Fowler & Edwards, 1973). The porcine oocyte takes about 40 hours to complete nuclear maturation to reach metaphase II stage (Hunter & Polge, 1966). Evidence in the rat suggests that, in this species, oocyte maturation is correlated to behavioral estrus (Odor, 1955) and the time

on the day of proestrus (Tsafriri & Kraicer, 1972).

In the earlier years, it was believed that LH was solely responsible for initiating ovulation and oocyte maturation *in vivo* (Tsafriri, 1978). This concept was based on the observations in the rat where administration of antiserum to the β subunit of LH on the afternoon of the day of proestrus prevented oocyte maturation and ovulation (Tsafriri *et al.*, 1976). However, it is becoming apparent now that other hormones can substitute for, or mimic the action of LH in induction of oocyte maturation and ovulation. Follicle-stimulating hormone, which is named for it role in follicular development, can induce ovulation by itself (Nuti *et al.*, 1974; Schwartz *et al.*, 1975; Schenken *et al.*, 1984). In fact, FSH preparations were found to be more potent in inducing ovulation than LH in the hamster (Greenwald & Papkoff, 1980). The ability of recombinant human FSH to induce follicular development and ovulation in hypophysectomized rats (Galway *et al.*, 1990), further demonstrated the capability of FSH to bring about these responses without the necessity of LH. Some investigators, however, believe that the demonstrated ability of FSH to induce oocyte maturation and ovulation is a non-physiological response to pharmacological doses of this hormone (Tsafriri, 1978). The observations that gonadotropin-releasing hormone (GnRH) can induce ovulation in hypophysectomized animals (Corbin & Bex, 1981; Eckholm *et al.*, 1981; Dekel *et al.*, 1983a; Koos & LeMaire, 1985; Minegishi & Leung, 1985), though faced with a contradictory report (de la Lastra & Leal, 1988), are unexpected and difficult to explain in terms of the known effects of GnRH on the pituitary. Given the fact that both FSH and LH are secreted during the preovulatory gonadotropin surge, it is tempting to believe that FSH and LH act together to initiate ovulation and oocyte maturation under normal conditions.

B Oocyte maturation *in vitro*

Pincus and Enzmann (1935) were the first to recognize that GV-stage rabbit oocytes, once liberated from their follicles and maintained under suitable conditions in hormone-free media, would resume meiosis spontaneously. These findings that extrafollicular oocytes can mature independently of any hormonal supplementation, have

subsequently been confirmed in many mammalian species (reviewed by Tsafriri, 1978; Masui & Clarke, 1979; Masui, 1985; Tsafriri, 1985). Studies using *in vitro* culture of isolated OCC have been used extensively to elucidate the mechanisms which regulate intrafollicular oocyte maturation. The major advantage of such an *in vitro* model system is its relative simplicity over other approaches. From ovaries of prepubertal animals, it is possible to obtain a large number of relatively synchronized oocytes without the complication of variations in stages of the oestrous cycle, thus reliably facilitating large-scale studies. Since only the oocyte and cumulus cells are involved, interpretation of results is relatively easier compared to more complex multicellular systems. However, *in vivo*, the oocyte is exposed to gonadotropins, steroids, growth factors, and other molecules, any or all of which may interact to regulate maturational changes that occur in the oocyte and its surrounding somatic follicular cells before ovulation. Therefore, the spontaneous maturation observed *in vitro* may not accurately reflect the physiological processes of oocyte maturation. It is evident that although the spontaneous maturation of oocytes *in vitro* upon liberation from the follicles results in morphologically normal secondary (i.e. matured) oocytes, the functional normality of such oocytes is not assured. Rabbit follicular oocytes, after spontaneous maturation outside the follicle, were incapable of transforming sperm nuclei into pronuclei in the ooplasm (Thibault, 1977). Similar deficiencies in pronucleus formation were reported in rats (Niwa *et al.*, 1976), hamsters (Leibfried & Bavister, 1983), pigs (Motlik & Fulka, 1974; Nagai *et al.*, 1984) and cows (Leibfried-Rutledge *et al.*, 1987). In instances where pronuclear development did occur, the rate of cleavage, frequency of preimplantation development, and development to live offspring were low compared to oocytes matured *in vivo* (Cross & Brinster, 1970; Niwa *et al.*, 1976; Moor & Trounson, 1977; Shalgi *et al.*, 1979; Leibfried-Rutledge *et al.*, 1987; Mattioli *et al.*, 1989). These deficiencies are attributed to inadequate cytoplasmic maturation of oocytes undergoing spontaneous maturation *in vitro*, even though apparently normal nuclear maturation is observed (Thibault, 1977; Leibfried-Rutledge *et al.*, 1987). It appears that follicular somatic cell secretory products, which are deficient in this culture system, are essential for complete

physiological maturation of the oocyte. Indeed the coculture of somatic follicular cells with OCC during *in vitro* maturation (IVM) has been shown to improve cytoplasmic maturation of the oocyte and will be discussed in detail in subsequent sections. Future studies aimed at refining culture conditions for IVM will lead to a broader use of this relatively easier system for large scale embryo production. Recent developments in techniques for *in vitro* oocyte maturation, fertilization and embryo culture in the cow has allowed preimplantation embryo production in proportions similar to those in laboratory animal species such as the mouse (Sirard *et al.*, 1988; Fukui & Ono, 1989; Kim *et al.*, 1990; Wiemer *et al.*, 1991; Rose & Bavister, 1992), thereby providing some optimism for similar approaches in other species.

Since oocyte maturation *in vivo* takes place within the whole follicle, an alternate approach of culturing isolated intact antral follicles has been shown to be a useful method for the study of follicular regulatory mechanisms of oocyte maturation. Hormonal stimulation is required to induce meiotic maturation in intrafollicular oocytes. Addition of gonadotropins to culture media containing carefully dissected follicles can induce resumption of meiosis in intrafollicular oocytes (rat: Tsafriri *et al.*, 1972; Hillensjo *et al.*, 1976; rabbit: Thibault & Gerard, 1973; sheep: Miller & Jagiello, 1973; Moor & Trounson, 1977; cow: Thibault *et al.*, 1975a,b; hamster: Gwatkin & Andersen, 1976; Mandelbaum *et al.*, 1977; monkey: Thibault *et al.*, 1976). In addition, several other substances have been shown to induce meiotic maturation in follicle-enclosed oocytes including prostaglandins (Tsafriri *et al.*, 1972; Lindner *et al.*, 1974), dibutyryl cAMP (Tsafriri *et al.*, 1972; Lindner *et al.*, 1974), forskolin (Dekel & Sherizly, 1983), GnRH (Hillensjo & LeMaire, 1980, Ekholm *et al.*, 1981; Dekel *et al.*, 1983a), EGF (Dekel & Sherizly, 1985) and transforming growth factor-β (Feng *et al.*, 1988). The advantage of this system is that it provides an *in vitro* model in which normal associations between the oocyte and somatic follicular components are maintained. Intrafollicular oocytes matured *in vitro* resembled more closely oocytes matured *in vivo* and were capable of supporting embryonic development after fertilization. Sperm head swelling was immediate in rabbit follicle-enclosed oocytes matured *in vitro* (Thibault & Gerard, 1973)

and these oocytes could sustain development up to the birth of live offspring (Thibault *et al.*, 1975). In sheep, birth of lambs was reported from follicular oocytes recovered after culture of large antral follicles in the presence of gonadotropins (Moor & Trounson, 1977). This indicates that the antral follicle is a physiological entity capable of supporting nuclear and cytoplasmic maturation of the oocyte *in vitro* and can ideally be used for studying the mechanisms underlying hormonal induction of oocyte maturation. But being multicellular, this model also suffers some major limitations, particularly for studies aimed to elucidate the site of hormonal actions or localization of the cellular response.

Culture of ovarian fragments from PMSG-treated mice yielded similar results (Neal & Baker, 1973, 1974, 1975; Neal *et al.*, 1975). These studies demonstrated a relationship between the stage of follicular development and the capacity of the oocyte to complete meiosis following hormonal stimulation. Whereas oocytes in large antral follicles completed meiosis and reached MII stage after hormonal stimulus, oocytes within small or medium-sized follicles remained arrested at metaphase I (MI) stage (Neal & Baker, 1973, 1974). Similar to the isolated follicle culture system, this model offers oocyte maturation comparable to maturation *in vivo* (Baker & Neal, 1972; Baker, 1979). At the same time, this system also suffers the disadvantage of being multicellular in which complex, intracellular interactions are difficult to interpret.

2.2.4 Follicular Support of Oocyte Maturation

Under normal physiological conditions *in vivo*, oocyte maturation takes place in an intrafollicular milieu that is determined by somatic follicular cells, in particular the cells of the cumulus oophorus and corona radiata with which the oocyte is in most intimate contact. The functions of these somatic cells are regulated by a variety of hormones, particularly the gonadotropins, as well as by other blood-borne substances that contribute to the intrafollicular environment. Therefore, it is not surprising that the follicle cells were found to support the oocyte for maintenance of normal metabolic activity and for the regulation of both nuclear and cytoplasmic maturation (Crosby *et al.*,

1981; Moor, 1983; Moor *et al.*, 1983). It has been demonstrated in a number of species that following an endogenous preovulatory gonadotropin surge *in vivo*, the oocyte undergoes an initial "inductive" phase of maturation within the follicle, which is essential for it to attain full physiological maturation (Iwamatsu & Chang, 1972; Thibault & Gerard, 1973; Niwa & Chang, 1975; Niwa *et al.*, 1976; Moor & Warnes, 1978). Moor and Trounson (1977) reported that sheep oocytes that undergo an "inductive" phase of maturation outside the follicle resume meiosis but show abnormalities at fertilization and blastulation. It has been shown that a relatively short period of maturation within the follicle is sufficient to increase the fertilizability of the oocyte. A period of six hours within the follicle after the initiation of maturation by hCG was sufficient to permit normal sperm head swelling in the rabbit (Thibault & Gerard, 1973). Similarly, sperm penetration occurred in rat oocytes only if they were maintained within the follicle for eight hours after hCG injection (Niwa & Chang, 1975). Moor and Warnes (1978) reported that in addition to six to eight hours of "inductive" phase within the follicle, sheep oocytes require a further six to twelve hours "synthetic" phase which may occur outside the follicle before normal fertilization and embryonic development can occur. Thus it appears that the "inductive" phase of oocyte maturation has specific intrafollicular requirements whereas the "synthetic" phase is independent of such follicular support.

The inability of OCC removed from antral follicles to attain full developmental competence after maturation *in vitro* may thus be explained by the lack of an initial obligatory phase inside the follicle. Several groups have reported that the addition of somatic follicular cells to maturation media containing gonadotropins and isolated OCC can improve fertilization rates and embryo development. The coculture of granulosa cells from follicles of oestrous cows during IVM with OCC from small antral follicles increased developmental competence following fertilization *in vitro* (Critser *et al.*, 1986; Lu *et al.*, 1987; Lutterbach *et al.*, 1987; Xu *et al.*, 1987). Similar findings were reported earlier for sheep oocytes (Staigmiller & Moor, 1984). Culture of porcine oocytes in the presence of extroverted follicles (i.e., OCC connected to the wall of the whole follicle turned inside out) improved the rate of fertilization and male pronucleus

formation (Mattioli *et al.*, 1988a,b). The cocultured somatic follicle cells may be providing some of the hormonal and cellular factors needed during the "inductive" phase, thereby enabling improved fertilization and embryonic development to occur. However, recent achievements in IVM-IVF due to improved techniques, particularly in the cow, seriously challenge this whole concept of "inductive phase". Blastocysts can now be obtained from the oocytes collected from slaughter house ovaries, and matured and fertilized *in vitro* in a chemically defined, serum-free medium without somatic cell conditioning (Pinyopummintr, 1990; Saeki *et al.*, 1991). In fact, it appears that inadequacies in the IVM culture media used for maturing the oocyte in earlier studies lead to the belief that a certain period of intrafollicular maturation was essential before a full developmental potential is acquired.

The somatic follicular cells play a fundamental role in the regulation of oocyte growth and development. By using a variety of techniques including electrophysiological recording, dye transfer, autoradiography and the movement of intracellular markers, evidence has been gathered which indicate the transmission of compounds from the somatic to germinal compartment through permeable gap junctions (Gilula *et al.*, 1978; Heller & Schultz, 1980; Moor *et al.*, 1980b). The role of follicle cells in providing nutrients for the oocyte was suggested by Biggers and colleagues (1967) who indicated that the granulosa cells provide energy substrate to the oocyte. The oocyte is dependent on follicular cells for its ability to utilize glucose or lactate (Masui & Clarke, 1979). The somatic compartment is also required for the transport of certain amino acids, nucleosides, and phospholipid precursors to the oocyte (reviewed by Moor, 1983). Follicle cells not only regulate the entry of RNA precursors but also provide stability to newly synthesized RNA in the oocyte by reducing its catabolism (Brower *et al.*, 1981). Apart from their nutritional role, the follicle cells also generate instructional signals which influence the nucleus and direct the synthesis of certain structural proteins (Osborn & Moor, 1982) and maturation specific proteins (Crosby *et al.*, 1981). Oocytes cultured within intact follicles exhibit patterns of protein synthesis more similar to those of *in vivo* matured oocytes than do oocytes removed from the follicle (Crosby *et al.*, 1981). The

signal for hormone-stimulated GVBD *in vitro* is also generated in cumulus cells and not in the oocyte itself (Downs *et al.*, 1989; Coskun & Lin, 1992), suggesting a regulatory role for follicular cells in nuclear maturation.

2.3 REGULATION OF OOCYTE MATURATION

Despite continuous efforts directed towards understanding the mechanisms which regulate the nuclear and cytoplasmic changes associated with oocyte maturation, identification of a universally accepted model is far from clear. That the follicle has a controlling influence on oocyte maturation is undisputed, but the precise nature of the somatic cell signal(s) is not known. The bulk of the evidence suggests that two separate events are involved in the regulation of oocyte maturation, these being alleviation of an inhibitory influence of the follicle on the oocyte, and the generation of an inductive stimulus in the follicular cells.

2.3.1 Inhibitors of Meiosis

A Follicular fluid, granulosa cells, OMI, and purines

Before the preovulatory gonadotropin surge, the follicle cells are responsible for inhibiting meiosis and maintaining the oocytes in the GV stage. This concept was based on the discovery by Pincus and Enzmann (1935) that oocytes enclosed within the follicle remain arrested at the dictyate stage, whereas oocytes removed from the follicle complete meiosis even in the absence of hormones. The first direct evidence for the presence of a putative oocyte maturation inhibitor (OMI) in the follicular fluid was provided by Chang (1955) and has subsequently been supported by some workers (Tsafriri & Channing 1975b; Gwatkin & Andersen, 1976) and disputed by others (Leibfried & First, 1980a,b; Racowsky & McGaughey, 1982a; Fleming *et al.*, 1983). The ability of follicular fluid to inhibit oocyte maturation does not seem to be species specific, since human follicular fluid inhibits maturation of porcine oocytes *in vitro* (Hillensjo *et al.*, 1979), bovine follicular fluid inhibits hamster oocytes (Gwatkin & Andersen, 1976), and porcine follicular fluid inhibits maturation of mouse and rat oocytes (Tsafiri *et al.*, 1977).

The activity of OMI appears to decline during the course of follicular development, being maximal in small and minimal in large antral follicles (Stone *et al.*, 1978). The inhibitory substance in follicular fluid was suggested to be a polypeptide with a molecular weight of less than 2 kDa (reviewed by Tsafriri *et al.*, 1982). The effects of OMI are apparently mediated through cumulus cells since it inhibits maturation of only cumulus-enclosed oocytes, but has no effect on oocytes devoid of cumulus (Hillensjo *et al.*, 1979).

A large body of evidence suggest that OMI is produced by granulosa cells of the follicle. Coculture of mural granulosa cells with oocytes result in density-dependent inhibition of oocyte maturation in the pig (Tsafriri & Channing, 1975b) and in the cow (Sirard & Bilodeau, 1990). This effect of granulosa cells is reversed *in vitro* by the addition of LH (Tsafriri *et al.*, 1976; Stone *et al.*, 1978). It has also been suggested that cellular contact between the oocyte and granulosa cells is not required, as granulosa cell extracts or conditioned medium can elicit similar inhibitory effects (Gwatkin & Andersen, 1976; Centola *et al.*, 1981; Sato & Koide, 1984; Anderson *et al.*, 1985). However, Sirard and Bilodeau (1990) concluded from their coculture studies with bovine oocytes that the inhibitory factor produced by granulosa cells does not accumulate in the follicular fluid in an active form and that a close contact between living granulosa cells and cumulus cells enhances the inhibition of meiosis. Similarly, Racowsky & Baldwin (1989) demonstrated that hamster OCC dislodged *in situ* from the mural granulosa cells, but contained in the antrum, resumed meiosis six hours after separation. By contrast, the OCC which re-established contact with the mural granulosa cells remained meiotically arrested. In addition, several other groups have demonstrated a complete ineffectiveness of granulosa cells in the maintenance of meiotic arrest in the oocyte (pig: Jagiello *et al.*, 1977; Leibfried & First, 1980a; Rice & McGaughey, 1980; cow: Jagiello *et al.*, 1977; Leibfried & First, 1980a; sheep: Jagiello *et al.*, 1977; mouse: Nekola & Smith, 1974). Surprisingly enough, Marc Sirard's group who earlier reported inhibition of meiotic maturation by granulosa cells (Sirard & Bilodeau, 1990), has recently (Richard & Sirard, 1994) reported that it is the theca, but not the granulosa cells that inhibit spontaneous

maturation of bovine oocytes. Recently, a Japanese group (Kotsuji *et al.*, 1994) has provided evidence that theca cells cooperate with granulosa cells to inhibit spontaneous maturation of bovine oocytes. Thus, many conclusions are open to criticisms and many results are difficult to repeat because the conditions of the biological material are not sufficiently well defined. The very existence of OMI has seriously been questioned in some quarters and, without additional biochemical support, remains a highly controversial subject.

Recent evidence suggests that purine bases and nucleosides may, in fact, be the low-molecular weight components of follicular fluid that prevent spontaneous oocyte maturation *in vitro*. Hypoxanthine and adenosine have been shown to be present in porcine (Downs *et al.*, 1985) and mouse (Eppig *et al.*, 1985) follicular fluids. These purines are present in millimolar concentrations within follicular fluid and inhibit meiotic resumption of mouse oocytes *in vitro* (Eppig *et al.*, 1985; Shim *et al.*, 1992) as well as *in vivo* (Downs & Eppig, 1987). Inhibition of oocyte maturation by hypoxanthine has also been reported in bovine (Sirard & First, 1988), monkey (Warikoo & Bavister, 1989) and rat (Tornell *et al.*, 1990) oocytes. However, no decrease in the levels of these purines was observed when meiosis was resumed after hCG injection (Eppig *et al.*, 1985). Later studies on uptake and metabolism of purines have not been able to clarify this paradoxical situation (Downs *et al.*, 1986).

B cAMP

It is widely believed that cAMP participates in the meiotic arrest of the oocyte (reviewed by Masui & Clark, 1979; Eppig & Downs, 1984; Eppig, 1989). This concept is largely based on the observations that spontaneous maturation of oocytes *in vitro* can be blocked by supplementing the culture media either with membrane-permeable analogs of cAMP, such as dibutyryl cAMP (dbcAMP) and 8-bromo-cAMP, or with the inhibitors of phosphodiesterase (PDE), such as isobutylmethylxanthine (IBMX) and theophylline (reviewed by Wassarman & Albertini, 1994). In addition, agents that increase cAMP levels by activating of adenylate cyclase, such as forskolin or FSH, can also maintain the

oocyte in meiotic arrest (reviewed by Eppig & Downs, 1984; Schultz, 1986). Furthermore, microinjection of an inhibitor of cAMP-dependent protein kinase A (PKA) initiates meiosis in oocytes maintained in GV stage with dbcAMP or IBMX (Bornslaeger *et al.*, 1986). In addition, a cAMP antagonist, Rp-AMPS, has been shown to reverse the meiotic inhibition maintained either by exogenous inhibitors (Downs, 1993), or by granulosa cells on *in vitro* grown, meiotically competent oocytes (Eppig, 1991a), resulting in GVBD. These data suggest that cAMP plays a central role in the maintenance of meiosis at the dictyate stage, such that a fall in intraoocyte cAMP levels could trigger reentry of oocytes into meiotic progression. Indeed, a decrease in oocyte cAMP has been shown to precede GVBD in the rat (Racowsky, 1984) and the mouse (Schultz *et al.*, 1983a).

It was proposed that cAMP in the oocyte is derived entirely from the cumulus cells through permeable gap junctions and that the termination of coupling between the two cell types leads to a fall in oocyte cAMP contents and thus GVBD (Dekel & Beers, 1978; Dekel *et al.*, 1981). Addition of cholera enterotoxin (Dekel & Beers, 1980) or forskolin (Dekel *et al.*, 1983b), both activators of adenylate cyclase, effectively inhibit spontaneous maturation of cumulus-enclosed, but not of denuded rat oocytes. Similarly, suboptimal concentrations of dbcAMP produce a greater inhibitory effect on meiotic maturation in cumulus-intact oocytes than in denuded oocytes (Eppig *et al.*, 1983). Furthermore, gonadotropins can reverse dbcAMP-induced meiotic inhibition in cumulus-enclosed rat oocyte but not in denuded oocytes (Dekel & Beers, 1978, 1980). These data, along with the knowledge that gonadotropin receptors are present on cumulus cells and not on the oocyte (Amsterdam *et al.*, 1975), support the concept that the oocyte is maintained in meiotic arrest either by the transfer of cAMP from cumulus cells to the oocyte to maintain high levels there or perhaps that cAMP generates and/or activates a maturation inhibiting factor(s) in cumulus cells that is transferred to the oocyte. A direct role for cAMP is supported by the observations that exposure of OCC to agents that increase cumulus cell cAMP levels also elevate the intraoocyte cAMP levels relative to their denuded counterparts similarly exposed (Racowsky, 1984, 1985a; Bornslaeger &

Schultz, 1985). However, the concept of a critical role for cAMP in maintaining the oocyte in the GV stage has some serious pitfalls. The onset of GVBD is not always preceded by a drop in intraoocyte cAMP levels (Moor & Heslop, 1981; Racowsky, 1985a,b; Hubbard, 1986). Furthermore, a decrease in intracellular coupling between the oocyte and cumulus cells can be induced independently of GVBD (Moor *et al.*, 1981; Eppig, 1982a). Alternatively, the intraoocyte cAMP levels can be elevated in sheep oocytes by cholera toxin without suppressing GVBD (Crosby *et al.*, 1985). In any event, a preponderance of evidence suggests that cAMP may be one of the components of an intricate system employed in the maintenance of meiotic arrest in the oocyte.

C Cumulus cell-oocyte communication

The zona pellucida imposes a physical barrier between the germinal and somatic compartments and discrete hydrophilic channels, known as gap junctions, provide the only means for bidirectional metabolic and ionic coupling between the two cell types. As described in the previous section, an inhibitory factor(s) transferred from the somatic cumulus cells to the oocyte is responsible for maintaining the oocyte in meiotic arrest. It was proposed that the endogenous preovulatory surge of LH dismantles oocyte-cumulus cell junctions and blocks the transfer of this suppressive factor, resulting in a fall in intraoocyte cAMP levels and consequent resumption of meiosis (Lindner *et al.*, 1974; Dekel & Beers, 1978; Gilula *et al.*, 1978; Moor *et al.*, 1980b; Larsen *et al.*, 1986). Indeed, studies in the rat and hamster indicate that metabolic uncoupling between oocytes and cumulus cells precedes GVBD (Dekel *et al.*, 1984; Dekel & Sherizly, 1985; Racowsky & Satterlie, 1985). However, several other studies have provided evidence to the contrary. In sheep oocytes, levels of cAMP do not decline at the onset of maturation but rather increase significantly at this time, due to gonadotropins acting on the follicular cells (Moor & Heslop, 1981; Crosby *et al.*, 1985). Studies in which ionic coupling (Dekel *et al.*, 1981) or choline or uridine transport (Moor *et al.*, 1980b; Eppig, 1982a) was measured indicate that the disruption of intercellular junctional contacts between the oocyte and follicle cells follows rather than precedes GVBD. Furthermore,

whilst FSH suppressed oocyte-cumulus cell coupling without inducing meiosis in ovine oocytes, LH induced GVBD in oocytes without affecting coupling with cumulus cells (Moor *et al.*, 1981). However, transport of ions, choline or uridine from cumulus cells to the oocyte does not necessarily translate into the transfer of inhibitory factor(s). There may be some quantitative or qualitative changes in the inhibitory signal from the cumulus cells to the oocyte preceding GVBD (Moor *et al.*, 1981). While it is clear that junctional associations of oocytes with cumulus cells are essential for maintaining their meiotic arrest *in vivo*, some measure of confusion still prevails surrounding the disruption of intercellular coupling by itself being a physiological trigger for the resumption of meiosis.

D Steroids

The role of steroids in the control of mammalian oocyte maturation is not yet fully resolved. The observations that the preovulatory gonadotropin surge induces a prompt rise in follicular steroid genesis (reviewed by Lindner *et al.*, 1974; Channing & Tsafriri, 1977) led to investigations of a possible role of steroids in the mediation of gonadotropin-stimulated oocyte maturation. However, the inhibition of the LH-induced rise in steroidogenesis did not block the stimulatory effect of this gonadotropin on the resumption of meiosis in rat oocytes (Lieberman *et al.*, 1976; Billig *et al.*, 1983), unsupportive of such an intermediatory role for these hormones. Rather, steroid hormones have been shown to block spontaneous maturation of oocytes *in vitro* in a number of species (McGaughey, 1977; Eppig & Koide, 1978; Rice & McGaughey, 1981; Racowsky, 1983), and this effect appears to be receptor-mediated (Barrett & Powers, 1993). The maturation-inhibiting action of dbcAMP on porcine oocytes can be augmented by androgens (Rice & McGaughey, 1981; Racowsky, 1983; Daniel *et al.*, 1986). Similarly, estradiol can directly inhibit spontaneous maturation of pig (McGaughey, 1977; Racowsky & McGaughey, 1982b) and hamster (Racowsky, 1985c) oocytes. However, it is difficult to ascertain whether this effect of estradiol is physiological or not, since estradiol inhibited porcine oocyte maturation only in the

absence of exogenous protein(s), an unphysiological state (Racowsky & McGaughey, 1982c). Addition of progesterone or testosterone can also elevate the inhibitory effects of cholera enterotoxin, forskolin, FSH or suboptimal concentrations of dbcAMP on maturation of denuded or cumulus-enclosed mouse oocytes (Eppig *et al.*, 1983; Schultz *et al.*, 1983b).

In vivo, the inhibition of oocyte maturation by follicular steroids may be important in delaying meiotic maturation to allow better synchrony of nuclear and cytoplasmic maturational processes. Indeed, evidence exists that follicular steroids play a vital role in the acquisition of developmental competence of oocytes (Moor *et al.*, 1980a; Osborn & Moor, 1983b), including the synthesis of male pronucleus growth factor (MPGF) (Thibault *et al.*, 1975; Moor, 1978; Fukui *et al.*, 1982). Supplementation of culture media during IVM with steroids, especially estradiol, together with gonadotropins improved nuclear and cytoplasmic maturation of sheep (Moor & Trounson, 1977; Moor *et al.*, 1980a) and cattle (Fukui *et al.*, 1982) oocytes. Moreover, the induction of selective alterations in the intrafollicular steroid environment with the use of specific inhibitors of steroidogenesis, resulted in anomalous nuclear and cytoplasmic maturation, manifested as gross abnormalities at fertilization (Moor *et al.*, 1980a; Osborn & Moor, 1983b; Osborn *et al.*, 1986). Inhibition of the 20α-hydroxycholesterol dehydrogenase with aminoglutethimide phosphate (AGP) terminated follicular steroidogenesis and led to a high incidence of abnormal sperm decondensation after penetration (reviewed by Moor *et al.*, 1983). The 17α-hydroxylase inhibitor (SU 10603) blocked androgen and estrogen production and greatly enhanced the production of progesterone and its metabolites. Under these altered steroid conditions the ability of the sperm to penetrate after attachment was totally impaired. However, the addition of exogenous steroids in the presence of the inhibitor increased the proportion of oocytes undergoing normal fertilization to about 50% Taking all the above information into consideration, it appears that steroids and gonadotropins may play separate but complementary roles during oocyte maturation.

The evidence from some *in vitro* studies suggests that steroids may mediate

actions of gonadotropins on cytoplasmic maturation of oocytes. The beneficial effect of FSH during IVM on subsequent fertilization of rat oocytes was abolished when steroidogenesis was blocked with AGP (reviewed by Armstrong *et. al.*, 1991). Subsequently, it was demonstrated that the addition of progesterone alone, at physiological concentrations during IVM, could substitute effectively for FSH in increasing the fertilization rate (Zhang & Armstrong, 1989).

E Mullerian inhibiting substance

Based on the observations that bovine mullerian inhibiting substance (MIS) inhibited GVBD in denuded and cumulus-enclosed rat oocytes *in vitro* (Takahashi *et al.*, 1986a), it was suggested that MIS may act as an oocyte meiosis inhibitor. In the cow, MIS has been detected in the follicular fluid of mature ovaries by RIA (Vigier *et al.*, 1984; Necklaws *et al.*, 1986) and was immunolocalized in the granulosa cells throughout reproductive life (Takahashi *et al.*, 1986b). The evidence from rat oocyte maturation studies in which addition of exogenous EGF during IVM reversed the inhibition of GVBD by MIS, and from other studies using cell lines (Ueno *et al.*, 1988 and other references cited there), indicates that MIS exerts its effects through interference with autophosphorylation of EGF receptors. Further studies are required to elucidate the exact role of MIS in oocyte and follicular physiology.

2.3.2 Stimulants of Meiosis

A Gonadotropins

As mentioned elsewhere, the role of gonadotropins as the major external trigger of *in vivo* maturation and ovulation is well established. However, their role during spontaneous maturation of cumulus-enclosed oocytes *in vitro*, including the impact on subsequent fertilization and embryonic development, has not been completely clarified. The bulk of information has come from studies with rodents, and in most of these studies oocytes were obtained from immature donors pretreated with gonadotropic hormones, usually PMSG, which possesses both FSH and LH activity. The balance of evidence

from these studies seems to suggest a beneficial effect of gonadotropins on oocyte maturation both *in vivo* and *in vitro*.

Luteinizing hormone, immunochemically pure FSH, or hCG can trigger the resumption of meiosis in rat follicles explanted on the day of proestrus. All of these hormones induce an immediate rise in cAMP accumulation in granulosa cells and a somewhat later increase in progesterone synthesis (Tsafriri *et al.*, 1972, 1973; Lindner *et al.*, 1974). Similar results were obtained by the culture of preovulatory follicles of immature PMSG-treated rats (Hillensjo, 1976). Gonadotropin-stimulated maturation of follicle-enclosed oocytes was also observed in sheep (Hay & Moor, 1973), rabbits (Thibault & Gerard, 1973), macaque monkeys (Thibault *et al.*, 1975), and hamsters (Gwatkin & Andersen, 1976).

Similar to the follicle-enclosed oocytes, FSH, but not LH or hCG, stimulated the resumption of meiosis in cumulus-enclosed mouse oocytes (Downs & Eppig, 1985; Downs *et al.*, 1988). The effect of FSH on GVBD was at first inhibitory and later stimulatory, and was not associated with a decline in metabolic coupling. This biphasic effect of FSH was attributed to the different thresholds of cAMP required for meiotic arrest and its stimulation, and will be discussed in detail under mechanisms of gonadotropin actions in Section 2.4. Eppig and Schroeder (1989) also found that addition of FSH during IVM of mouse oocytes after their *in vitro* growth in the presence of IBMX increased the incidence of GVBD and first polar body formation. The inability of LH or hCG to induce maturation in mouse OCC was attributed to the presence of only limited LH receptors on cumulus cells compared to the mural granulosa cells (Downs *et al.*, 1988). Since LH is the primary gonadotropin implicated with meiotic induction *in vivo* and is able to induce maturation in follicle enclosed oocytes *in vitro* (see above), it was proposed that mural granulosa cells mediate the stimulatory effect of LH on oocyte maturation (Downs *et al.*, 1988). However, in the rat, LH has been shown to induce resumption of meiosis in cumulus-enclosed oocytes kept *in vitro* in meiotic arrest with a low molecular weight fraction of porcine follicular fluid (reviewed by Tsafriri *et al.*, 1982). These different effects of LH in the rat and the mouse may be due to species

differences.

In addition to their stimulatory effects on spontaneous maturation, gonadotropins have also been shown to influence cytoplasmic maturation. Supplementation of the culture medium with high concentrations of LH greatly increased the developmental ability of sheep oocytes cultured outside the follicle (Crosby *et al.*, 1981). During IVM of bovine oocytes obtained from immature follicles, addition of FSH and LH together with estradiol to the culture media increased the proportion of embryos that cleaved normally *in vitro* and *in vivo* after fertilization *in vitro* (Sirard *et al.*, 1988). On the other hand, follicular oocytes from a gonadotropin-deficient mutant strain of mouse (hpg/hpg) displayed retarded meiotic maturation, and markedly reduced rates of fertilization and development to blastocyst stage in culture. However, when the hypogonadal mice were primed with PMSG *in vivo*, these deficiencies disappeared (Schroeder & Eppig, 1989). Since hypogonadal mice used in these studies lack both FSH and LH, and PMSG contains activity for both of the gonadotropins, it is difficult to draw conclusions regarding which of these two pituitary gonadotropins is responsible for the beneficial effect *in vivo*.

Efforts have also been made to elucidate the roles of the separate gonadotropins in oocyte maturation. Addition of FSH to the culture medium during IVM of oocytes from gonadotropin-primed (PMSG) mice increased the frequency of preimplantation development (Downs *et al.*, 1986; Schroeder *et al.*, 1988). Studies with the rat have provided further information to clarify the roles of FSH and LH separately on cytoplasmic maturation of oocytes, as assessed by their developmental potential *in vivo* after IVM. The rat oocytes that were matured *in vitro* as OCC in the presence of LH exhibited greater frequency of development *in vivo* upon transfer to recipients than those matured in the absence of this hormone (Shalgi *et al.*, 1979). Subsequently, using a similar approach, Dekel *et al* (1988) demonstrated that both FSH and LH could be equally effective in this regard given that the oocytes were used from small and large follicles of PMSG primed rats. However, only FSH, but not LH, was effective when the source of oocytes was only small follicles from nonprimed immature rats. Therefore,

it appears that the efficacy of gonadotropins is determined by the state of differentiation of cumulus and granulosa cells with respect to FSH and LH receptors. By looking at the fertilization and cleavage potentials of oocytes obtained from adult rats at different stages of the oestrous cycle, Armstrong *et al* (1991) provided further evidence to support the role of FSH in oocyte cytoplasmic maturation. The oocytes obtained on the days of proestrus and estrus, when circulating FSH levels are highest, yielded higher fertilization and cleavage rates than those obtained on the days of metestrus and diestrus, when FSH levels are low (Butcher *et al.*, 1974).

B Maturation-promoting factor (MPF) and cytostatic factor (CSF)

Extensive research using amphibian and star-fish model systems has demonstrated that an activity known as MPF appears in the cytoplasm of the oocyte that is responsible for the transition of the oocyte from the G phase to the M Phase (reviewed by Masui & Clarke, 1979; Masui & Shibuya, 1987). It is now well established that this MPF brings about GVBD and the subsequent maturational events in oocytes of amphibians (Masui & Markert, 1971; Smith & Ecker, 1971), starfish (Kishimoto & Kanatani, 1976), mouse (Sorensen *et al.*, 1985; Hashimoto & Kishimoto, 1988), and pig (Fulka, 1983; Fulka *et al.*, 1985; Mattioli *et al.*, 1991). Such a factor was also shown to be a regulator of the cell cycle in mitotically dividing somatic cells of many species, ranging from yeast to man (Sunkara *et al.*, 1979; Nelkin *et al.*, 1980; Kishimoto *et al.*, 1982; Tachibana *et al.*, 1987). Since somatic cell extracts can induce GVBD in the meiotically incompetent oocyte (Balakier, 1978; Sunkara *et al.*, 1979), it seems likely that both mitotically dividing somatic cells and meiotically dividing germ cells share a common MPF. Furthermore, transfer of ooplasm from meiotically mature oocytes of one species into meiotically incompetent oocytes of another species induce maturation in the recipient oocytes (Kishimoto & Kanatani, 1977; Dettlaff & Felgengauer, 1980; Kishimoto *et al.*, 1982, 1984), indicating a species-nonspecific nature of MPF.

Purified MPF from *Xenopus* oocytes has been shown to consist of two major peptides of 34 and 45 kDa (Lohka *et al.*, 1988). The 34-kDa protein is the homolog product of the fission yeast cell cycle control gene $cdc2^+$ and is a serine/threonine protein

kinase, whose activity, using histone H1 as a substrate, increases as the cell enters M phase (Draetta & Beach, 1988; Dunphy *et al.*, 1988; Gautier *et al.*, 1988; Labbe *et al.*, 1988). On the other hand, the 45 kDa protein probably corresponds to *Xenopus* cyclin (Langan *et al.*, 1989) and as in other cell systems is complexed with $p34^{cdc2}$ (Booher *et al.*, 1989; Draetta *et al.*, 1989). In the prophase-arrested *Xenopus* oocytes, MPF is present in an inactive form called pre-MPF (Cyert & Kirschner, 1988).

It is generally believed that the levels of the active form of MPF in the ooplasm oscillate according to the stage of the cell cycle, with peaks at each metaphase in the oocytes of starfish (Doree *et al.*, 1983), amphibians (Gerhart *et al.*, 1984), mice (Hashimoto & Kishimoto, 1988), and pigs (Mattioli *et al.*, 1991). Thus it would appear that the inductive signal for oocyte maturation entails activation of MPF resulting in chromatin condensation, GVBD, and spindle formation in maturing oocytes. In this respect, studies with starfish (Clark & Kanatani, 1975), cow (Jagiello *et al.*, 1978) and pig (Kubelka *et al.*, 1988) oocytes suggest that the first step involves the post-translational modification of pre-existing MPF molecules by proteolysis resulting in its activation. This activated pre-existing MPF, however, is capable of only promoting chromatin condensation, whereas the ability of MPF to disintegrate the nuclear membrane (GVBD) requires new protein synthesis and its subsequent proteolysis (Kuberka *et al.*, 1988). The regulation of MPF by various factors which influence nuclear and cytoplasmic maturation of mammalian oocytes both *in vivo* and *in vitro* is unknown.

After completion of the first meiotic division, the oocyte is arrested at MII stage until sperm penetration or parthenogenetic activation. Observations in *Rana pipiens*, *Xenopus laevis*, and mouse oocytes suggest that a cytoplasmic factor, called "cytostatic factor" (CSF), may mediate this meiotic arrest by stabilizing MPF (Masui & Markert, 1971; Balakier & Czolowska, 1977; Meyerhof & Masui, 1979; Newport & Kirschner, 1984; Sagata *et al.*, 1989). Its activity remains high in the unfertilized oocyte until soon after fertilization, when it gets degraded (Meyerhof & Masui, 1977, 1979; Sagata *et al.*, 1989). Although the chemical nature of CSF is not fully clear, some evidence suggests

that it is a product of the c-mos proto-oncogene, a protein uniquely expressed in meiotic cells (Sagata *et al.*, 1989). Biochemical studies of Xhao *et al.* (1991) on the interaction of c-mos protein with tubulin suggests that c-mos/CSF may be integrally involved in regulating microtubule organization during meiotic maturation. This concept is further supported by results from c-mos anti-sense injection experiments in which depletion of c-mos in maturing mouse oocytes following polar body emission, lead to signs of parthenogenetic activation (O'Keefe *et al.*, 1989). Thus it appears that MPF and CSF regulate oocyte maturation with the cyclical change in their activities coinciding with the stage of nuclear maturation in the oocyte. Nevertheless, the relationship between MPF, CSF and the hormonal induction of oocyte maturation both *in vivo* and *in vitro*, particularly in large domestic species, remains to be explored.

C Role of calcium

Several studies in invertebrates and lower vertebrates have suggested that the control points of the cell division cycle in oocytes, eggs, and embryos are triggered by increases in intracellular calcium (Poenie *et al.*, 1985; Swann & Whitaker, 1986; Kleis-San Francisco & Schuetz, 1987; Kline, 1988; Smith, 1989; Steinhardt, 1990; Whitaker & Patel, 1990). However, such a role for calcium in resumption and subsequent progression of meiosis in the mammalian oocyte has not yet been clearly established. Verapamil, a calcium transmembrane channel blocker, exerts a synergistic effect with dbcAMP in maintaining mouse oocytes in meiotic arrest (Powers & Paleos, 1982). In addition, calcium levels, as revealed by measurements of total intracellular calcium, increase in rat oocytes prior to onset of GVBD (Batta & Knudsen, 1980). Furthermore, calcium ionophore (A_{23187}) stimulates meiotic resumption in follicle-enclosed rat oocytes (Tsafriri & Bar-Ami, 1978) and can override dbcAMP-maintained meiotic arrest in mouse (Powers & Paleos, 1982) and hamster (Racowsky, 1986) oocytes. However, the results from these ionophore experiments should be interpreted with caution, since this ionophore not only increases intracellular calcium to nonphysiological levels but can also act as a magnesium ionophore at high concentrations (Campbell, 1983).

Recent studies have provided some convincing evidence to implicate calcium in

the regulation of meiotic maturation. Direct blockage of intracellular calcium transients with either BAPTA or Quin2, two chelators of intracellular calcium, inhibited GVBD in cow (Homa, 1991), mouse (De Felici *et al.*, 1991) and pig (Kaufman & Homa, 1993) oocytes. In the pig study, it was concluded that mobilization of intracellular calcium is essential for the initiation of GVBD and that external calcium influx is not a direct requirement for GVBD, although it may be needed for the progression of meiosis past metaphase I. This idea is further supported by the observations that microinjection of inositol trisphosphate, which mobilizes calcium from intracellular stores (Rickords & White, 1993), relieves cAMP-maintained meiotic arrest in bovine oocytes (Homa *et al.*, 1991). However, to further clarify the role of this ion in the regulation of oocyte maturation, a spatial and temporal analysis of free intracellular calcium levels during different stages of meiotic maturation is required.

D Role of peptide growth factors in oocyte maturation

The involvement of peptide growth factors in follicular regulation has received much attention in recent years, and there are several reports of stimulatory effects on oocyte maturation primarily with rodent oocytes. Epidermal growth factor stimulated maturation of rat oocytes cultured within follicles (Dekel and Sherizly, 1985) and prevented inhibition of rat oocyte maturation *in vitro* by mullerian inhibiting substance (Ueno *et al.*, 1988). Epidermal growth factor also induced GVBD in isolated cumulus cell-enclosed mouse oocytes in which meiotic arrest was maintained with purines, cAMP, or IBMX (Downs *et al.*, 1988). Since both EGF and FSH elevated intracellular cAMP levels in OCC and had no effect on GVBD in denuded oocytes, the authors suggested that the hormones generate a positive signal in cumulus cells that acts upon the oocyte to stimulate GVBD. This suggestion is further supported by the observations that acute, but not chronic, exposure of follicle-enclosed oocytes to dbcAMP can stimulate GVBD (Hillensjo *et al.*, 1978; Dekel *et al.*, 1982). It was therefore postulated that oocyte maturation is modulated according to the duration and magnitude of elevated cAMP levels, and this aspect will be discussed in details in Section 2.4. In addition to EGF,

some other growth factors have also been shown to influence resumption of meiosis by the oocyte. Not surprisingly, TGF-α, which binds to the EGF receptor, augmented meiotic maturation of cumulus cell-enclosed mouse oocytes *in vitro* (Brucker *et al.*, 1991). The effects of TGF-β on the resumption of meiosis in the oocyte are conflicting. Feng *et al* (1988) found TGF-β to stimulate the maturation of both follicle- and cumulus cell-enclosed rat oocytes. However, this group failed to demonstrate effective stimulation of oocyte maturation by TGF-β when meiotic arrest was maintained with inhibitors such as cAMP or IBMX. On the other hand, Tsafriri *et al.* (1989) found no effect of TGF-β on spontaneous maturation of rat oocytes when added alone, but it partially suppressed the LH-stimulated GVBD in cultured follicles. In the mouse, TGF-β had no effect on the spontaneous maturation of cumulus-enclosed oocytes (Downs, 1989). Although not as potent as EGF, insulin and fibroblast growth factor were also found to stimulate GVBD in mouse oocytes (Downs, 1989).

In addition to their effects on spontaneous nuclear maturation, some preliminary reports suggest that growth factors may also influence oocyte cytoplasmic maturation. Epidermal growth factor improved the developmental potential of *in vitro* matured bovine oocytes to the eight-cell stage (Coskun *et al.*, 1991) and to the blastocyst stage (Harper & Brackett, 1993). A recent report in the pig (Ding & Foxcroft, 1994) indicated a beneficial effect of EGF on pronuclear formation, when added with gonadotropins during IVM; EGF alone, however, had no such effect. Evidence from porcine IVF studies in our own laboratory indicate that the addition of insulin-like growth factor-I (IGF-1) during IVM is beneficial to early embryonic development (Xia *et al.*, 1994). All these data suggest that these small peptides may play an important role in the acquisition of full developmental potential by the oocyte.

2.3.3 Signal Transduction Pathways in Oocyte Maturation

A In gonadotropin regulation

The mechanism by which the resumption of meiosis in the oocyte is brought about by gonadotropins is not yet fully clear. Prevailing opinion holds that, as in many other

cell types, the cAMP-dependent protein kinase A (PKA) is the second messenger pathway for gonadotropin-induced oocyte maturation (Dekel & Kraicer 1978; Eppig, 1979a; Downs *et al.*, 1988). Both LH and FSH stimulated oocyte maturation in rat follicle-enclosed oocytes and augmented cAMP accumulation in the follicle (reviewed by Tsafriri, 1978). Furthermore, microinjection of dbcAMP into the follicle antrum induced the resumption of meiosis of the oocytes within the injected follicles (Tsafriri *et al.*, 1972). In isolated mouse OCC, FSH-stimulated spontaneous oocyte maturation was accompanied by increased intracellular cAMP accumulation (Downs *et al.*, 1988). Since FSH had no effect on spontaneous maturation in denuded oocytes, it was concluded that this gonadotropin generates a positive signal in the cumulus cells to induce maturation in the oocyte. That the gonadotropins can reverse dbcAMP-induced meiotic inhibition in cumulus-enclosed rat oocyte but not in denuded oocytes (Dekel & Beers, 1978, 1980), further supports the concept that the inductive signal is generated in the cumulus cells. This is also consistent with the observation that the gonadotropin receptors are present on cumulus cells but not on the oocyte (Amsterdam *et al.*, 1975).

However, cAMP has also been implicated with the maintenance of meiotic arrest in the oocyte as the membrane-permeable analogs of cAMP, such as dbcAMP and 8-bromo-cAMP, or the inhibitors of PDE, such as IBMX and theophylline inhibit spontaneous maturation in the oocyte (reviewed by Wassarman & Albertini, 1994). In addition, agents that increase cAMP levels by activating adenylate cyclase, such as forskolin or FSH, can also maintain the oocyte in meiotic arrest (reviewed by Eppig & Downs, 1984; Schultz, 1986). Furthermore, microinjection of an inhibitor of cAMP-dependent PKA initiates meiosis in oocytes maintained in GV stage with dbcAMP or IBMX (Bornslaeger *et al.*, 1986). In addition, a cAMP antagonist, Rp-AMPS, has been shown to reverse the meiotic inhibition maintained either by exogenous inhibitors (Downs, 1993), or by granulosa cells on *in vitro* grown, meiotically competent oocytes (Eppig, 1991a), resulting in GVBD. Therefore, it appears that cAMP is involved with the maintenance of meiotic arrest in the oocyte rather than with the onset of gonadotropin-stimulated GVBD. Indeed, a decrease in oocyte cAMP has been shown to

precede GVBD in the rat (Racowsky, 1984) and the mouse (Schultz *et al.*, 1983a).

In vitro studies with the mouse have helped to provide some clarification to this paradoxical situation. Follicle-stimulating hormone produced first an inhibition of oocyte maturation, followed by a stimulation of GVBD when cumulus-enclosed mouse oocytes were cultured in the presence of maturation inhibitors such as dbcAMP (Downs & Eppig, 1985; Downs *et al.*, 1988). This transient inhibition of oocyte maturation by FSH was correlated with a transient rise in cAMP in the OCC (Schultz *et al.*, 1983b). Further increase in cAMP levels by FSH was, however, correlated with the stimulation of GVBD. It was, therefore, proposed that the initial inhibitory action of FSH on oocyte maturation may be the result of elevated cAMP levels and that once the amount of cAMP reaches the maturation threshold, a positive signal is generated in cumulus cells which make this gonadotropin stimulatory (Downs & Eppig, 1985; Downs *et al.*, 1988). Consistent with this idea was the induction of GVBD by a brief exposure of OCC to a high concentration (1 mM) of the dbcAMP (Downs *et al.*, 1988). Similarly, spontaneous oocyte maturation is stimulated by acute, but not chronic exposure of explanted follicles in culture to dbcAMP (Hillensjo *et al.*, 1978; Dekel *et al.*, 1981). Thus the evidence suggests that oocyte maturation is modulated according to the duration and magnitude of elevated cAMP levels.

B In paracrine and autocrine regulation

As evident from the discussion in Section 2.3.2 D, EGF is the only member among autocrine-paracrine factors that has clearly been implicated with the stimulation of GVBD. The role of other members in this preovulatory process is either not fully resolved or is controversial. Therefore signal transduction pathways pertaining only to EGF will be discussed in this section. To my knowledge, the mechanism of EGF-induced oocyte maturation has not yet been fully investigated. In general, EGF stimulation of the intrinsic tyrosine kinase activity of its receptor is considered the most likely event in the generation of second messengers (Oberg *et al.*, 1990). EGF stimulation of the tyrosine kinase has been shown to result in hydrolysis of phosphatidyl

inositol-4, 5-bisphosphate to form inositol-1, 4, 5-trisphosphate and diacylglycerol (DAG) in A-431 cells (Wahl & Carpenter, 1988). These products in turn liberate calcium from endoplasmic reticulum and activate calcium-dependent protein Kinase C (PKC) respectively (Abdel-Latif, 1986). However, tyrosine kinase stimulation is not always an exclusive event in the generation of second messengers by EGF. Bovine luteal cells possess an aberrant form of EGF-R which is devoid of intrinsic TK activity (Chakravorty *et al.*, 1993). The ligand activates other signalling systems, such as mitogen activated protein (MAP) kinase, by modulating critical post-receptor phosphorylation/dephosphorylation events in the luteal cells. Furthermore, EGF stimulated cAMP accumulation in mouse OCC, indicating that EGF-induced oocyte maturation may, in fact, be mediated through cAMP-dependent PKA pathway (Downs *et al.*, 1988). This cAMP stimulation in OCC is consistent with observations in A-431 cells where activation of the EGF-R resulted in increased intracellular cAMP levels (Ball *et al.*, 1990). Like FSH, EGF generated a positive signal in the cumulus cells to induce oocyte maturation as it had no effect on GVBD in denuded oocytes. All these data suggested that EGF and FSH may share the same cAMP-PKA pathway to induce oocyte maturation. This concept is further supported by the observations that EGF and FSH had no additive or synergistic actions to stimulate GVBD (Downs *et al.*, 1988).

However, not all the actions of EGF on mouse OCC are consistent with those of FSH. For instance, FSH stimulation of cAMP was much greater (15-fold) compared to that by EGF (4-fold). The effect of EGF on oocyte maturation was stimulatory at all the times studied, whereas the effect of FSH was at first inhibitory and later stimulatory (Downs *et al.*, 1988). Furthermore, EGF actions in follicle cells are often inconsistent with those of cAMP. For instance, EGF failed to stimulate cAMP synthesis in rat granulosa cells and antagonized FSH-stimulated cAMP accumulation (Knecht & Catt, 1983). In addition, EGF attenuated cAMP-mediated LH receptor induction in rat and pig granulosa cells (Schomberg *et al.*, 1983) and inhibited FSH-, dbcAMP- or cholera toxin-stimulated steroidogenesis in rat granulosa cells (Hsueh *et al.*, 1981). Clearly, further studies are needed to elucidate the signal transduction pathways for EGF-induced

oocyte maturation.

2.4 CUMULUS EXPANSION

2.4.1 Description of the Process

Following the preovulatory gonadotropin surge, the cumulus cells surrounding the oocyte undergo a dramatic conformational change. The compact layers of cumulus cells expand due to deposition of mucoelastic material in the extracellular matrix. The ultrastructure of the mucified matrix is a fibrillar network of which hyaluronic acid, a nonsulfated glycosaminoglycan, is an integral component (Yudin *et al.*, 1988). Specific hyaluronidases alter this network (Talbot, 1984; Yudin *et al.*, 1989) and dissociate the cumulus mass into individual cells (McClean & Rowlands, 1942). Cumulus expansion *in vitro,* but not hyaluronic acid synthesis, was shown to be dependent upon the presence of some high molecular weight (> 10,000 daltons) component of serum (Eppig, 1979a, 1980a). When the freshly isolated mouse OCC were cultured with FSH in the absence of fetal calf serum (FCS), the mucoelastic matrix did not form. Under these culture conditions, cumulus cells lost contact with each other and settled individually on the culture dish. However, total hyaluronic acid synthesis by FSH-stimulated OCC remained unaltered (Eppig 1980a; Salustri *et al.*, 1989). In the presence of FCS, ~ 80% of the synthesized hyaluronic acid is retained in the matrix, whereas the same proportion is released into the medium in the absence of FCS. It was, therefore, suggested that the role of serum in cumulus expansion *in vitro* is to promote the retention of hyaluronic acid within the OCC (Eppig, 1980a). The serum factor has recently been identified as a protein belonging to the inter-α-trypsin inhibitor family (IαI) with an apparent Mr 150,000 (Chen *et al.*, 1992). Addition of purified IαI at 1 μg/ml enabled cumulus cells to expand *in vitro* in response to FSH in the absence of serum, while OCC failed to expand when the serum was depleted of IαI, further confirming the identity of the factor. This factor was detected in mouse follicular fluid collected 6 hours after hCG injection to stimulate ovulation, but not in unstimulated mice (Chen *et al.*, 1992). Immunocytochemical staining, using anti-human IαI IgG, also revealed anti-IαI-positive

epitopes in the cumulus extracellular matrix of mouse preovulatory follicles. Recently, Camaioni *et al* (1993) reported that the serum factor acts as a structural component of the matrix and that its specific binding to hyaluronic acid is essential for successful organization of the OCC matrix.

In the pig, FSH-induced cumulus expansion *in vitro* was shown to be accompanied by significant changes in biosynthetic activities of the OCC (Ball *et al.*, 1985). Overall protein synthesis was markedly increased and the total DNA synthesis was significantly reduced, while the RNA synthesis remained unchanged. The authors, however, cautioned that the lack of a significant increase in RNA synthesis in response to FSH should not be interpreted as evidence that the observed increase in protein synthesis is mediated solely by post-transcriptional mechanisms. Rather, the lack of a significant effect of FSH on RNA accumulation was attributed to the fact that the measured uridine incorporation reflected the synthesis of stable RNA species (principally rRNA) rather than mRNAs. However, by using α-amanitin, an inhibitor of heterogeneous nuclear RNA synthesis, Meinecke and Meinecke-Tillmann (1993) have recently shown that *de novo* mRNA synthesis within the cumulus cells is essential for gonadotropin-induced porcine cumulus expansion *in vitro*. It appeared that the mRNA synthesis was completed within the first two hours of culture as the addition of α-amanitin after this period had no effect on gonadotropin-induced cumulus expansion. Thus the failure to detect a significant increase in mRNA synthesis in the earlier study (Ball *et al.*, 1985) may be due to the longer culture period (24 hours) used for RNA measurements.

The time sequence of cumulus expansion in relation to maturational changes in the oocyte varies among species. In the mouse (Eppig, 1980b) and rat (Dekel *et al.*, 1979) it follows, but in the pig (Motlik *et al.*, 1986) it precedes the onset of GVBD. *In vivo*, this morphological transformation of cumulus cells is temporally associated with a reduction in the number of gap junctions between oolemma and cumulus cells leading to decreased ionic and metabolic coupling with the oocyte (Gilula *et al.*, 1978; Heller & Schultz, 1980; Eppig, 1982a). The induction of cumulus cell expansion, however, is not a prerequisite for loss of coupling between the germ cell and somatic cell compartments.

For instance, although FSH is a very effective uncoupling agent *in vitro* (Moor *et al.*, 1981; Eppig, 1982b), oocyte-cumulus cell uncoupling *in vitro* does not require FSH-stimulated cumulus expansion (Eppig & Ward-Bailey, 1982; Salustri & Siracusa, 1983). A recent study in the cow (Sutovsky et al., 1993) has provided further insight into the gap junctional communication during cumulus expansion *in vitro*. The connexin 43-positive gap junctions localized between corona radiata cell projections and oolemma disappeared shortly before the expansion of the cumulus became fully detectable morphologically. Concomitantly, the OCC lost the ability to transfer a fluorescent dye, Lucifer Yellow, from cumulus cells to the oocyte. However, the functional gap junctions among the cumulus cells were maintained until the second meiotic arrest. Both the changes in corona-oocyte coupling and cumulus expansion were preceded by the redistribution of F-actin in the cytoplasm of cumulus cells. These changes in F-actin distribution were attributed to create and maintain, rather than disrupt, functional intercellular communication among cumulus cells during expansion. Thus it appears that, although communication is lost between the oocyte and cumulus cells during cumulus expansion, functional communication is maintained among the expanded cumulus cells.

2.4.2 Regulation of Cumulus Expansion

Cumulus expansion is triggered *in vivo* by the endogenous preovulatory surge of gonadotropins. Cumulus expansion can also be induced in PMSG-primed animals by the injection of hCG (Dekel *et al.*, 1979; Eppig, 1980b; Leibfried & First, 1982). *In vitro,* cumulus expansion can be induced in isolated OCC by FSH (Eppig, 1979a), EGF (Downs, 1989), cAMP analogs (Buccione *et al.*, 1990), forskolin (Prochazka *et al.*, 1991), or prostaglandin E_2 (Eppig, 1981a). Eppig (1979a, 1980b) has consistently shown that purified LH is unable to induce cumulus expansion in the mouse *in vitro*. However, studies in the rat (Hillensjo *et al.*, 1976; Dekel & Kraicer, 1978) and in the pig (Hillensjo & Channing, 1980) have indicated that LH is capable of inducing cumulus expansion in these species *in vitro*. This difference in the rat and pig, and the mouse

may, therefore, be attributed to species specificity. Recently, mitogenic lectins, such as concanavalin A, have been shown to induce cumulus expansion in mouse OCC *in vitro* (Fagbohun & Downs, 1990). Since non-mitogenic lectins such as wheat germ agglutinin had no effect on cumulus expansion, it was suggested that the lectin-generated signal(s) for cumulus expansion may be similar, or identical, to the stimulus that mediates the mitogenic action of these ligands. While lectins cannot be considered physiological inducers of cumulus expansion, they nevertheless may serve as useful tools in elucidating the mechanism(s) involved. In general, due to its consistent and potent effects, FSH is used more commonly than any other agent for carrying out cumulus expansion studies *in vitro* (Buccione *et al.*, 1990; Salustri *et al.*, 1990; Prochazka *et al.*, 1991).

Although FSH is present in the follicular fluid of developing Graafian follicles and is potentially available to the cumulus cells prior to the preovulatory gonadotropin surge, the cumulus cells do not expand before the surge. Furthermore, the cumulus cells do not expand when the immature mice are primed with PMSG even though the isolated OCC are capable of expanding in response to the PMSG *in vitro* (Eppig, 1980b). It was proposed in the mouse that some component of the antral follicle inhibits the response of the cumulus cells until after the LH surge (Eppig, 1980b). Since LH in the mouse is unable to induce expansion by itself (Eppig, 1979a, 1980b), it was further postulated that LH acts indirectly to stimulate cumulus expansion *in vivo* by terminating the inhibitory action of the antral follicle that prevents the cumulus expansion in response to FSH. Although LH is able to induce expansion by itself in species such as the pig and the rat (Hillensjo *et al.*, 1976; Dekel & Kraicer, 1978; Hillensjo & Channing, 1980), the follicular FSH is unable to induce expansion before the LH surge even in these species. Therefore, it is tempting to believe that a similar inhibitory action of the follicle may also prevail in these species.

Based on the observations that the sulfated glycosaminoglycans (GAGs) inhibit FSH-stimulated cumulus expansion and hyaluronic acid synthesis in the isolated mouse OCC *in vitro*, it was suggested that these GAGs may function *in vivo* to regulate the action of FSH on cumulus cells (Eppig, 1981b). The relative potencies of sulfated GAGs

in inhibiting FSH-stimulated cumulus expansion and hyaluronic acid synthesis were heparin > heparin sulfate ~= chondroitin sulfate B > chondroitin sulfate C > chondroitin sulfate A (Eppig, 1981b). Since GAGs also inhibited dbcAMP-stimulated cumulus expansion, it was concluded that GAGs inhibit FSH-stimulated cumulus expansion at some step after the generation of intracellular cAMP (Eppig, 1981c). Chondroitin sulfate B (dermatan sulfate) and heparin-like molecules, particularly heparin sulfate, are the principal GAGs produced by rat granulosa cells (Yanagishita & Hascall, 1979). In the pig, GAGs were found in the follicular fluid at concentrations in the range of 1 to 5 mg/ml and their concentrations declined dramatically during follicular maturation (Ax & Ryan, 1979). Furthermore, LH significantly decreased the synthesis of sulfated GAGs *in vitro* (Gebauer *et al.*, 1978), further supporting the hypothesis that the LH surge *in vivo* may alleviate the follicular inhibition, thereby allowing FSH to induce cumulus expansion. Although the above data support a role for GAGs in the regulation of cumulus expansion *in vivo,* clearly further studies are needed to implicate these chemicals more precisely in this preovulatory process.

Recent studies indicate that the oocyte participates in cumulus cell expansion *in vitro* in the mouse by enabling the cumulus cells to expand in response to a hormonal stimulus. This aspect of cumulus expansion will be discussed in detail in Chapter 6.

2.4.3 Signal Transduction Pathways in Cumulus Expansion

A In gonadotropin regulation

Like oocyte maturation, gonadotropin-stimulated cumulus expansion is believed to be mediated through cAMP-dependent PKA pathway (Dekel & Kraicer, 1978; Eppig, 1979a). This belief is largely based on the observations that FSH treatment of OCC *in vitro* induces a quick rise in intracellular cAMP levels (Salustri *et al.*, 1985; Downs *et al.*, 1988; Buccione *et al.*, 1990; Prochazka *et al.*, 1991). In addition, membrane permeable analogs of cAMP, activators of adenylate cyclase, and the inhibitors of PDE also stimulate hyaluronic acid synthesis in OCC *in vitro* (Salustri *et al.*, 1989). All these observations indicate a direct role for cAMP in the mediation of gonadotropin-stimulated

cumulus expansion. Although it appears that the gonadotropins stimulate the preovulatory processes of both oocyte maturation and cumulus expansion through a cAMP-dependent pathway, *in vitro* studies have demonstrated that the two physiological events are not causally related. For instance, addition of sulfated GAGs to FSH-treated mouse OCC completely suppressed cumulus expansion, yet failed to prevent spontaneous oocyte maturation (Eppig, 1981b, c). Future studies are needed to determine if oocyte maturation and cumulus expansion are mediated through separate transduction signals downstream from the cAMP generation or if those events reflect different thresholds for the same signal.

As previously mentioned, PGE_2 stimulates cumulus expansion in the isolated mouse OCC *in vitro* (Eppig, 1981a). In the follicle, PGE_2 has been shown to be produced both by the theca and the granulosa cells (Evans *et al.*, 1983). In addition, the gonadotropins stimulate prostaglandin synthesis by the granulosa cells (Erickson *et al.*, 1977; Clark *et al.*, 1978; Evans *et al.*, 1983). Based on this information, it was hypothesized that PGE_2 may be an obligatory intermediate in the FSH-stimulated hyaluronic acid synthesis by the cumulus cells both *in vivo* and *in vitro* (Eppig, 1981a). However, indomethacin, a prostaglandin synthetase inhibitor, failed to inhibit FSH-stimulated cumulus expansion in isolated mouse OCC (Eppig, 1981a), eliminating the possibility of any such intermediary role for PGE_2.

B In autocrine-paracrine regulation

Out of ten known growth factors tested, only EGF was able to stimulate cumulus expansion in the mouse OCC *in vitro* and was more potent in this regard than FSH (Downs, 1989). The failure of the other growth-promoting agents to provoke cumulus expansion demonstrated a profound specificity for EGF and suggested a physiological role for the growth factor in this preovulatory event. However, other than that described in Section 2.3.3 B under EGF-induced oocyte maturation, no separate information is available on the signal transduction pathways for EGF-induced cumulus expansion. As in the case of gonadotropins, *in vitro* studies have clearly demonstrated that EGF

stimulates oocyte maturation and cumulus expansion via different mechanisms. Although heparin completely suppressed EGF-stimulated cumulus expansion in mouse OCC *in vitro*, it had no effect on EGF-stimulated GVBD (Downs, 1989). Consistent with this idea are the observations that although several other growth factors stimulated GVBD, only EGF brought about cumulus expansion (Downs, 1989). Clearly, further studies are needed to determine if EGF-stimulated oocyte maturation and cumulus expansion are mediated by separate transduction signals or if those events reflect different thresholds for the same signal.

2.4.4 Possible Physiological Significance of Cumulus Expansion

The process of cumulus expansion and its physiological significance to reproduction in mammals is a subject of current interest. The "quality" of the cumulus, particulary the degree of its expansion, is often cited as a major criterion for the selection of oocytes for IVF protocols both in human and farm animals (Ball *et al.*, 1983; Laufer *et al.*, 1984; Foote, 1987). For normal ovulation to occur, optimal expansion of the cumulus mass appears to be essential. For instance, the inhibition of cumulus expansion with intraperitoneal injection of 6-diazo-5-oxo-1-norleucine (DON) inhibited ovulation in PMSG-primed immature mice that were stimulated to ovulate (Chen *et al.*, 1993). The increased hyaluronic acid synthesis during cumulus expansion may promote the detachment of OCC from the membrana granulosa, as has been suggested in the case of detachment of 3T3 BALB cells from the substratum in culture (Abatangelo *et al.*, 1982). The hyaluronic acid-rich expanded cumulus and the development of a semisolid mucoid mass may also provide a vehicle required for protrusion of the OCC from the follicle (Chen *et al.*, 1993). After ovulation, the expanded cumulus surrounding the oocyte provides a large mass capable of being picked up and transported by the fimbria of the oviduct (Mahi-Brown & Yanagimachi, 1983).

In the fallopian tubes, the mucified matrix and other cumulus cell secretory products appear to provide an appropriate environment for sperm penetration and fertilization (Gwatkin *et al.*, 1972; Schuetz & Dubin, 1981; De Felici & Siracusa, 1982;

Meizel, 1985). Anecdotal evidence reported over many years suggests that the presence of expanded cumulus plays some role in the acrosome reaction and capacitation of the fertilizing spermatozoa (Ball *et al.*, 1983; Tesarik, 1985; Mattioli *et al.*, 1988a; Stock *et al.*, 1989), thereby improving their functional efficiency (Soupart & Morgenstern, 1973). Components of the expanded cumulus matrix might serve as a filter, weeding out abnormal spermatozoa that are unable to swim strongly and might lead to maldevelopment (reviewed by Yanagimachi, 1994). The expanded cumulus may impart physiological stability or viability to the oocyte by delaying aging of the oocyte (Marston & Chang, 1963; Longon, 1980), and/or preventing hardening of the zona pellucida (Gianfortoni & Gulyas, 1985). Other proposed functions of the cumulus include enhancement of sperm motility, facilitation of fertilization by offering a greater target area for spermatozoa to act, preventing acrosome reacted spermatozoa from swimming away from the zona surface, and providing mechanical aid during sperm penetration by inhibiting zona rotation with the cumulus (reviewed by Yanagimachi, 1994). Further support to the role of expanded cumulus in fertilization and sperm penetration comes from the observations that the preincubation of ovulated mouse OCC with different concentrations of anti-OCC specific to the preovulatory cumulus oophorus led to dose-dependent impairment of fertilization rates as well as to a decrease in the number of spermatozoa attached to the zona pellucida (Amiel *et al.*, 1993). In addition to the effects on sperm penetration and fertilization, increased developmental potential has been ascribed to the expansion of cumulus oophorus (Ball *et al.*, 1983; Fukui, 1990). A close correlation between the expansion of cumulus mass and the rate of development to the two-cell stage following IVF was observed in the mouse (Chen *et al.*, 1993). Furthermore, the inhibition of hyaluronic acid synthesis and expansion of the cumulus extracellular matrix *in vivo* reduced the fertility of these *in vivo* matured oocytes (Chen *et al.*, 1993). Although, the precise cellular and molecular mechanisms underlying positive effects of cumulus expansion on embryonic development are not known, it appears that the normal expansion of cumulus supports the cytoplasmic maturation of the oocyte.

In spite of the evidence discussed above, some investigators consider the cumulus as unnecessary junk. This belief is based on the observations that the eggs of some eutherian species do not have a cumulus or have only remnants of the cumulus oophorus at the time of fertilization, and that the oocytes of most species can be fertilized *in vitro* without an intact cumulus or cumulus components (Fraser *et al.*, 1971; Fraser, 1983; Mahadevan & Trounson, 1985). However, since expansion of the cumulus is a physiological event *in vivo* that accompanies the release of the oocyte during ovulation in most mammalian species, it is tempting to believe that the expanded cumulus plays a beneficial role during fertilization.

2.5 *IN VITRO* FERTILIZATION (IVF)

The strongest motivation for development of systems for fertilization and production of embryos *in vitro* in domestic animals comes from the realization that development of the biotechnologies for producing transgenic offspring or for multiplication *in vitro* of lines of genetically superior offspring are dependent on volume production of precisely staged embryos with high developmental potential. In the human, the development of suitable IVF systems provides the only hope for infertile couples to fulfil their desire for babies. The whole process of IVF includes a complex series of events including the maturation, capacitation and activation of the sperm and its union with the female gamete whose details are beyond the scope of this discussion. However, a brief summary of the process of fertilization as it occurs *in vivo* will help to understand the results from IVF experiments in the later part (Chapter 9) of the thesis.

2.5.1 Sperm Capacitation, Acrosome Reaction, Formation of Pronuclei, and Syngamy

In order to successfully penetrate oocytes, mature spermatozoa must undergo two types of maturational changes: capacitation and the acrosome reaction. Capacitation normally occurs in the female reproductive tract following ejaculation and the term itself refers to the conditioning or sensitization of spermatozoa. Capacitation can also be accomplished *in vitro* in chemically defined media (reviewed by Yanagimachi, 1994).

There has been much speculation about the conditions or factors which directly control capacitation in the female reproductive tract *in vivo*. *In vitro*, some changes associated with capacitation have been identified including changes in intracellular ions, adenylate cyclase-cAMP systems, metabolism, the nucleus, and the plasma membrane (reviewed by Yanagimachi, 1994). However, it is not clear which of these changes is necessary for capacitation.

The anterior portion of the sperm nucleus is covered by a membrane-bound, cap-like structure called the acrosome. During the sperm-egg interaction, there is an orderly breakdown of the plasma membrane and the outer acrosomal membrane leading to the formation of vesicles and fenestrations. Membrane fenestrations allow the release of lytic enzymes, particularly important are hyaluronidase and acrosin, which apparently facilitate the entry of the sperm into the oocyte (reviewed by Yanagimachi, 1994). In the rabbit, this process can occur within 30 minutes but varies among species (Singhas & Oliphant, 1978). While the ability of the zona pellucida to induce the acrosome reaction is undisputed (reviewed by Meizel, 1985), some evidence also suggests that components of cumulus cells may promote this reaction in mammalian spermatozoa (Bavister, 1982; Tasarik, 1985; Westrick *et al.*, 1985). Although many factors have been reported to facilitate the acrosome reaction *in vitro* (see Yanagimachi, 1994 for details), there is a general agreement that calcium ions are essential (Yanagimachi & Usui, 1974).

Acrosome-reacted spermatozoa appear to use both mechanical and enzymatic means to cross through the zona pellucida (reviewed by Yanagimachi, 1988). Once through the zona, the equatorial membrane of the sperm head fuses with the oolemma. The process of sperm-egg fusion is concluded by the gradual incorporation of first the sperm head and then the entire tail (reviewed by Gaddum-Rosse, 1985). The entry of the spermatozoon activates the oocyte resulting in the exocytosis of cortical granule contents and the re-initiation of meiosis (for the second time). Cortical granules are small membrane-bound vesicles which contain hydrolytic enzymes and saccharide components (Gulyas, 1980). They exist in the cortex of mature unfertilized eggs of all mammalian species studied so far including the human (reviewed by Yanagimachi, 1994). Once liberated, the contents of cortical granules act on the zona and alter its physical and

chemical characteristics whereby the zona becomes impenetrable to other spermatozoa. This alteration in the nature of the zona is called the zona reaction and is responsible for blocking polyspermy in such species as hamsters, dogs, sheep, humans and pigs (Austin & Braden, 1956; Barros & Yanagimachi, 1972; Wolf, 1981; Sathananthan & Trounson, 1985; Cran & Cheng, 1986). The oocyte plasma membrane seems to present an alternative mechanism to block polyspermy in species such as the rabbit and mole (Austin & Braden, 1956; Wolf, 1981).

Within ooplasm, the nuclear envelope of the sperm head disintegrates and the sperm nucleus decondenses resulting in its transformation into the male pronucleus. The transformation of the sperm nucleus appears to depend on a putative cytoplasmic factor, called male pronucleus growth factor (MPGF) (Thibault & Gerard, 1973; Yanagimachi, 1981). Meanwhile the reinitiation of meiosis in the oocyte results in the haploid nucleus (with the extrusion of the second polar body) which gets transformed into the female pronucleus. Eventually, the male and female pronuclei migrate towards each other, their nuclear envelopes disintegrate and their chromosomes intermix resulting in a diploid zygote. The fusion of male and female pronuclei, called syngamy, marks the end of fertilization and the beginning of embryonic development.

2.5.2 Developmental Potential Following IVF with Particular Reference to the Pig

The first report of fertilization of mammalian oocytes *in vitro* validated by the birth of offspring was provided in the rabbit by Chang in 1959 where he inseminated tubal ova with uterine-capacitated spermatozoa. Since then successful IVF has been achieved in the hamster (Yanagimachi & Chang, 1963), mouse (Iwamatsu & Chang, 1969), cat (Hamner *et al.*, 1970), guinea pig (Yanagimachi, 1972), squirrel monkey (Gould *et al.*, 1973), rat (Miyamoto & Chang, 1973), dog (Mahi & Yanagimachi, 1976), cow (Iritani & Niwa, 1977) and pig (Iritani *et al.*, 1978). Although human oocytes were fertilized *in vitro* much earlier (Edwards *et al.*, 1969), the birth of Louise Brown in July, 1978 marked the first successful case in the human where an oocyte recovered from a

women's ovary was fertilized *in vitro*, developed in culture and developed to term after transfer to the uterus (Steptoe & Edwards, 1978).

In spite of success in achieving fertilization *in vitro*, the whole IVF procedure is still faced with some serious limitations and there is much room for improvement in the future. Generally oocytes that are matured *in vitro* exhibit much lower fertilization rates and survive to viable offspring in proportions much lower than those of *in vivo* matured oocytes (Cross & Brinster, 1970; Niwa *et al.*, 1976; Moor & Trounson, 1977; Shalgi *et al.*, 1979; Leibfried-Rutledge *et al.*, 1987). In addition, experiments involving IVF frequently lack reproducibility of results (Blandau, 1980; Quigley, 1980). Furthermore, comparisons of results among different IVF reports are difficult as the end points vary greatly. While monospermy and male pronucleus formation are essential for normal development, they do not accurately predict the quality of the resulting embryo. The use of early cleavage stages alone is not sufficient to assess fertilization accurately since parthenogenetic activation or fragmentation can confound the evaluation. The birth of live offspring is obviously the most definitive proof of successful fertilization *in vitro*, but is prohibitively costly. Furthermore, the frequency of obtaining live offspring following IVF is very low in many species and thus can not provide any meaningful comparison. The evaluation of proportions of oocytes/embryos yielding pregnancies following IVF is also very difficult as the various investigators publish their pregnancy rates in different ways. Depending upon attractiveness, results are published as per treatment cycle, per laparoscopy/ultrasound or per embryo transfer.

In spite of the inherent limitations mentioned above, IVF has met with a promising success in some species such as mice and hamsters (reviewed by Rogers, 1978). The recent development of techniques for *in vitro* oocyte maturation, fertilization and embryo coculture in the cow has allowed production of preimplantation embryos in numbers sufficient to afford biochemical analyses that were previously possible only with embryos of laboratory species such as the mouse (Sirard *et al.*, 1988; Fukui & Ono, 1989; Kim *et al.*, 1990; Wiemer *et al.*, 1991; Rose & Bavister, 1992). Such progress in the bovine system offers some optimism that improvement of the culture conditions

may yield similar IVF results in other species where the success of IVF is now questionable.

The pig may prove to be an excellent model to produce large scale embryos *in vitro* both for research and commercial purposes. The large size of the ovaries and the fact that multiple ovulations are physiological in this species make it feasible to obtain relatively large number of OCC. Suitable tissue is readily available from abattoirs: gilts (nulliparous females) are normally slaughtered shortly before their first ovulation, providing a relatively synchronized population of follicles without the complication of variations in stages of the estrous cycle. However, despite continuous efforts, *in vitro* maturation and fertilization in the pig has met with a very limited success. Polyspermy is a major problem encountered in pig oocytes matured and fertilized *in vitro*, and as high as 80% of the oocytes have been reported to be polyspermic in some studies (Cran and Cheng, 1986; Mattioli *et al.*, 1988b, 1989). Furthermore, only a very low proportion of these *in vitro* matured and fertilized oocytes are capable of transforming sperm into the male pronucleus (Nagai *et al.*, 1984; Mattioli *et al.*, 1988b; Yoshida *et al.*, 1990; Nagai, 1994). These problems seem to result from deficiencies in the culture media used for IVM leading to inadequate maturation of the oocyte. Indeed reports using media with different compositions indicate that the selection of IVM media influences the outcome of porcine IVF (Wang *et al.*, 1992; Yoshida *et al.*, 1992a; Nagai, 1994). Supplementation of IVM media with follicular fluid also improved the fertilizing and subsequent developmental capacity of porcine oocytes *in vitro* (Yoshida *et al.*, 1992b). The importance of an adequate culture system is further revealed by the observations that the presence of whole follicular wall or isolated follicular cells in the IVM medium improve fertilization, embryonic development and subsequent developmental capacity in this species (Mattioli *et al.*, 1988b; Nagai *et al.*, 1993). Thus it would appear that the somatic cells secrete undefined factor(s) into the culture medium which facilitate oocyte maturation and subsequent embryonic development. Deficiencies in oocyte maturation *in vitro* are further revealed by the facts that the *in vivo* matured porcine oocytes exhibit less incidence of chromosomal anomalies and polyspermy, and higher

rates of development following IVF than those matured *in vitro* (Yoshida *et al.*, 1990)

Recent efforts to optimize culture media for oocyte maturation has greatly improved the success of IVM-IVF in the pig. Full developmental potential of pig embryos derived from oocytes matured and fertilized *in vitro* has been demonstrated (Yoshida *et al.*, 1993). However, the proportion of piglets from IVM-IVF oocytes is extremely low and much further research aimed at refining culture conditions is needed before later stages of embryos such as blastocysts can be obtained on a reliable and reproducible basis.

CHAPTER 3 RATIONALE

Mammalian ovaries contain hundreds of thousands of germ cells at birth, the majority of which never ovulate, being lost before puberty or in adult life by atretic processes at various stages of follicle development. In domestic animals, this excess supply of female germ cells is a huge potential source of genetic material not currently being used efficiently in selection of traits of economic importance, due to the lack of efficient and reliable IVM-IVF systems. Although it is possible to obtain morphologically normal secondary oocytes with expanded cumuli oophori on a large scale *in vitro*, the functional normality of these oocytes is not assured. Evidence suggests that such oocytes often fail to transform sperm into the male pronucleus and their ability to support subsequent embryonic development is limited compared to oocytes matured *in vivo*. These deficiencies have been shown to result from inadequate cytoplasmic maturation of oocytes undergoing spontaneous maturation *in vitro*, even though apparently normal nuclear maturation is observed. Therefore, a more efficient utilization of this enormous reproductive wastage will depend upon the improvement of techniques for maturing oocytes *in vitro*. Identification of factors which influence various aspects of oocyte maturation including initiation of meiosis, induction of cumulus cell expansion, and completion of nuclear and cytoplasmic maturation is essential to optimize the culture media. *In vivo* oocyte maturation takes place in an intrafollicular milieu that is determined by the surrounding somatic cells of the follicle. The importance of somatic cells in oocyte maturation is also exemplified *in vitro* by the evidence that the presence of somatic follicular cells during IVM increased fertilizability and subsequent developmental potential of oocytes following IVF. Thus it would appear that the somatic cells secrete undefined factor(s) which facilitate the oocyte maturation and subsequent embryonic development. Indeed, increasing evidence has appeared which indicates that somatic follicular cells are the site of production, secretion and action of several different autocrine-paracrine factors. Although a number of growth factors has been implicated in ovarian physiology, EGF was the only one of ten known growth factors tested in a

pilot study in the mouse, which stimulated oocyte maturation and cumulus cell expansion *in vitro* (Downs, 1989). Similar results were obtained in the rat, suggesting the specificity for this growth factor in these preovulatory processes. However, there is very little or no information about EGF regulation of oocyte maturation and cumulus expansion in domestic species such as the pig. Furthermore, most of these studies in rodents were pilot studies in which effects of exogenous EGF were tested only on resumption of meiosis and cumulus cell expansion in isolated OCC. This, being only one aspect of oocyte maturation, by no mean indicates the completion of nuclear and cytoplasmic maturation. In addition, there is virtually no information regarding the interactions of this growth factor with other substances present in the follicular environment, like gonadotropins and ovarian steroids which are known to influence oocyte maturation. Neither is there any information on influences of EGF on cytoplasmic maturation of oocytes, its mechanisms of action, or its local production in the ovarian follicle. The present study was aimed to investigate, in detail, the role of EGF in the regulation of oocyte maturation and cumulus cell expansion in the pig using an *in vitro* model system. The pig has several advantages as a model for these studies. The overall process of meiotic maturation in the pig takes considerably longer than in rodents offering greater opportunities to determine the accurate temporal sequence of intermediate events. The large size of the ovaries and the fact that multiple ovulations are physiological in this species make it feasible to obtain OCC in relatively large numbers. Nulliparous females (gilts) are normally slaughtered before their first ovulation and thus provide a relatively synchronized population of follicles.

The specific aims of this research project were:

1) To examine the effects of EGF and its various combinations with gonadotropins and ovarian steroids on the initiation of meiotic maturation and cumulus cell expansion.
2) To determine the role of the oocyte in EGF-induced cumulus cell expansion.
3) To test local follicular production of EGF and the presence of its receptors in the different components of the ovarian follicle by detecting the mRNAs and immunoreactive peptides.

4) To elucidate the mechanisms of EGF- and FSH-induced cumulus expansion and meiotic maturation.

5) To investigate the influence of EGF on cytoplasmic maturation of the oocyte as revealed by the success of fertilization and by the changes in the pattern of protein synthesis in the oocyte and cumulus cells.

6) In addition to EGF, TGF-α, another ligand for EGF receptor, has been studied extensively for its potential roles in follicular cell functions. Like EGF, it is a potent stimulant of meiotic maturation in cumulus enclosed mouse oocytes (Brucker *et al.*, 1991). Therefore, it was considered important to test follicular production of TGF-α by localizing its mRNA and the peptide.

CHAPTER 4 GENERAL MATERIALS AND METHODS

4.1 Isolation and Culture of Oocyte-Cumulus Cell Complexes (OCC)

For all experiments, ovaries were collected from prepubertal gilts at JM Schneider Inc., Kitchener, Ontario, washed in sterile saline and transported to the laboratory at room temperature in HEPES-buffered Hanks balanced salt solution (HBSS) containing 1% (w/v) bovine serum albumin (BSA). Intact OCC were isolated by rupturing 4-6 mm non-atretic follicles (follicles which appeared translucent, but not opaque, when light was shone through them) in HBSS under a stereoscopic microscope. Only oocytes that were completely surrounded by at least 4-5 layers of unexpanded cumulus oophorus cells and had a homogeneous cytoplasm were selected for the experiments. The OCC were washed with the culture medium, pooled and then cultured in 35 mm plastic dishes containing 2 ml of medium and between 25-40 OCC per dish. Culture medium was prepared according to the description of Tsafriri and Channing (1975b) with few modifications and consisted of Medium 199 with Earle's salts (Gibco, Burlington, Ontario) supplemented with 25 mM HEPES buffer (Gibco), 15% (v/v) fetal calf serum (Gibco or Flow, Mississauga, Ontario), 1 mM L-glutamine (Gibco), 0.03 mM sodium pyruvate (Gibco), 2.5 mM sodium lactate (Fisher Scientific, Unionville, Ontario) and 100 μg/ml gentamycin (Gibco). Incubations were carried out at 39°C in an atmosphere of 95% air and 5% CO_2 for different time periods depending upon the experimental protocol.

4.2 Assessment of Cumulus Expansion

The cumulus expansion was scored on the commonly used subjective scale of 0 to 4 (Downs, 1989; Buccione *et al.*, 1990) in which 0 indicates no observable expansion; +1 indicates outer 1-2 layers expanded; +2 indicates outer half of the cumulus expanded; +3 indicates all layers expanded other than last layers of corona radiata and +4 indicates all layers expanded including corona radiata.

4.3 Assessment of Oocyte Maturation, Fertilization and Pronuclei Formation

In the experiments with GVBD as the end point, the cumulus cells were removed from oocytes by repeated pipetting using a fine-bore pipette (~ 100 μm) in the presence of 0.1% hyaluronidase (bovine testis: Sigma Chemical Co., St. Louis, MO). For the fertilized oocytes, any attached cumulus or adhering excessive sperm were removed with a fine-bore pipette. The oocytes were then washed in Dulbecco's phosphate-buffered saline (DPBS; Gibco) to remove any media protein. The oocytes were examined as whole mounts after fixation and staining using a modification of Tarkowski's technique (1971), which I have adapted for porcine oocytes. Briefly, the oocytes were fixed in Heidenchein fixative (2:2:1; saturated aqueous solution of mercuric chloride: MilliQ water: formalin alkanized with calcium carbonate) for five minutes or longer, washed with DPBS and mounted on a precleaned microscopic slide in a drop of egg white (egg white filtered through a nylon gauze and containing 0.01% thimerosal can be stored in a refrigerator up to many months). Excess of egg white was carefully withdrawn with a mouth pipette and the remainder holding oocytes was fixed with alcohol vapour under a stereoscopic microscope by inverting the slide on a 35 mm plastic dish containing absolute ethanol (ETOH). The slides were then immersed in 50% ETOH for at least 5 minutes (although at this point, if necessary, the slides can be stored for up to a week) and stained with haematoxylin for 2-3 minutes. The desired staining colour (pink or blue) was obtained by lowering the slides in two coplin jars containing a few drops of concentrated HCL and NH_3 respectively in 50% ETOH. Thereafter, the slides were treated as regular histological slides. The slides were dehydrated through ascending grades of alcohol (70%, 95% and two changes in 100% ETOH, each change for five minutes) and stored in xylene for at least overnight. The slides were mounted with permount and examined under oil immersion. This technique was preferred over all the staining procedures in current use for the highly pigmented pig oocyte (Hunter & Polge, 1966; McGaughey & Polge, 1971; Motlik & Fulka, 1976; Daniel *et al.*, 1986; Mattioli *et al.*, 1988b) as it gave permanent preparations and higher accuracy of nuclear and fertilization events.

CHAPTER 5 EFFECTS OF EGF ON THE RESUMPTION OF MEIOSIS AND CUMULUS CELL EXPANSION

5.1 Introduction

As mentioned previously in Chapters 2 and 3, *in vitro* studies with rodents have implicated EGF as a potent stimulant of the resumption of meiosis and cumulus cell expansion. EGF stimulated maturation of rat oocytes cultured within follicles (Dekel & Sherizly, 1985) and also prevented inhibition of rat oocyte maturation *in vitro* by mullerian inhibiting substance (Ueno *et al.*, 1988). It also induced GVBD in isolated cumulus cell-enclosed mouse oocytes in which meiotic arrest was maintained with purines, cAMP, or IBMX (Downs *et al.*, 1988). In fact, EGF was the only one of ten known growth factors tested in the mouse, which stimulated oocyte maturation and cumulus cell expansion *in vitro* (Downs, 1989), suggesting the specificity for this growth factor in these preovulatory processes. Transforming growth factor-alpha, which binds to the EGF receptor, also augmented meiotic maturation of cumulus cell-enclosed mouse oocytes (Brucker *et al.*, 1991). However, there is very little or no information about EGF regulation of oocyte maturation and cumulus expansion in domestic species such as the pig. Furthermore, the oocyte is exposed *in vivo* to an ever-changing milieu of gonadotropins, steroids, growth factors, and other molecules, any or all of which may interact to regulate maturational changes that occur in the oocyte and its surrounding cumulus cells during the periovulatory period. However, to date no study looking at the interactions among growth factors, gonadotropins and steroids in the regulation of oocyte maturation and cumulus expansion has been undertaken for any species. Within the follicle, the activity of EGF appears to be closely linked to gonadotropins and ovarian steroids. EGF has an antagonistic effect on FSH-induced formation of both LH receptors and estrogen biosynthesis (Hsueh *et al.*, 1981; Jones *et al.*, 1983; Schomberg *et al.*, 1983; Steinkampf *et al.*, 1988; Mason *et al.*, 1990). On the contrary, FSH appears to induce and maintain EGF receptors in the follicle (Siebers *et al.*, 1985; Feng *et al.*, 1987; Buck and Schomberg, 1988; Fujinaga *et al.*, 1992). These observations strongly

suggest that interactions among EGF, gonadotropins and ovarian steroids may play a crucial role in the determination of the overall course of oocyte development.

The present study was undertaken to examine the effects of EGF and its various combinations with FSH, LH, androstenedione (A_4), and estradiol-17β (E_2) on the initiation of meiotic maturation and cumulus expansion in the pig *in vitro*. These factors were chosen for the study based on reports of either their presence in the ovary or effects on oocyte maturation. The pig system is of particular interest in that meiotic resumption takes a longer time than in rodents and maturation-related changes are easily analyzed without adding the meiotic inhibitors routinely used in rodent studies.

5.2 Materials and Methods

The OCC were isolated and cultured as described in Chapter 4. The culture were supplemented with various combinations of human recombinant EGF (Gibco), FSH (NIH-oFSH-14), LH (USDA-bLHB5) (both generously provided by NIH hormone distribution office, Baltimore, MD), and A_4 and E_2 (both from Sigma Chemical Co.). At the end of the culture period, the cumulus expansion was recorded and the oocytes examined for GVBD, using the protocols outlined in Chapter 4. The oocytes were classified according to the description of Hunter and Polge (1966) as having an intact germinal vesicle (GVI) or having undergone germinal vesicle breakdown (GVBD). Each experiment was replicated at least three times to confirm consistency of results. Data were assessed by analysis of variance (ANOVA) using arcsin transformation of the proportions (Snedecor, 1956). Differences between means for multiple comparisons were assessed by Duncan's New Multiple Range test (Steel & Torrie, 1960). For presentations, data were expressed as percent mean $\pm$ the standard error of the mean (SEM).

5.3 Results

5.3.1 Establishment of a suitable culture period

In order to test the stimulatory effect of any substance on the resumption of meiosis in the oocyte, it is important to keep the GVBD levels in the control groups as

low as possible. This experiment was, therefore, designed to determine the time-course of initiation of nuclear maturation in porcine oocytes cultured in the absence of any hormonal supplementation. The OCC were cultured in the medium containing only serum for 12, 24 and 44 hours as previously described. The oocytes were examined for the status of the germinal vesicle after removal of the cumulus cells. As can be seen in Text Figure 5-1, large proportions of the oocytes remained in GVI stage until 24 hours of culture. There was no significant difference in the proportions of oocytes undergoing GVBD between 12 and 24 hour culture periods. However, after 44 hours, more than 50% of the oocytes had undergone GVBD even in the absence of any hormonal treatment. Based on these observations, a 24 hour culture period was used for testing the effects of various hormones on the resumption of meiosis in the remainder of this Chapter.

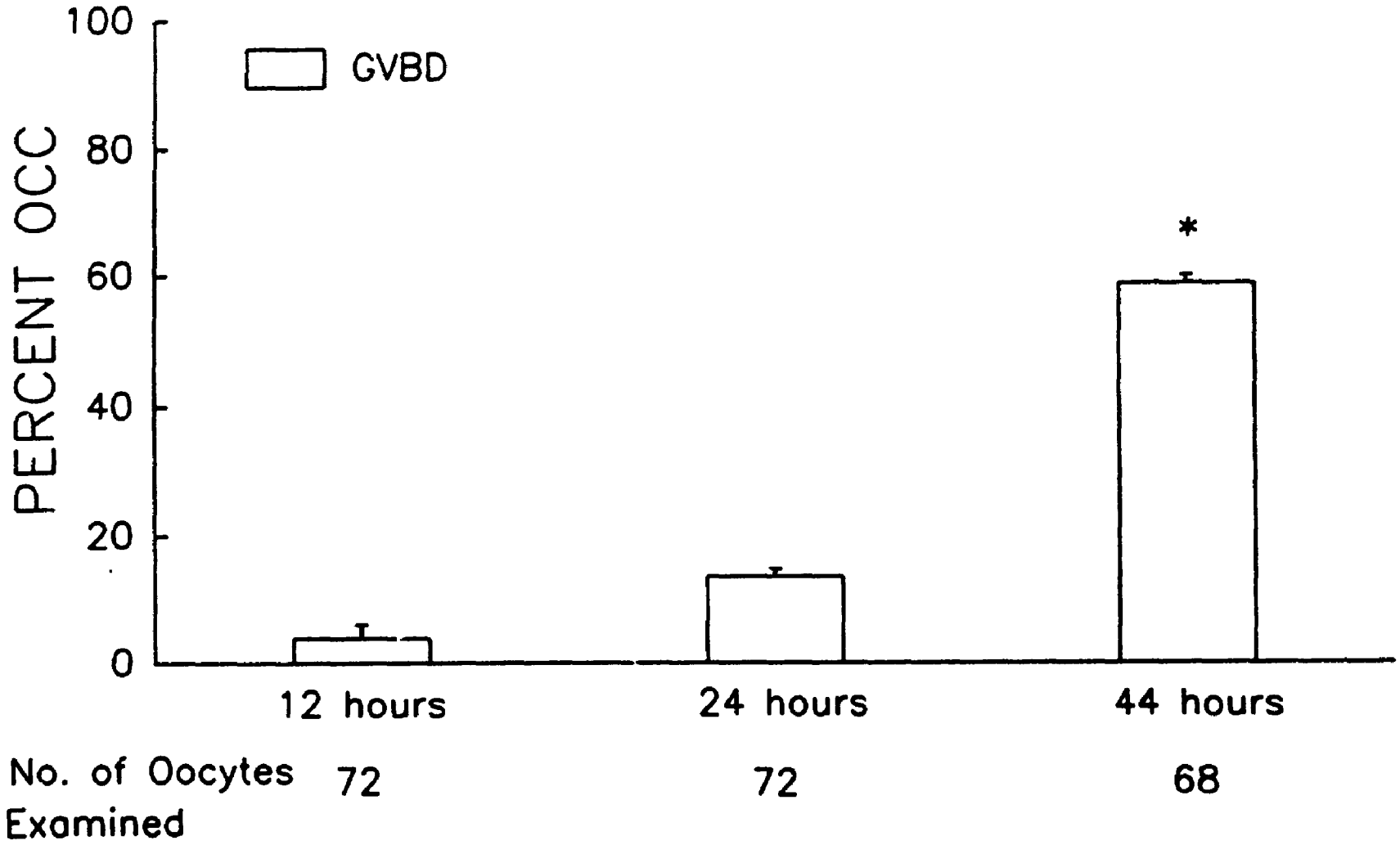
5.3.2 Effects of EGF on oocyte maturation and cumulus expansion

In this experiment, OCC were cultured with different concentrations of EGF from 1 pg/ml to 10 ng/ml for 24 hours and examined for GVBD and cumulus expansion. As shown in Text Figure 5-2, EGF stimulated GVBD in a concentration dependent fashion. A significantly higher proportion of oocytes exhibited GVBD at 100 pg/ml (37%) compared to the control (12%), with maximal stimulation at 1 ng/ml (56%). The maximal degree of cumulus expansion observed after 24 hours of culture was +3 (Figure 5-1). Due to the low number of complexes exhibiting +1 and +2 degrees of cumulus expansion, only the complexes with +3 expansion were used for comparisons among various hormonal treatments. A significantly higher percentage of OCC exhibited +3 expansion in the presence of 1 ng or 10 ng/ml EGF compared to other groups (Table 5-1). No OCC exhibited +3 expansion when cultured in the control medium without EGF.

5.3.3 Effects of FSH, LH and E_2 on oocyte maturation and cumulus expansion

In this experiment, the effects of different combinations of FSH, LH and E_2 on the resumption of oocyte maturation and cumulus expansion were observed. This was done in a factorial experiment with the factors being 1) gonadotropins (control, FSH,

Text Figure 5-1. Time-course of resumption of meiosis in porcine oocytes cultured in the absence of any hormonal supplementation. Oocyte-cumulus cell complexes were incubated in the medium containing only serum for 12, 24 and 44 hours, and assessed for GVBD. Data represent percent mean $\pm$SEM of three independent experiments. Asterisk indicates significant difference ($P < 0.05$) compared to the other groups.


100
80
60
40
20
0
PERCENT OCC
GVBD
*
12 hours
24 hours
44 hours
No. of Oocytes Examined
72
72
68

Text Figure 5-2. Concentration-response study of EGF effect on GVBD. Oocyte-cumulus cell complexes were incubated in the medium containing varying concentrations of EGF, and GVBD was assessed after 24 hours. Data represent percent mean $\pm$SEM of three independent experiments. Asterisks indicate significant difference ($P < 0.05$) compared to the control.

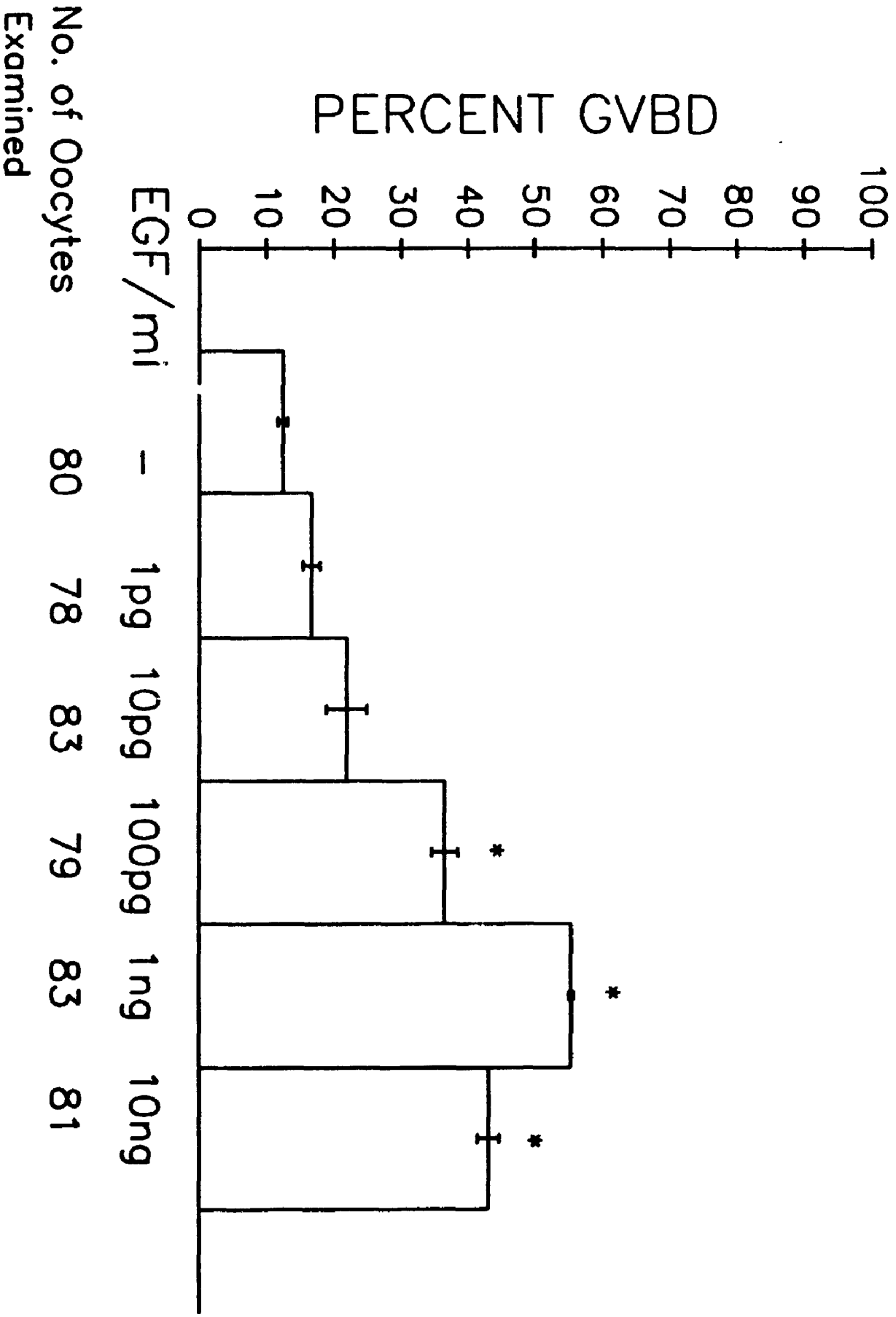

PERCENT GVBD
0
10
20
30
40
50
60
70
80
90
100
EGF/ml
No. of Oocytes Examined
–
80
1pg
78
10pg
83
100pg
79
1ng
83
10ng
81
*
*
*

Figure 5-1. Epidermal growth factor-induced cumulus expansion *in vitro*. Oocyte-cumulus cell complexes were cultured for 24 hours in the control medium (A) or in medium containing 1 ng/ml EGF (B).

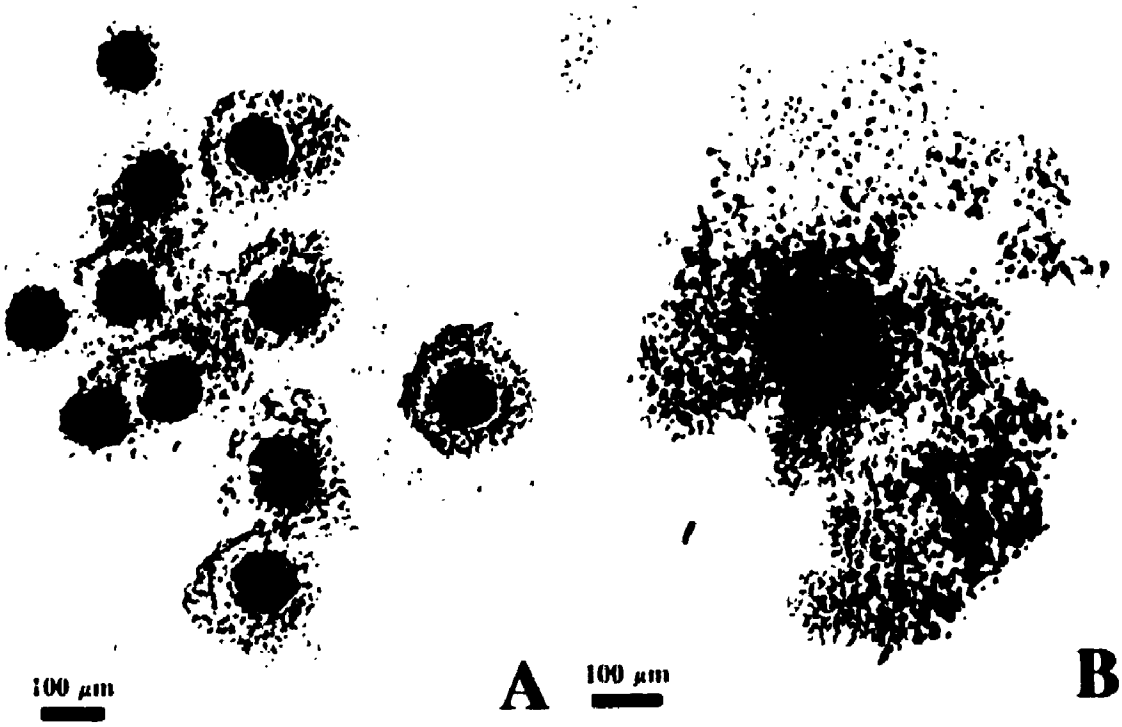

100 μm
A
100 μm
B

Table 5-1. Cumulus expansion observed in porcine OCC after 24 hours of culture with varying concentrations of EGF.

Treatment	Total number of OCC examined*	Percent ± SEM with +3 expansion
Control	87	0[a]
EGF (1 pg/ml)	89	2 ± 2.1[a]
EGF (10 pg/ml)	87	18 ± 2.3[b]
EGF (100 pg/ml)	88	30 ± 2.4[c]
EGF (1 ng/ml)	89	51 ± 2.1[d]
EGF (10 ng/ml)	90	46 ± 1.1[d]

* Data pooled from three independent experiments. Values with different superscripts are significantly different ($P<0.05$) by ANOVA.

LH, LH + FSH) and 2) E_2 (absent or present). The concentrations were similar to those used in previous studies of IVM of pig oocytes (Mattioli *et al.*, 1988b) for IVF, i.e. 1.5 μg/ml NIH-oFSH-i4 (LH contamination: 0.04 times NIH-LH-S1 by OAAD bioassay), 2.0 μg/ml USDA-bLHB5 and 1 μg/ml E_2, and OCC cultured for 24 hours. As can be seen in Text Figure 5-3, both FSH and FSH+LH significantly increased the proportion of oocytes undergoing GVBD, while LH alone had no significant effect. At the concentration used, the percentage of oocytes with GVBD was significantly higher in the presence of FSH+LH than of either of those alone and this effect appeared to be additive (Text Figure 5-3). Addition of E_2 had no effect by itself but significantly suppressed the increase in GVBD stimulated by FSH and FSH+LH (Text Figure 5-3). Even though both FSH and LH alone were effective in inducing cumulus expansion, FSH induced +3 expansion in a significantly higher proportion of OCC than LH (88% vs 57%) and was as effective as FSH+LH (Table 5-2). Estradiol had no effect on expansion by itself, nor did it influence the expansion induced by FSH. However, it significantly decreased the percentage of OCC exhibiting +3 expansion in response to LH (Table 5-2).

5.3.4 Effects of FSH, LH, and E_2 on EGF-induced GVBD and cumulus expansion

In this experiment, the effects of FSH (1.5 μg/ml), LH (2.0 μg/ml), and E_2 (1 μg/ml) were individually examined on EGF (1 ng/ml)-induced GVBD and cumulus expansion. As is shown in Text Figure 5-4, the addition of FSH to EGF significantly increased the percentage of the oocytes undergoing GVBD compared to EGF alone (84% vs 51%), while LH and E_2 had no significant effect. The percentage of OCC undergoing +3 expansion in response to EGF was significantly increased by FSH, while LH and E_2 had no effect (Table 5-3).

5.3.5 Effect of A_4 on EGF- and FSH-induced oocyte maturation and cumulus expansion

This was also assessed in a factorial experiment, with the factors being 1) control, EGF (1 ng/ml), FSH (1.5 μg/ml); and 2) A_4 (1 μg/ml) absent or present. As is shown in Text Figure 5-5, A_4 significantly decreased the percentage of oocytes undergoing

Text Figure 5-3. Effect of various combinations of FSH, LH and E_2 on meiotic maturation of porcine oocytes. Oocyte-cumulus cell complexes were cultured in the medium containing different combinations of FSH (1.5 μg/ml), LH (2.0 μg/ml), and E_2 (1 μg/ml), and GVBD was assessed after 24 hours. Data represent percent mean $\pm$ SEM of three independent experiments. Bars with different superscripts indicate significant difference ($P<0.05$).

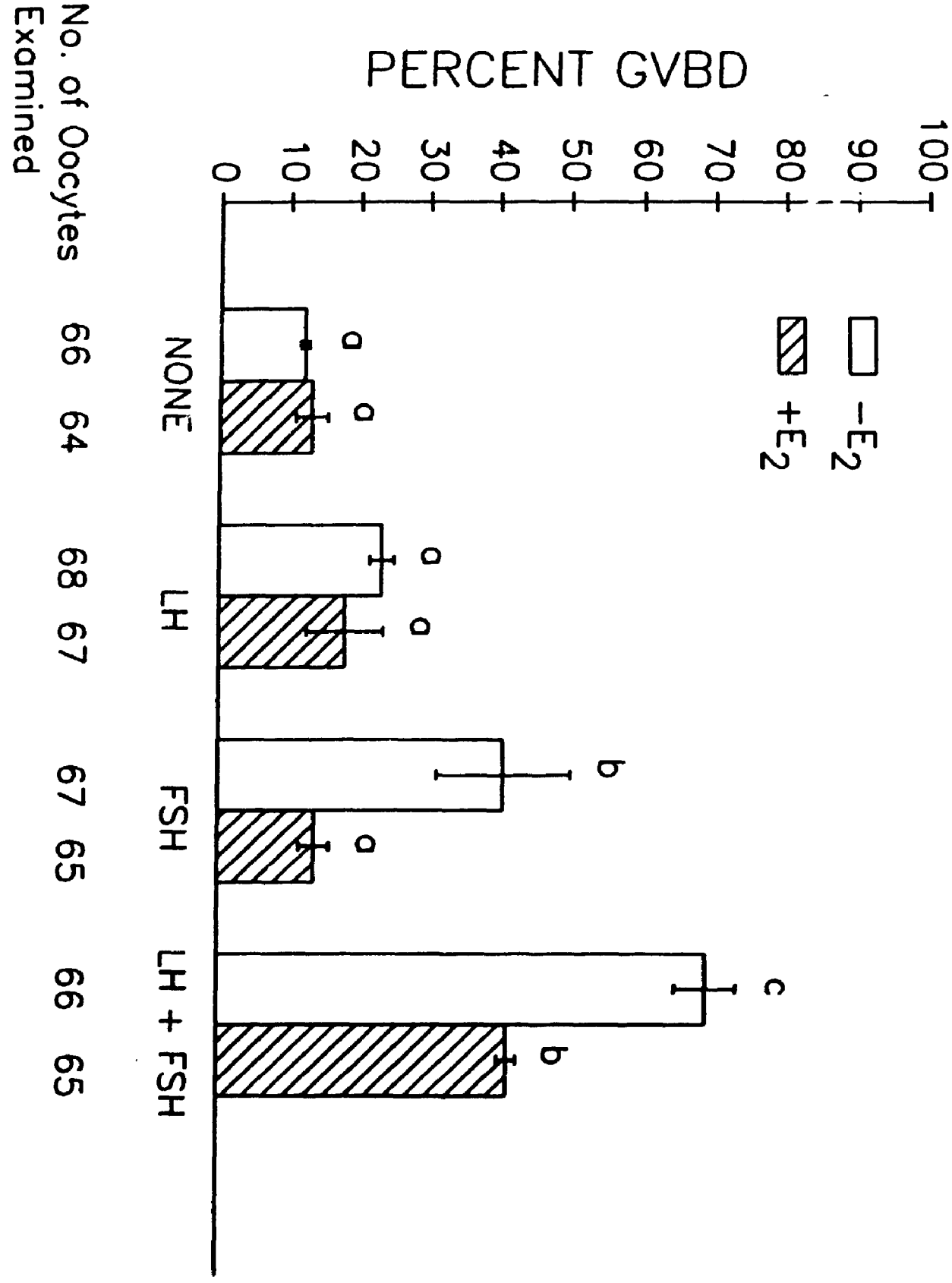
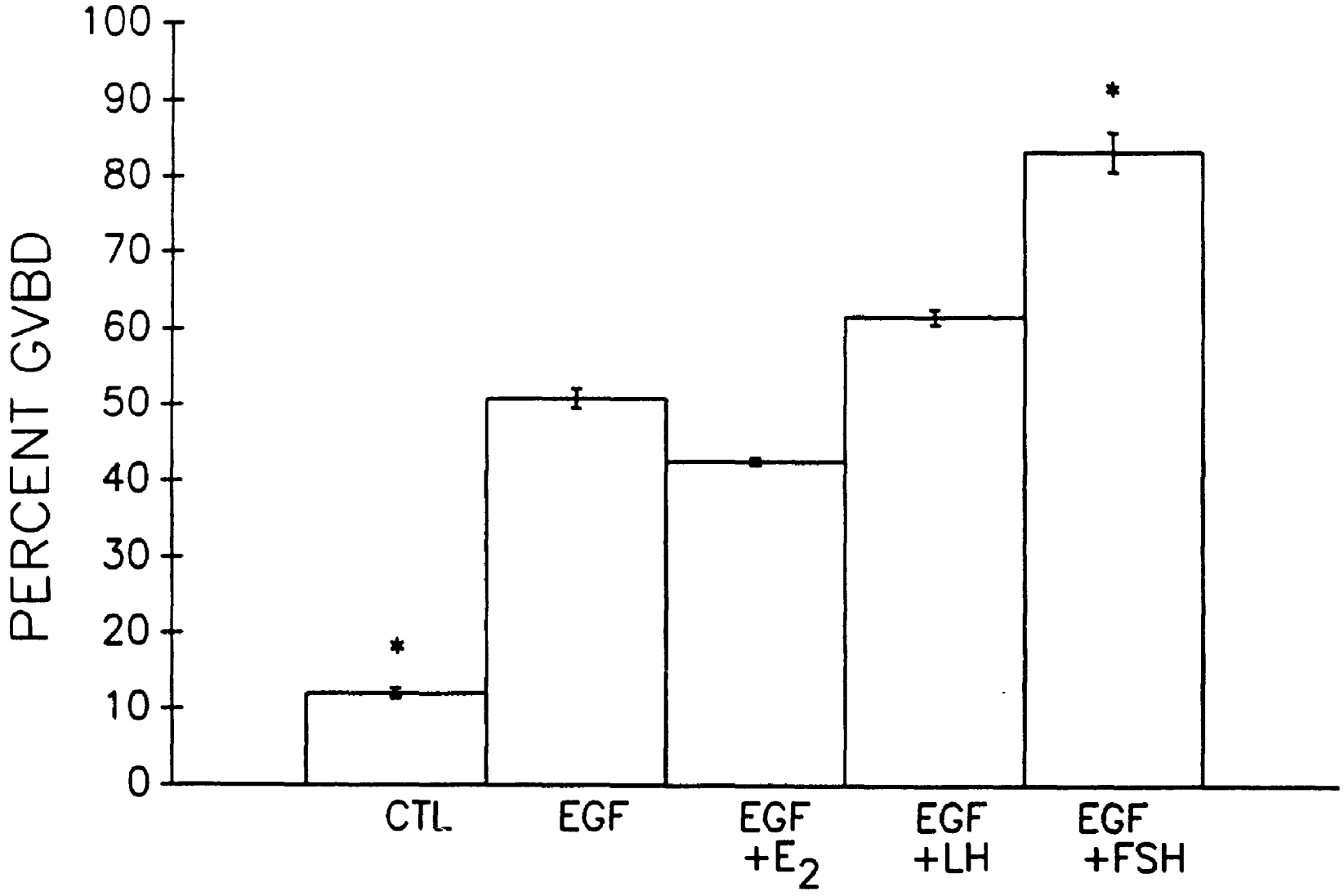

PERCENT GVBD
0
10
20
30
40
50
60
70
80
90
100
$-E_2$
$+E_2$
NONE
LH
FSH
LH + FSH
a
a
a
a
b
a
c
b
No. of Oocytes Examined
66 64
68 67
67 65
66 65

Table 5-2. Cumulus expansion observed in porcine OCC in response to various combinations of FSH (1.5 μg/ml), LH (2 μg/ml) and E_2 (1 μg/ml) after 24 hours of culture.

Treatment	Total number of OCC examined*	Percent ± SEM with +3 expansion
Control	70	0[a]
LH	69	57 ± 7.1[b]
FSH	69	88 ± 3.9[c]
LH + FSH	69	90 ± 3.6[c]
E_2	70	0[a]
LH + E_2	68	28 ± 11.5[d]
FSH + E_2	69	78 ± 7.4[c]
LH + FSH + E_2	69	78 ± 7.9[c]

* Data pooled from three independent experiments. Values with different superscripts are significantly different ($P<0.05$) by ANOVA.

Text Figure 5-4. Effects of FSH (1.5 μg/ml), LH (2.0 μg/ml), and E_2 (1.0 μg/ml) on EGF (1 ng/ml)-induced GVBD. Oocyte-cumulus cell complexes were cultured in the control medium and in medium containing EGF or EGF plus one of the above-mentioned hormones, and GVBD was assessed after 24 hour. Data represent percent mean $\pm$ SEM of three independent experiments. Asterisks indicate significant difference ($P<0.05$) compared to the EGF group.



	CTL	EGF	EGF +E_2	EGF +LH	EGF +FSH
No. of Oocytes Examined	66	65	66	65	58

Table 5-3. Effects of FSH (1.5 μg/ml), LH (2 μg/ml) and E_2 (1 μg/ml) on EGF (1 ng/ml)-induced cumulus cell expansion after 24 hours of culture.

Treatment	Total number of OCC examined*	Percent ± SEM with +3 expansion
Control	70	0[a]
EGF	70	51 ± 4.2[b]
EGF + E_2	70	50 ± 4.2[b]
EGF + LH	70	66 ± 3.9[b]
EGF + FSH	70	86 ± 1.7[c]

* Data pooled from three independent experiments. Values with different superscripts are significantly different ($P<0.05$) by ANOVA.

GVBD in the control (with no added hormones) and FSH groups. However, A_4 did not affect EGF-induced GVBD (Text Figure 5-5) and had no effect on cumulus expansion by itself or when added with EGF or FSH (data not shown).

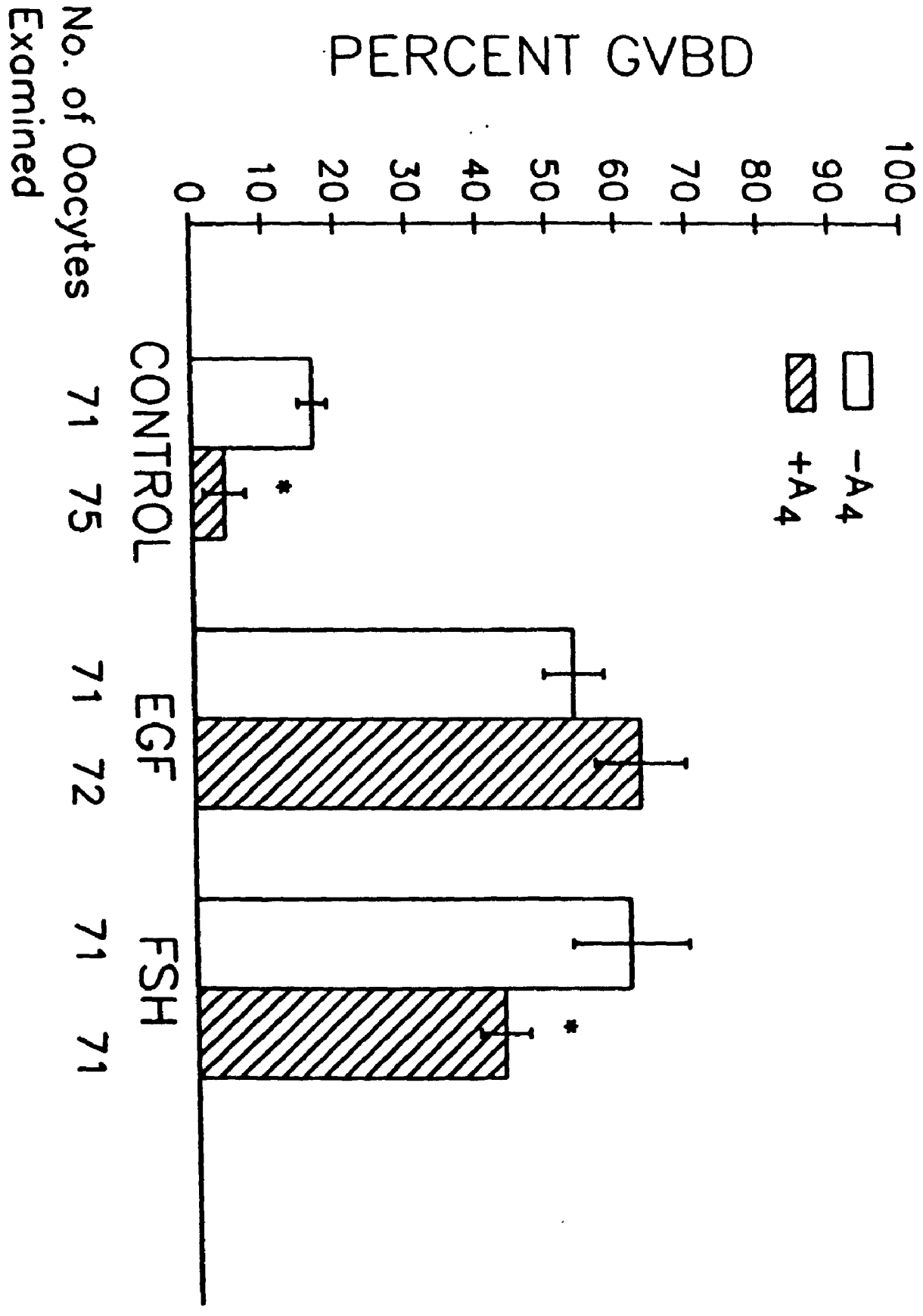
5.4 Discussion

5.4.1 Effects on initiation of meiotic maturation

Oocyte maturation has been judged by many different criteria according to the goals of specific studies. In studies testing the ability of factors to stimulate the meiotic process, the end point is usually the occurrence of GVBD. In the present study EGF significantly stimulated GVBD of porcine oocytes. This is consistent with studies in the rat and mouse, where EGF was shown to be a potent inducer of GVBD (Dekel and Sherizly, 1985; Downs *et al.*, 1988; Ueno *et al.*, 1988; Downs, 1989). Similar to studies with rodents in which FSH effectively stimulated GVBD (Downs *et al.*, 1988; Downs, 1989), purified ovine FSH significantly increased the proportion of oocytes undergoing GVBD in the present study. The stimulatory effect of EGF+FSH on GVBD was greater than EGF alone. This may be explained on the basis of observations that FSH can induce and maintain EGF receptors in the follicle (Siebers *et al.*, 1985; Feng *et al.*, 1987; Buck and Schomberg, 1988; Fujinaga *et al.*, 1992). LH had no significant effect on oocyte maturation at the concentrations used. Addition of LH and FSH together, however, resulted in significantly higher proportions of oocytes undergoing GVBD than was observed in response to FSH or LH alone. Even though FSH and LH concentration-response relationships were not studied, this greater effect of LH+FSH than FSH or LH alone may be due to the fact that cumulus cells have limited LH receptors in the absence of FSH (Erickson *et al.*, 1979; Lawrence et al., 1980; Channing et al., 1981). The greater effect on oocyte maturation due to FSH-induced LH receptors in cumulus cells may be further supported by the fact that gonadotropins induce oocyte maturation by generating a positive maturational signal in the cumulus cells (Downs et al., 1988).

The proportion of oocytes undergoing GVBD after 24 hours in the control

Text Figure 5-5. Effects of A_4 on EGF- and FSH-induced GVBD. Oocyte-cumulus cell complexes were cultured in the medium containing no added hormone (control), EGF (1 ng/ml), or FSH (1.5 μg/ml), with A_4 (1.0 μg/ml) absent or present, and GVBD was assessed after 24 hours. Data represent percent mean $\pm$ SEM of three independent experiments. In each group, asterisk indicates significant difference ($P < 0.05$).


PERCENT GVBD
0
10
20
30
40
50
60
70
80
90
100
-A4
+A4
CONTROL
EGF
FSH
No. of Oocytes Examined
71 75
71 72
71 71
*
*

cultures was lower in the present study compared to some previous porcine studies (Racowsky & McGaughey, 1982c; Racowsky, 1983; Daniel *et al.*, 1986). This difference may largely be attributed to different compositions of the culture media used in those studies. In the above mentioned porcine studies, insulin was routinely added to the culture medium at very high concentrations (12.5 mU/ml). Downs (1989) has reported significant stimulation of GVBD by insulin in the mouse. Thus the lower GVBD observed in the present study may have resulted from the absence of insulin in the cultures. Another contributing factor may be the use of fetal calf serum in this study, whereas pig serum was used in the previous studies.

It has been shown that oocytes require a specific intrafollicular steroid environment for the completion of the full maturation process and that alterations to the steroid profile during maturation lead to gross abnormalities at fertilization (Moor *et al.*, 1980a). Similar to the case in other studies in the pig (Racowsky & McGaughey, 1982c), E_2 inhibited the FSH- and FSH+LH-induced resumption of meiosis. Yoshimura *et al* (1989) concluded that addition of E_2 to IVM media supplemented with gonadotropins and prolactin increased the percentage of rabbit oocytes developing to morulae and blastocysts. The inhibitory effect of E_2 on FSH- and FSH+LH-induced meiotic maturation may be of importance in delaying meiotic maturation to allow better synchrony of nuclear and cytoplasmic maturational processes. However, E_2 did not significantly inhibit EGF-induced meiotic maturation, suggesting that EGF may be acting through a different mechanism than does FSH.

Androstenedione is the principle aromatizable C_{19}-steroid produced by isolated theca cells of the follicle in most animal species (Gore-Langton & Armstrong, 1988). The results from studies of the effects of androgens on oocyte maturation are conflicting. Porcine oocyte maturation was not affected by androstenedione (Schaerf *et al.*, 1982), testosterone or dihydrotestosterone (Schaerf *et al.*, 1982; Racowsky, 1983). In other studies testosterone (Rice & McGaughey, 1981) and 19-norandrostenedione (Daniel et al., 1986) inhibited maturation of cumulus-enclosed porcine oocytes *in vitro*. In the present study, A_4 inhibited GVBD whether added alone or in the presence of FSH, but

had no effect on EGF-stimulated GVBD. Rice and McGaughey (1981) suggested that, since androgens are converted to estrogens by porcine cumulus cells, estrogens may mediate the inhibitory effect of androgens on porcine oocyte maturation. Thus the inhibitory effects of A_4 observed in this study may have been mediated by estrogens, and the absence of A_4 inhibition in the EGF group may be explained on the basis that E_2 has no effect on EGF-stimulated GVBD.

5.4 2 Effects on cumulus expansion

Similar to previous studies with rodents (Dekel & Kraicer, 1978; Downs, 1989) both EGF and FSH effectively stimulated cumulus expansion in the present study. However, in contrast to the mouse study, in which EGF was more effective than FSH in stimulating cumulus expansion (Downs, 1989), FSH appeared to be more effective in the present study. The lesser effect of EGF on porcine cumulus expansion may be due to the use of human EGF in this study, while homologous murine EGF was used in the mouse study. The greater effect of EGF+FSH than EGF alone (Table 5-3) on porcine cumulus expansion may be explained on the basis of greater FSH effect on cumulus expansion. *In vitro* studies using highly purified LH indicated that LH cannot directly stimulate cumulus expansion in the mouse and that it allows the cumulus cells to respond to FSH (Eppig, 1980b). However, LH was shown to stimulate cumulus expansion in the pig (Hillensjo & Channing, 1980). In the present study, purified bovine LH significantly stimulated cumulus expansion in porcine OCC, although at the concentration used, the effect was significantly lower than that of FSH. The ability of LH to stimulate expansion in the pig, but not in the mouse, may be attributed to species differences. Addition of LH has no effect on EGF-induced cumulus expansion, suggesting that EGF and LH may be acting through a similar mechanism.

In summary, the observations that growth factors may influence oocyte maturation and cumulus expansion are rather recent and their possible role in these preovulatory processes should not be viewed in isolation. It is likely that these peptides interact with gonadotropins and steroids to regulate meiotic maturation of the oocyte and other

follicular functions. Studies in this Chapter have provided evidence that EGF is a potent inducer of meiotic maturation and cumulus expansion of the porcine oocyte. The facts that prepro-EGF message has been detected in the mouse ovary (Rall *et al.*, 1985) and that EGF receptors are present in the follicle and are modulated by gonadotropins (Fujinaga *et al.*, 1992), suggest that EGF could have a physiological role in the regulation of meiotic maturation and cumulus expansion during the periovulatory period. The inhibitory effects of E_2 and A_4 on gonadotropin-induced meiotic maturation may help to synchronize nuclear and cytoplasmic maturation. The observations that E_2 and A_4 had no affect on EGF-stimulated GVBD suggest that EGF may act through a different mechanism to induce GVBD than does FSH. Further support to this comes from the observations that FSH significantly increased the proportion of oocytes undergoing GVBD in response to EGF. *In vitro* studies of these complex interactions among growth factors, gonadotropins and steroids in oocyte maturation may lead to improved techniques for IVM and thus to increased fertilization and embryonic development.

CHAPTER 6 ROLE OF THE OOCYTE IN EGF- AND FSH-INDUCED PORCINE CUMULUS EXPANSION

6.1 Introduction

Cellular interactions between the oocyte and somatic cell components of the mammalian ovarian follicle are crucial for its normal development and function (reviewed by Eppig, 1991b). These bidirectional interactions between the somatic and germ cell compartments appear to be mediated by both membrane gap junctions and paracrine factors. The dependence of the oocyte on somatic follicular cells for its normal development and function is undisputed. The intimate association of the oocyte with its companion somatic cells is essential for oocyte growth (Eppig, 1977, 1979b; Herlands & Schultz, 1984; Buccione *et al.*, 1987) and the regulation of meiotic maturation (Tsafriri *et al.*, 1982; Eppig *et al.*, 1983; Schultz *et al.*, 1983b; Downs *et al.*, 1988). However, little is known about the role of the oocyte in the development and function of somatic cells, in particular the cumulus cells with which the oocyte is in intimate contact. It is known that the oocyte initiates the formation of the ovarian follicle; no follicles form without oocytes (reviewed by Eppig, 1991b). In addition, some experiments in which oocytes were removed from antral follicles of ovaries *in situ*, suggest that the oocyte might play a role in preventing the spontaneous luteinization of granulosa cells (El Fouly *et al.*, 1970; Nekola & Nalbandov, 1971). However, in general terms, the role of the oocyte in hormonal-induced differentiation and function of cumulus cells is still unknown.

As reviewed in Chapter 2, the compact layers of cumulus cells surrounding the oocyte expand in response to the endogenous preovulatory gonadotropin surge, due to deposition of mucoelastic material in the extracellular matrix. The mucified cumulus cells appear to provide an appropriate environment for sperm penetration and fertilization (Meizel, 1985). The ultrastructure of the mucified cumulus cell matrix is a fibrillar network of which hyaluronic acid is an integral component (Yudin *et al.*, 1988). Cumulus expansion can be induced *in vitro* by either EGF or FSH, and is thought to be

mediated by cAMP (Dekel & Kraicer, 1978; Eppig, 1979a; Downs, 1989; Results from Chapter 5). It has been reported that expansion of mouse cumuli induced by EGF, FSH or cAMP analogs such as 8-bromo cAMP or dbcAMP, is dependent upon a specific factor(s) secreted by the oocyte (Buccione *et al.*, 1990; Salustri *et al.*, 1990). For this factor(s) to act, contact between the oocyte and the cumulus cells is not necessary since the oocyte-conditioned medium can enable the cumulus cells to expand in response to a hormonal stimulus (Buccione *et al.*, 1990; Salustri *et al.*, 1990). The factor(s) appears to be specific to the oocyte as the conditioned media from various somatic cells, such as granulosa cells, fibroblasts, sertoli cells, mixed male germ cells, or spermatozoa, were ineffective in this ability (Buccione *et al.*, 1990). Furthermore, in the oocyte, production of this factor is developmentally regulated; significant amounts are normally secreted only by oocytes competent to undergo GVBD (Vanderhyden *et al.*, 1990). The putative factor possibly acts downstream from the generation of cAMP since cumulus expansion was not stimulated by cAMP analogs in the absence of the oocyte (Buccione *et al.*, 1990; Salustri *et al.*, 1990). This factor is not hyaluronic acid as the denuded oocytes do not synthesize detectable amounts of hyaluronic acid, but enable cumulus cells to increase hyaluronic acid synthesis in cocultures (Salustri *et al.*, 1990). Although the exact chemical nature is still obscure, a preliminary characterization indicated that the factor probably is a highly unstable, heat labile protein, or depends upon a protein for its activity (Eppig *et al.*, 1993).

In contrast to the mouse, FSH- and forskolin-stimulated cumulus expansion and cAMP production in the pig has been shown to be independent of the oocyte (Prochazka *et al.*, 1991). EGF induces cumulus expansion by a mechanism which at least initially appears to be different from that of FSH (Downs *et al.*, 1988). However, it is not known whether EGF also requires the oocyte to induce cumulus expansion in the pig. Furthermore, although FSH-stimulated expansion of porcine cumuli is not dependent on the oocyte, it is not known if the porcine oocyte is capable of secreting the factor(s) that enables the mouse cumuli to expand. The present study has two-fold objectives: 1) to test the dependence of porcine cumuli on the oocytes for EGF-stimulated expansion; and

2) to determine if the porcine oocyte secretes the putative cumulus expansion-enabling factor that is required for hormone-stimulated mouse cumulus expansion.

6.2 Materials and Methods

6.2.1. To test the dependence of porcine cumulus oophorus on the oocyte for EGF-induced expansion

Ovaries were collected from prepubertal gilts and intact oocyte-cumulus cell complexes (P-OCC) were isolated in HBSS as described in Chapter 4. Oocytectomized oocyte-cumulus cell complexes (P-OOX) were obtained by removing oocyte contents of isolated P-OCC by microsurgical procedures under a stereoscopic microscope. Briefly, the P-OCC were approached with a 10-15 μm pipette and just sufficient negative pressure was applied through the micropipette with the help of an attached 20 ml syringe to let the micropipette penetrate cumulus cells and zona. As the pipette crossed the zona, contents of the oocyte were drawn into the pipette due to negative pressure. Upon withdrawal of the micropipette, the cumulus mass returned to a normal shape due to resilience of the zona pellucida. To exclude participation of small fragments of ooplasm observed in a few P-OOX after manipulation, and to test if the original structure and orientation of the cumulus cells around the zona pellucida is required for their expansion, intact clumps of the cumulus cells (P-CCC) were obtained by removing zona intact oocytes from the isolated P-OCC with a micropipette. In order to obtain P-CCC, oocytes in P-OCC were approached gently with a 10-15 μm micropipette and just sufficient negative pressure was applied with the mouth to hold the oocytes while pulling them out from the P-OCC. The P-OCC, P-OOX, and P-CCC were cultured at 39°C for 24 hours with or without 1 ng/ml human recombinant EGF or 1.5 μg/ml NIH-oFSH-14 as previously described in Chapter 4. The concentration of EGF used in this study represented the concentration required to elicit a maximal cumulus expansion and oocyte maturation in porcine OCC (Chapter 5) and that of FSH was similar to that used in previous studies of IVM of pig oocytes (Mattioli *et al.*, 1988). At the end of the culture period, the cumulus expansion was similarly scored on an arbitrary scale of 0 to +4 (see Chapter 4). In the P-CCC where corona radiata disappeared due to removal of the zona

intact oocytes, +3 indicated expansion of all layers of the cumulus.

6.2.2. To determine the production of cumulus expansion-enabling factor by the porcine oocyte

Mouse ovaries were collected from 3-4 weeks old swiss CD-1 mice injected with 4 IU PMSG 44 hours before the time of sacrifice. All the mice were purchased from Charles River Canada Inc., St. Constant, Quebec and were housed in air-conditioned quarters with free access to food and water. Lighting was provided for 14 hours daily with the lights on from 0500 to 1900. Mouse oocyte cumulus cell complexes (M-OCC) and cumulus cell clumps (M-CCC) were obtained using procedures similar to those used for the pig. The P-OCC were isolated as mentioned earlier and pig denuded oocytes (P-DO) were obtained by removing cumulus and corona cells from P-OCC by repeated pipetting using a fine bore (~ 80 μm) pipette. Ten-15 M-CCC were cocultured with 35-40 P-DO in 50 μl of TCM 199 under oil with or without 1.5 μg/ml FSH at 37°C for 24 hours (conditions sufficient for intact M-OCC to expand). Cumulus expansion was similarly scored on a scale of 0 to +4.

Statistical analysis: All the experiments were replicated at least three times to confirm the consistency of results. As described in Chapter 5, data were assessed by ANOVA using arcsin transformation of the proportions (Snedecor, 1956). Differences between means for multiple comparisons were assessed by Duncan's New Multiple Range test (Steel & Torrie, 1960). Significance was inferred at $P < 0.05$. For presentations, data were expressed as percent mean $\pm$ SEM.

6.3 Results

6.3.1. Effects of the oocyte on EGF- and FSH-induced expansion of porcine cumulus cells

At the end of 24 hour culture periods, both EGF (1 ng/ml) and FSH (1.5 μg/ml) induced +3 expansion in P-OCC, P-OOX, and P-CCC compared to 0 for the controls (Figure 6-1; Table 6-1). Similar to the case in the previous Chapter, due to an insignificant number of complexes exhibiting +1 and +2 degrees of cumulus expansion,

Figure 6-1. Similar degree of cumulus expansion (+3) observed in porcine oocyte-cumulus cell complexes (P-OCC), oocytectomized oocyte-cumulus cell complexes (P-OOX) and intact cumulus cell clumps (P-CCC) in response to FSH. The P-OCC, P-OOX and P-CCC were cultured at 39°C for 24 hours in the absence or presence of 1.5 μg/ml FSH, and examined for expansion of cumulus cells: A) non-expanded P-OCC cultured in the absence of FSH (control), B) an expanded FSH-treated P-OCC, C) non-expanded control P-OOX, D) an expanded FSH-treated P-OOX, E) non-expanded control P-CCC, F) an expanded FSH-treated P-CCC.

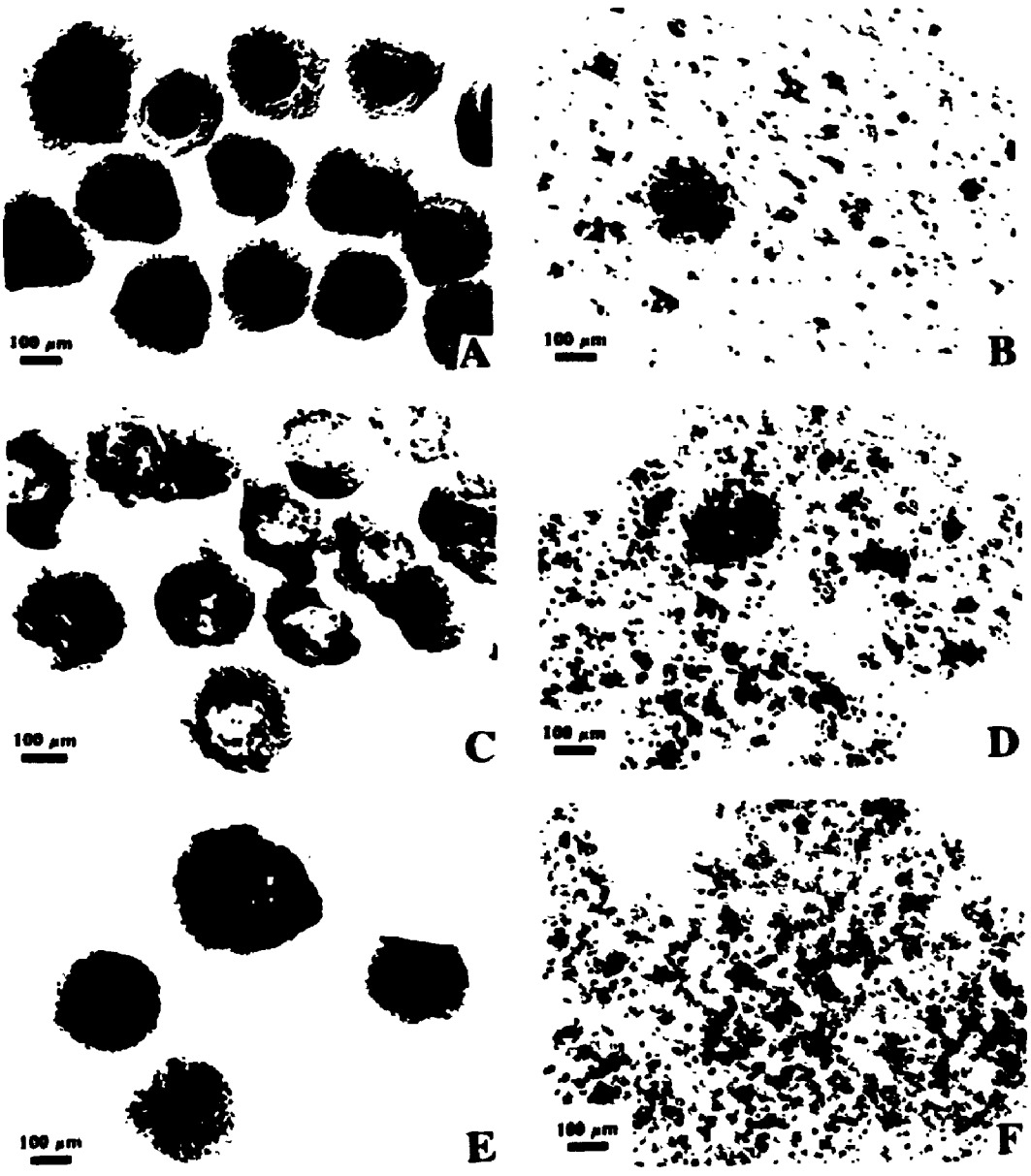

100 µm
A
100 µm
B
100 µm
C
100 µm
D
100 µm
E
100 µm
F

Table 6-1. Cumulus cell expansion observed in P-OCC, P-OOX and P-CCC in response to EGF and FSH after 24 hours of culture.

Group	Treatment	Total number examined*	Percent ± SEM with +3 expansion
P-OCC	Control	67	0[a]
P-OOX		73	0[a]
P-CCC		65	0[a]
P-OCC	EGF (1ng/ml)	69	57 ± 9.5[b]
P-OOX		75	56 ± 1.9[b]
P-CCC		63	60 ± 2.2[b]
P-OCC	FSH (1.5μg/ml)	71	94 ± 3.1[c]
P-OOX		74	91 ± 1.3[c]
P-CCC		67	91 ± 5.5[c]

* Data pooled from three independent experiments. Values with different superscripts are significantly different ($P<0.05$) by ANOVA.

only the complexes with +3 expansion were used for comparisons among various treatment groups. FSH induced +3 cumulus expansion in a significantly higher proportion of complexes compared to EGF (91-94% vs 56-60%; Table 6-1). However, there was no difference in the proportion of complexes with +3 expansion among P-OCC, P-OOX and P-CCC with EGF or FSH (Table 6-1).

6.3.2. Ability of the porcine oocyte to secrete the putative cumulus expansion-enabling factor

FSH induced +3 expansion in intact M-OCC after a 24 hour culture period compared to 0 for the control (Figure 6-2). FSH failed to induce expansion in M-CCC cultured alone after 24 hours (Table 6-2). However, upon coculture of M-CCC with P-DO, 79% of the complexes exhibited +3 expansion in response to FSH, similar to that observed in intact M-OCC (76%) cultured in the presence of FSH (Table 6-2). No expansion was observed when the M-CCC were cocultured with P-DO in the absence of FSH (Figure 6-2). The percentage of P-OCC undergoing +3 expansion was significantly higher (96%) compared to M-OCC (76%) and M-CCC + P-DO (79%) (Table 6-2).

6.4 Discussion

Several reports indicate that FSH initiates expansion of cumulus cells by inducing the synthesis of hyaluronic acid (Dekel & Kraicer, 1978; Eppig, 1980b; Ball *et al.*, 1985; Salustri *et al.*, 1989). In the present study, purified ovine FSH at 1.5 μg/ml induced +3 expansion in 94% P-OCC after 24 hours in culture. This is similar to the previous porcine studies in which ~ 90% OCC exhibited +3 expansion in response to purified porcine FSH (Prochazka *et al.*, 1991) or forskolin (Racowsky, 1985b). However, in contrast to the studies in the mouse where oocytectomized complexes (Buccione *et al.*, 1990) and isolated cumulus cells (Salustri *et al.*, 1990) failed to expand in response to FSH, P-OOX and P-CCC exhibited similar expansions (+3) in response to FSH. These observations are in agreement with a previous report in the pig (Prochazka *et al.*, 1991)

Figure 6-2. Cumulus expansion observed in mouse oocyte-cumulus cell complexes (M-OCC) and mouse cumulus cell clumps (M-CCC) cocultured with porcine denuded oocytes (P-DO) in the absence/presence of FSH: A) non-expanded M-OCC cultured without FSH, B) FSH-treated M-OCC showing +3 expansion, C) non-expanded M-CCC cocultured with P-DO in the absence of FSH, D) M-CCC cocultured with P-DO in the presence of FSH showing +3 expansion.

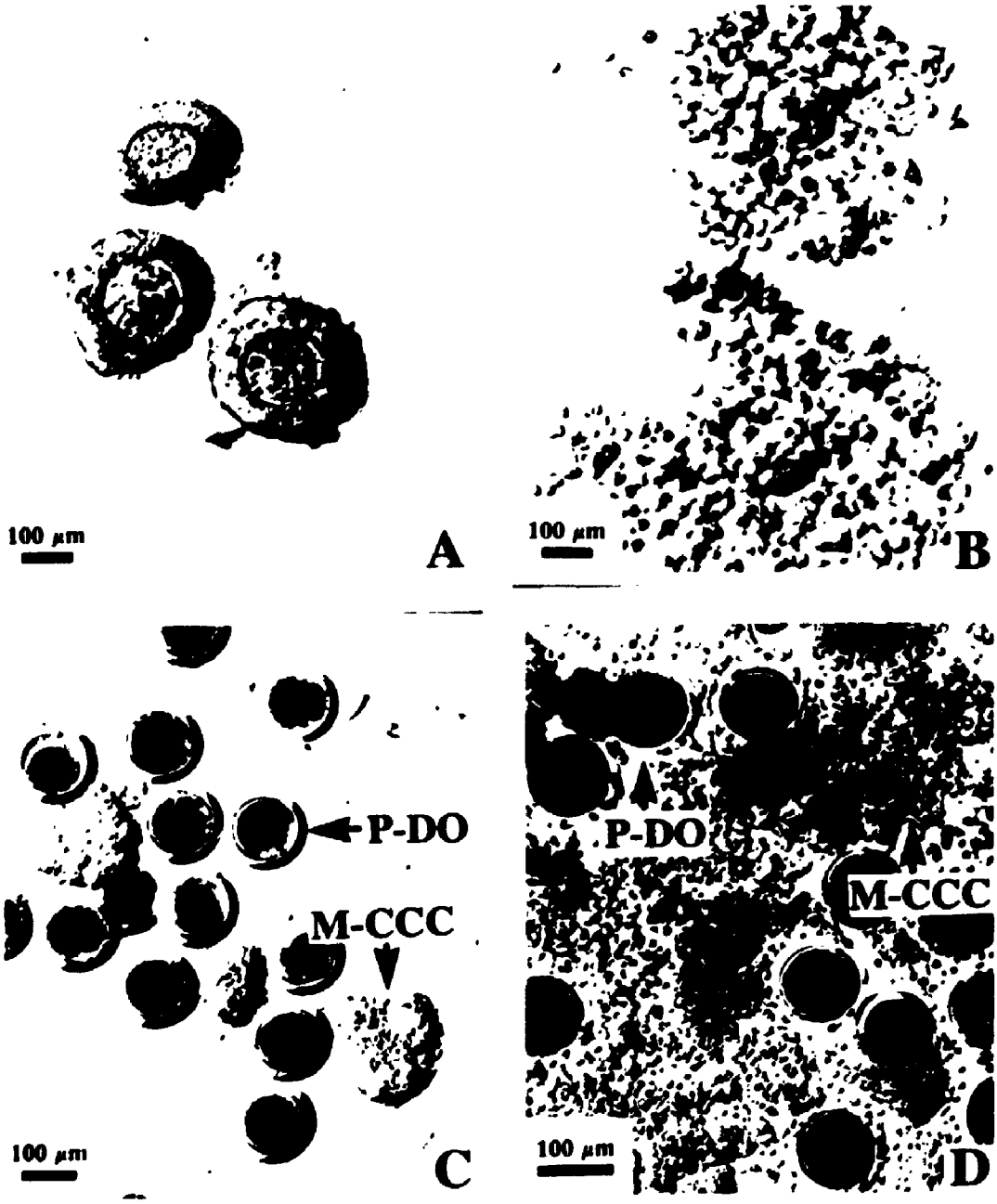

100 μm
A
100 μm
B
P-DO
M-CCC
100 μm
C
P-DO
M-CCC
100 μm
D

Table 6-2. Cumulus cell expansion observed in M-OCC, P-OCC, M-CCC and M-CCC cocultured with P-DO in response to FSH after 24 hours of culture.

Group	Treatment	Total number examined*	Percent ± SEM with +3 expansion
M-OCC	Control	90	0[a]
P-OCC		105	0[a]
M-CCC		70	0[a]
M-CCC + P-DO		89	0[a]
M-OCC	FSH (1.5μg/ml)	97	76 ± 4.7[b]
P-OCC		105	96 ± 3.4[c]
M-CCC		70	0[a]
M-CCC + P-DO		95	79 ± 4.9[b]

* Data pooled from three independent experiments. Values with different superscripts are significantly different ($P<0.05$) by ANOVA.

and confirm that FSH-induced expansion of porcine cumuli *in vitro* is not dependent on the oocyte. In the mouse, EGF was shown to be a potent inducer of cumulus cell expansion *in vitro* and was more effective in this regard than FSH (Downs, 1989). This effect appeared to be specific to EGF since it was the only one out of several known growth factors tested which possessed such an ability (Downs, 1989). In the previous Chapter, I have shown that EGF is an effective inducer of cumulus expansion in the pig, although the proportion of OCC that underwent expansion in response to EGF was less compared to FSH. EGF seems to induce expansion by a mechanism which is at least initially different from that of FSH (Downs *et al.*, 1988). Binding sites for EGF have been demonstrated in the granulosa cells of the pig (Fujinaga *et al.*, 1992). Additionally, prepro-EGF message has been detected in the mouse ovary by direct hybridization using a [^{32}P] cDNA probe suggesting local ovarian production of this growth factor (Rall *et al.*, 1985). Like FSH, EGF failed to induce cumulus expansion in oocytectomized mouse cumulus cell complexes (Buccione *et al.*, 1990). In the present study, EGF induced similar expansion in P-OCC, P-OOX, and P-CCC indicating that like FSH, EGF-stimulated porcine cumulus expansion is independent of the oocyte. Similar expansion observed in P-CCC also eliminated the possible participation of the residual fragments of ooplasm observed after manipulation in the P-OOX expansion and indicated that maintenance of original structure and orientation of the cumulus cells around the zona pellucida is not required for their expansion.

FSH induced +3 expansion in M-OCC compared to 0 in the absence of FSH. However, in agreement with the previous reports (Buccione *et al.*, 1990; Salustri *et al.*, 1990), M-CCC did not expand in response to FSH, confirming the dependency upon the oocyte for mouse cumulus cell expansion. Even though the porcine cumulus expansion is not dependent on the oocyte (Prochazka *et al.*, 1991, present study), coculture of M-CCC with P-DO enabled FSH to stimulate cumulus expansion in isolated M-CCC, indicating that the porcine oocyte secretes a soluble cumulus expansion-enabling factor and that this factor is not species specific. The findings that the coculture of P-DO failed to induce expansion in M-CCC in the absence of FSH indicate that both the factor and

FSH are required to induce expansion in mouse cumulus cells. That the porcine cumulus cells underwent expansion in response to EGF or FSH in the absence of the oocyte (Table 6-1; Figure 6-1) suggests that the porcine cumulus cells may possess the ability to sequester the oocytic factor(s). An alternate explanation may be that the porcine cumulus cells do not require the oocyte factor(s) for expansion, and that the porcine oocyte secretory product which happens to affect mouse cumulus expansion may have some other function in the pig.

In vivo, FSH is present in the follicle before the resumption of meiosis, yet the cumulus cells do not expand before the preovulatory LH surge (Eppig, 1980b). It was proposed for the mouse model that granulosa cells suppress the secretion of the expansion-enabling factor until the induction of oocyte maturation by the endogenous LH surge (Eppig, 1991b). It was also hypothesized that the liberation of GV-stage oocytes from the follicles *in vitro* might trigger the precocious release of the factor from the oocyte, thereby enabling OCC to expand in response to FSH. All this suggested that LH plays an indirect role in FSH-induced cumulus expansion *in vivo* by terminating the inhibitory effect of granulosa cells on the secretion of the oocytic factor(s). Such a concept is also consistent with the observations both *in vivo* and *in vitro* that purified LH is unable to induce cumulus expansion in mice OCC by itself, but rather it allows the cumulus cells to respond to FSH (Eppig, 1980b). However, the present findings in the pig, indicating that the porcine cumulus cells can expand in response to EGF or FSH in the absence of the oocyte, do not support the prevalence of such LH regulatory system in this species. In fact, the data suggest that if the oocytic factor(s) plays any role at all in the cumulus expansion in this species, it is already sequestered in cumulus cells, and therefore produced constitutively in the follicle before the LH surge. Furthermore, purified LH alone can induce cumulus expansion in porcine OCC *in vitro* (Hillensjo & Channing, 1980; studies here in Chapter 5), suggesting a direct, rather than indirect, role for this gonadotropin in the preovulatory process. Thus in the pig, it appears that the suppression of cumulus expansion before the LH surge may, in fact, be exerted on hormonal induction of cumulus expansion. Future experiments aimed at testing the

ability of porcine follicular fluid in enabling isolated M-CCC to expand in response to hormonal stimuli may confirm the presence of a biologically active oocytic factor(s) in the follicle before the gonadotropin surge.

The proportion of porcine complexes undergoing expansion in response to 1.5 μg/ml purified ovine FSH was significantly greater than those of mice (Table 6-2). However, in a previous mouse study (Buccione *et al.*, 1990), 1 μg/ml purified ovine FSH induced cumulus expansion in at least 90% OCC. This difference may partially be attributed to the use of a different culture medium with completely different composition in that study (Eagle's minimum essential medium compared to TCM-199 in the present study). The other contributing factor may be that the FSH concentration-dependent response was not determined for the mouse OCC and that the concentration best suited for the pig may not necessarily be the best for the mouse. In any event, the proportion of mouse complexes (both M-OCC and M-CCC) exhibiting +3 expansion was significant enough to support the findings.

In summary, the results from this study indicate that even though the porcine cumulus expansion *in vitro* is not dependent on the oocyte, the porcine oocyte is capable of secreting cumulus expansion-enabling factor(s). Although the physiological significance of cumulus expansion-enabling factor of oocyte origin in species other than mouse remains a subject of speculation, the presence of this activity in porcine oocytes may facilitate further studies aimed at elucidating its chemical structure and mechanism of action.

CHAPTER 7 EGF AND EGF-R GENE EXPRESSION AND PEPTIDE LOCALIZATION IN THE OVARIAN FOLLICLE

7.1 Introduction

Studies described in the previous Chapters suggest that EGF may have a physiological role in follicular functions. It stimulates the resumption of meiosis and cumulus cell expansion *in vitro*, the two vital physiological events associated *in vivo* with gonadotropin surge. It has also been implicated in the regulation of somatic cell functions including differentiation, proliferation, and steroidogenesis, all of which are indispensable for follicular development. However, in order for EGF to be implicated in follicular physiology, it should be locally present and there should be receptors for it present in somatic and/or germ cells of the follicle. The reports indicating the presence of prepro-EGF message (Rall *et al.*, 1985) and EGF binding sites (Vlodavsky *et al.*, 1978; St-Arnaud *et al.*, 1983; Chabot *et al.*, 1986; Buck & Schomberg, 1988; Fujinaga *et al.*, 1992) in the ovary provide, indeed, some support in this direction.

Despite all this, reports concerning the presence of EGF and EGF-R in the ovary are controversial. Using an antiserum against human urogastrone, an equivalent of human EGF (Carpenter & Cohen, 1979), Elder *et al* (1978) failed to demonstrate any immunostaining in the human ovary; however, using human EGF antiserum, Kasselberg *et al* (1985) localized faint EGF-immunostaining in primordial follicles from newborn human females. On the other hand, Maruo *et al* (1993), using monoclonal antibodies against human EGF and EGF-R, failed to detect the ligand and the receptor in primordial follicles of women with regular menstrual cycles. Instead, they localized EGF and EGF-R only in the oocyte of primary follicles, and in the oocyte, granulosa and theca interna cells of antral follicles. Hsu *et al* (1987) reported the presence of EGF-like activity in porcine follicular fluid using the A-431 cell membrane radioreceptor assay, whereas Vaughan *et al* (1992a) found no detectable immunoreactive EGF in porcine follicular fluid using a homologous radioimmunoassay. Although EGF has been shown to influence porcine theca cell proliferation (May *et al.*, 1992), the binding sites for EGF

were exclusively localized in granulosa cells and were totally absent in theca cells (Fujinaga *et al.*, 1992). Furthermore, localization of peptides alone by such immunoreactive methods can not distinguish between local synthesis and sequestration from other sources. To date no study looking at the localization of both the mRNAs and peptides for EGF and its receptor in the ovarian follicle has been reported for any mammalian species. The present study aimed to determine the expression of genes for EGF and EGF-R in various components of medium-sized ovarian follicles by reverse transcription-polymerase chain reaction (RT-PCR) and to localize EGF and EGF-R during folliculogenesis by immunocytochemistry in the pig.

7.2 Materials and Methods

7.2.1 Separation and collection of follicular components

Ovaries were collected from prepubertal gilts at a local abattoir and medium-sized (4-6 mm) follicles were dissected out from the intact ovarian stroma. The isolated follicles were bisected with scissors, and granulosa cells were scraped from the basement membrane using a heat-bent Pasteur pipette. The OCC were collected from the dishes with a mouth-controlled micropipette. Oocytes and cumulus cells were separated by removing cumulus cells from the OCC with a fine bore (~80 μm) pipette. The granulosa cells were dispersed by repeated pipetting and theca interna cells were prepared using the technique described by Tsang *et al* (1979) and modified by Hunter and Armstrong (1987). Briefly, theca interna was separated mechanically by peeling off the inner layer and dispersed enzymatically with 0.25% collagenase (Type II, Sigma Chemical Co., St. Louis, MO), 0.05% hyaluronidase (Type I, Sigma) and 0.05% dispase (Boehringer-Mannheim Canada, Laval, Quebec). All cells were washed 5-7 times with D-PBS containing 0.3% polyvinyl pyrrolidine (PVP) (Sigma). Pools of 100 oocytes, cumulus cells from 100 OCC, 1 million granulosa and 1 million theca cells were stored in 10 μl of D-PBS at -70°C until RNA isolation.

7.2.2 Extraction of RNA

Total RNA was extracted according to the method of Rappolee *et al* (1989) as

modified by Valdimarsson *et al* (1993). Briefly, the oocyte and somatic cell samples were solubilized at room temperature by the addition of 100 μl of solution D (4 M guanidine thiocyanate, 0.5% Sarkosyl, 100 mM 2-mercaptoethanol, 25 mM sodium citrate, pH 7.0), to which 20 μg E. coli rRNA (Boehringer-Mannheim) was added to act as a carrier. The lysates were carefully layered over 100 μl of 5.7 M CsCl in 0.1 M EDTA, pH 7.5, in polyallomer ultracentrifuge tubes. The samples were spun at 80,000 rpm and 20°C in a TLA-100 rotor in a Beckman TL-100 tabletop ultra centrifuge for 4 hours. The RNA pellets were dissolved in 100 μl of 2.5 M ammonium acetate and precipitated with ethanol overnight at -20°C. The following day, the RNA was air-dried after washing twice with 70% ethanol.

7.2.3 RT-PCR

The RNA pellets were redissolved in water, and total RNA was reverse-transcribed into cDNA in a volume of 20 μl containing 2 units/μl recombinant RNasin (Promega, Madison, WI), 20 ng/μl oligo-dT primer (Boehringer-Mannheim), 1 mM each dATP, dCTP, dGTP, and dTTP (Boehringer-Mannheim), 1.5 units/μl AMV reverse transcriptase (Boehringer-Mannheim) in 50 mM Tris (pH 8.3), 60 m KCl, 3 mM $MgCl_2$, and 10 mM dithiothreitol. The RT reaction was carried out at 42°C for 1.5 hours, after which it was stopped by boiling for 10 min, followed by quenching on ice. The RT preparation was stored at -70°C after dividing into suitable aliquots.

PCR was carried out in a total volume of 50 μl using a Perkin Elmer-Cetus DNA Thermal Cycler. Each reaction contained 1-2 μl cDNA solution, 50 pmol of each primer, 5 μl of 10X PCR buffer (Promega), 2 μl of 5 mM dNTPs, and water to 50 μl. The mixture was overlaid with 75 μl of paraffin oil and heated to 94°C for 5 min prior to the addition of 1-2 units of Taq polymerase. The amplification profile consisted of 35 cycles: at 94°C for 0.3 min (dissociation); at 55°C for 0.5 min (annealing) for EGF-R and 65°C for 0.5 min (annealing) for EGF; and at 72°C for 1.0 min (extension). The final cycle included a further 5 min at 72°C for complete strand extension. All RT-PCR reactions were carried out at least twice for each individual RNA sample.

7.2.4 PCR primers

The primers for EGF and EGF-R containing an 8 bp 5'-extension incorporating a *BamHI* restriction site for cloning purposes were synthesized on a Biosearch 8750 four-column DNA synthesizer (Biosearch, San Rafael, CA) as previously described by Vaughan *et al* (1992b) and Kennedy *et al* (1993). Briefly, the upstream primer for EGF (5' -TCTGAACCCGGACGGATT TG- 3') is identical to nucleotide 6-25 bp upstream from the 3'-end of the pig EGF gene exon 19, and the downstream primer (5' -GACATCGCTCGCGAACGTAG- 3') represents the reverse complement of nucleotides 12-31 of exon 21. This primer pair predicts a DNA fragment of 214 bp. The upstream primer for EGF-R (5'- TGGAGGA(G/A)AAGAAAG- 3') represents nucleotides 260-274 of the human cDNA and the downstream primer (5'- GAT(A/G)ATCTG (G/C)AGGTT-3') represents the reverse complement of nucleotides 494-508. This primer pair predicts a DNA fragment of 264 bp. A primer pair for β-actin was included as an internal control, since transcripts for cytoplasmic β-actin are expected to be present in all cell types. Both the upstream (5'-CGTGGGCCGCCCTAGGCACCA-3') and downstream (5'-TTGGCCTTAGGGTTCAGGGGGG-3') primers were based on the mouse β-actin cDNA sequence (Tokunaga *et al.*, 1986). All these primer pairs span known introns which help to distinguish amplifications of mRNA reverse transcribed to cDNA from amplifications of any contaminating genomic DNA.

7.2.5 Confirmation of PCR products

The identity of PCR products was confirmed either by cutting the DNA bands with a diagnostic restriction enzyme (*RsaI* for EGF and *AvaII* for EGF-R) according to the supplier's instructions (Gibco, BRL), or by a combination of restriction digestion and Southern Analysis. For Southern Analysis, aliquots of RT-PCR reaction material were separated by electrophoresis through 1.5% agarose gels and transferred to nylon membrane (Hybond-N, Amersham, Oakville, Ontario). cDNA probes were [^{32}P]-radiolabelled by the random primer method (Feinberg & Vogelstein, 1983). High stringency hybridization and washing conditions were employed as described for

Northern blots by Valdimarsson *et al* (1991).

7.2.6 Immunocytochemistry

Anti-porcine EGF (pEGF) serum was raised in a rabbit injected with recombinant 53-residue pEGF produced by expression of a synthetic gene in yeast and was the generous gift of Dr. K.D. Brown (AFRC Institute of Animal Physiology and Genetics Research, Babraham, Cambridge, UK). This antiserum shows no cross-reactivity with IGF-1, PDGF, and TGF-α or -β, and less than 1% cross-reactivity with mouse EGF (Vaughan *et al.*, 1991). Anti-EGF-R was an affinity-purified rabbit polyclonal antibody raised against a peptide corresponding to residues 1005-1016 of the human EGF-R peptide, and was purchased from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA).

Small pieces ($\sim$0.5 X 0.5 cm) of freshly excised ovaries were fixed in paraformaldehyde (4%) and glutaraldehyde (0.5%) fixative for 4-6 hours at room temperature and then stored in 70% ETOH. Further processing of the tissue, including dehydration, embedding in paraffin and cutting sections of 5-7 μm thickness, was done by the technicians in the Department of Pathology, University Hospital, London, Ontario, using routine histological procedures. Streptavidin-Biotin immunostaining was performed as outlined in the Histostain-SP kit (Zymed Laboratories Inc., San Francisco, CA) for rabbit primary antibodies with few modifications. Briefly, the sections were deparaffinized, rehydrated, and endogenous peroxidase was blocked with 3% hydrogen peroxide in methanol at room temperature for 10 min. The slides were then incubated overnight at 4°C with 1:400 anti-pEGF or 1:200 anti-EGF-R in D-PBS containing 10% pig serum. For negative controls, the primary antibody was either replaced with rabbit pre-immune serum (EGF) or pre-absorbed with the control peptide (EGF-R). The slides were then washed twice in D-PBS, and biotinylated secondary antibody was applied over the section for 10 min at room temperature. After two washes, streptavidin peroxidase conjugate was applied at room temperature for 5 min followed by washing in D-PBS. The reactions were completed by the addition of freshly prepared substrate-chromogen mixture for 10-15 min at room temperature. The slides were then counter-stained with haematoxylin, and the results were recorded by photography.

7.3 Results

7.3.1 EGF mRNA

A very strong signal for EGF mRNA was detected in the oocyte (lane O in Figure 7-1A). In contrast, the expression of EGF mRNA was very weak but detectable in cumulus, granulosa, and theca cells (lanes C, G & T in Figure 7-1A). Only the predicted 214 bp EGF band was detected in all of the samples, demonstrating the specificity of the amplification procedure. The identity of the amplified product in the oocyte was further confirmed by cutting the band with the diagnostic restriction enzyme *RsaI* which yielded the expected fragments of 119 and 95 bp (Figure 7-1B). Furthermore, no amplified product was detected when the oocyte or somatic cells were absent from the rinse medium (lane R in Figure 7-1A), or when the cDNA was replaced with water (lane W in Figure 7-1A). Also, no signal was observed when rat kidney cDNA was used (lane K in Figure 7-1), indicating the specificity of the primers for the pig.

7.3.2 EGF-R mRNA

A very strong EGF-R mRNA signal was observed in cumulus, granulosa, and theca cells, whereas the signal in the oocyte was very weak (Figure 7-2A). In addition to the 264 bp band corresponding to EGF-R, some additional bands were observed in cumulus, granulosa, and theca cells (lanes C, G & T in Figure 7-2A). However, only a single band corresponding to the 264 bp was observed in rat kidney (lane K in Figure 7-2A), which was used as a positive control. The identity of this amplified product in rat kidney was confirmed by cutting the band with the restriction enzyme *AvaII*, which yielded the expected fragments of 150 and 114 bp (Figure 7-2B). This PCR amplified rat kidney cDNA was then radiolabelled and used as a probe for Southern analysis in the oocyte, cumulus, granulosa, and theca cells, as described in the Materials and Methods. Southern analysis confirmed only one band corresponding to 264 bp as EGF-R in these cells (Figure 7-2C).

7.3.3 EGF peptide

The immunolocalization of EGF in porcine ovarian follicles is shown in Figure

Figure 7-1. Expression of EGF mRNA in the porcine ovarian follicle. **A:** Total RNA was isolated from oocytes (O), cumulus (C), granulosa (G), and theca (T) cells, submitted to RT-PCR using primers specific for pEGF, and the amplified cDNA fragments analyzed by PAGE. For the negative controls, oocytes or somatic cells were absent from the rinse medium (R), or else the cDNA was replaced with water (W). Since the primers were specifically designed against the porcine EGF sequence, their specificity was tested by using cDNA from rat kidney (K). **B:** The PCR-amplified product in the oocyte was confirmed after digestion with the restriction enzyme *RsaI*, which yielded the expected 119 and 95 bp products. U, uncut; C, cut. Molecular weight markers (L) are a 1 kb ladder containing vector DNA fragments that range from 75 to 1,636 bp (Gibco, BRL). The size (bp) of predicted amplified product is indicated.

Figure 7-2. Expression of EGF-R mRNA in the porcine ovarian follicle. **A:** Total RNA was isolated from oocytes (O), cumulus (C), granulosa (G), and theca (T) cells, submitted to RT-PCR using primers specific for EGF-R, and the amplified cDNA fragments analyzed by PAGE. Since EGF-R is fairly conserved among species, rat kidney cDNA (K) was used as the positive control. For the negative controls, oocytes or somatic cells were absent from the rinse medium (R), or else the cDNA was replaced with water (W). **B:** Confirmation of the PCR-amplified product in rat kidney by digesting with the restriction enzyme *AvaII*, which yielded the expected 150 and 114 bp products. U, uncut (an additional band observed in this control lane have resulted due to the use of cDNA from a different kidney sample that has previously displayed two bands); C, cut. Molecular weight markers (L) are a 1 kb ladder containing vector DNA fragments that range from 75 to 1,636 bp (Gibco, BRL). The size (bp) of predicted amplified product is indicated. **C:** Southern analysis in which the rat kidney PCR-amplified cDNA was used as a probe, confirmed only the band corresponding to 264 bp as being EGF-R in cumulus (C), granulosa (G), and theca (T) cells. A very intense band is also observed in the rat kidney (K), whereas the band is hardly visible in the porcine oocyte (O).

Fig. 7-1

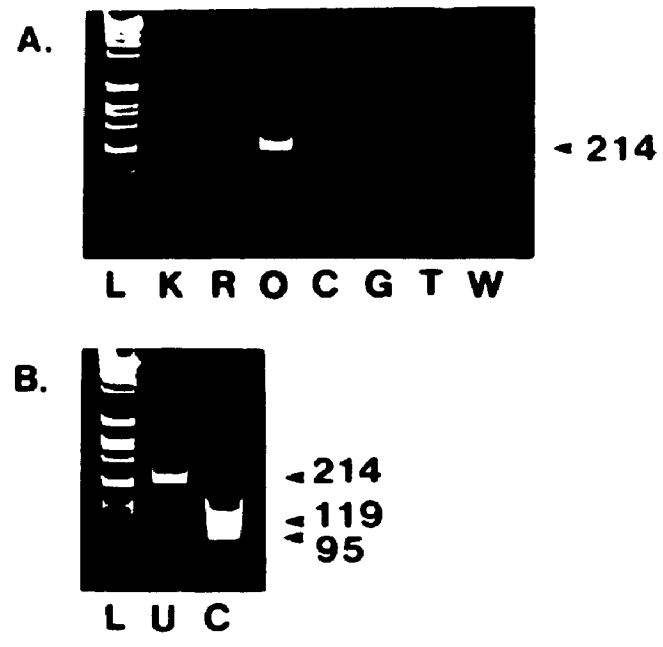
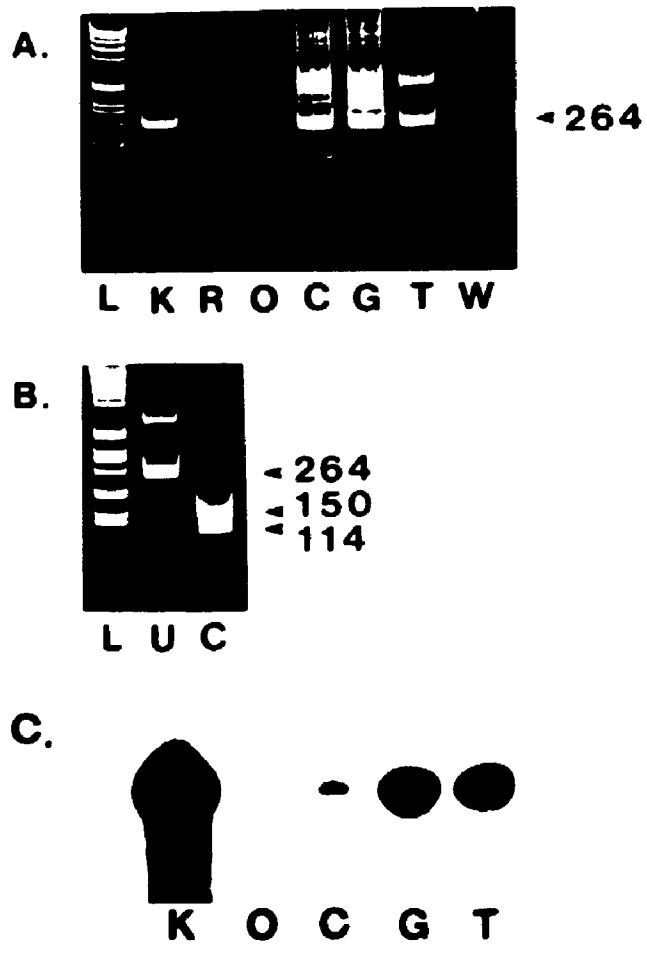

A.
214
L K R O C G T W
B.
214
119
95
L U C

Fig. 7-2


A.
264
L K R O C G T W
B.
264
150
114
L U C
C.
K O C G T

7-3A-E. EGF was localized in the oocyte, cumulus, and granulosa cells of all stages of follicle (Figure 7-3B, C). In the oocyte, the intensity of immunostaining was more pronounced in primordial and primary follicles than in antral follicles. In large antral follicles, granulosa cells showed intense immunostaining, whereas theca cells exhibited little or no detectable staining for EGF (Figure 7-3D). However, in some early antral follicles (a part of one shown at the top of Figure 7-3B), theca cells exhibited some staining for EGF. EGF staining was also observed in the cumulus and granulosa cells of follicles undergoing atresia (Figure 7-3E). EGF-immunostaining was substantially reduced when the primary antibody was replaced with rabbit preimmune serum (Figure 7-3A).

7.3.4 EGF-R peptide

The localization of EGF-R in the porcine ovarian follicle is depicted in Figure 7-4A-F. EGF-R immunostaining was observed in the oocytes of primordial and primary follicles (Figure 7-4B, C), and cumulus, granulosa, and theca cells of all stages of follicle including atretic follicles (Figure 7-4C-F). In large antral follicles, the immunostaining was more pronounced in theca cells than in granulosa cells (Figure 7-4D, E), and the oocyte showed little or no detectable staining (the oocyte not present in Figure 7-4D, E). No immunostaining was observed when the antibody was pre-absorbed with the control peptide (Figure 7-4A).

7.4 Discussion

RT-PCR studies indicated a very strong EGF mRNA signal in the oocyte, whereas the signal in the cumulus, granulosa, and theca cells was very weak but detectable. The expression of EGF gene in the pig oocyte is consistent with a previous report in which only one component of the follicle, the denuded oocytes were examined (Vaughan *et al.*, 1992b). Although the PCR method used in this study was not a quantitative one, the much stronger signal for EGF mRNA in the oocyte compared to somatic follicular cells

Figure 7-3. Immunocytochemical localization of EGF in the porcine ovarian follicle. **A:** Absence of immunostaining in primordial (PL), primary (PR), and antral (AL) follicles when the primary antibody was replaced with pre-immune serum. A, antrum; G, granulosa cells; TI, theca interna, **B:** Similar section of ovary as in **A**, incubated with anti-pEGF serum showing EGF staining in primordial (PL), primary (PR), and antral (AL) follicles. **C:** Portion of an antral follicle showing staining in the oocyte (O), cumulus (C), and granulosa (G) cells. Some staining can also be observed in the antrum (A); however, theca interna (TI) is negative. **D:** Part of a very large antral follicle showing EGF staining in granulosa cells (G), whereas theca interna (TI) and theca externa (TE) show no staining. **E:** Portion of a antral follicle undergoing atresia, showing EGF staining in cumulus cells (C). **A** and **B**, X160; **C**, **D**, and **E**, X250.

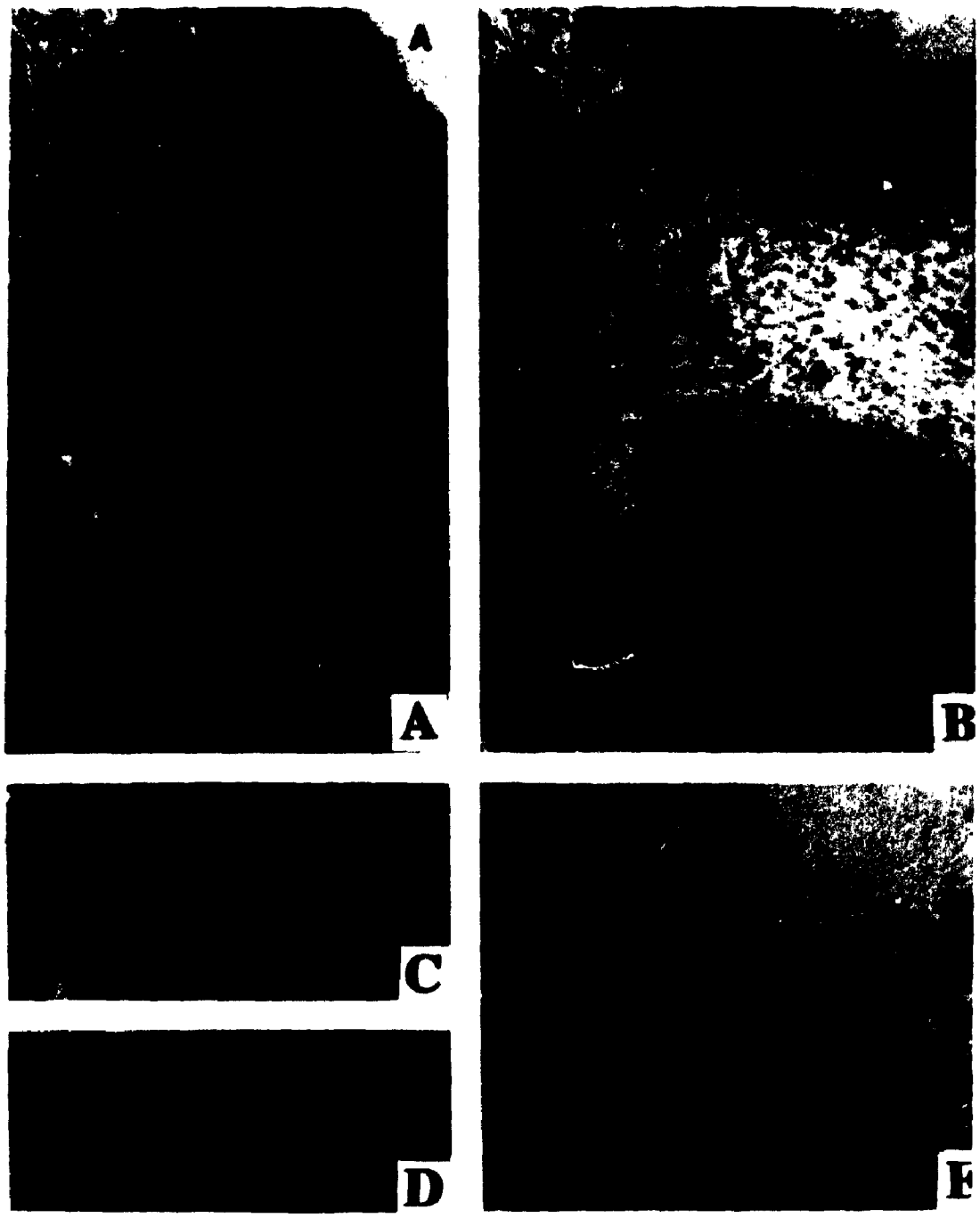
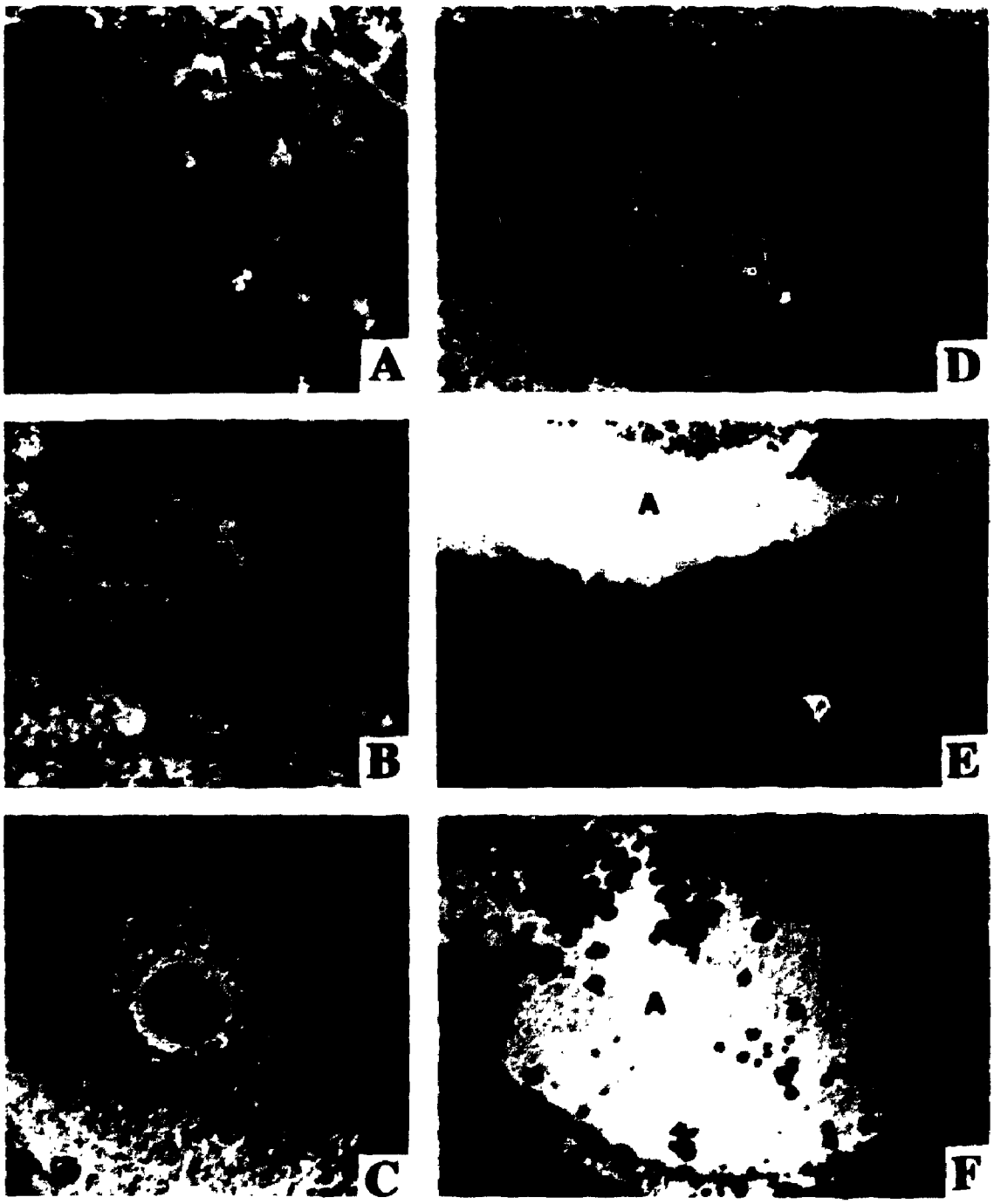

A
B
C
D
E

Figure 7-4. Immunocytochemical localization of EGF-R in the ovarian follicle. **A:** Absence of EGF-R immunostaining in primordial follicles (PL) when the primary antibody was preabsorbed with the control peptide. **B:** Similar section of ovary as in **A**, incubated with EGF-R a ıtibody showing intense EGF-R staining in primordial follicles (PL). **C:** A primary follicle showing EGF-R immunostaining in the oocyte (O) and granulosa cells (G). **D:** An early antral follicle showing a more pronouced EGF-R staining in theca interna (TI) cells compared to granulosa cells (G). **E:** Portion of an antral follicle showing intense staining in theca interna (TI) and theca externa (TE). The granulosa cells (G) also show some staining. **F:** Portion of an antral follicle undergoing atresia, showing EGF-R staining in granulosa cells (G). **A-F,** *Y*250.


A
D
B
A
E
C
A
F

may suggest a greater abundance of EGF mRNA in the oocyte compared to surrounding somatic cells. This is supported by the fact that the number of oocytes used for isolating total RNA was substantially lower than that of the somatic cells (see Materials and Methods). Although quantitative measurements of total RNA were not done before PCR among various groups, consistency of strong EGF expression in the oocyte in two separate cell preparations support such a notion. However, further studies involving quantitative techniques are needed to confirm this possibility.

The cellular distribution of EGF-R gene expression was the opposite to that of EGF, in that a very strong signal for EGF-R mRNA was detected in cumulus, granulosa, and theca cells, whereas a very weak signal was observed in the oocyte. The limited EGF-R mRNA signal in the oocyte may be due to the use of lesser amount of starting RNA for the oocyte, as the total number of oocytes used for RNA extraction was much lower than somatic cells (see Materials and Methods). Alternatively, since PCR is an extremely sensitive technique, the hardly detectable signal in the oocyte may have resulted from the presence of gap-junctional projections from the cumulus cells which transverse the zona. The observation of additional bands in somatic follicular cells of the pig, and only one band in the rat kidney in this gel, may be due to partial sequence homology of EGF-R mRNA with other mRNAs in the porcine ovarian follicle. Southern analysis, however, confirmed the identity of the band corresponding to 264bp as EGF-R. To my knowledge this is the first report indicating the expression of genes for EGF and EGF-R in ovarian follicles for any mammalian species.

Immunocytochemical studies localized EGF peptide in the oocyte, cumulus cells, and granulosa cells of all stages of follicles including atretic follicles. Although immunocytochemical staining alone cannot distinguish between local synthesis and sequestration from other sources, the presence of mRNA in the oocyte, cumulus, and granulosa cells suggest synthesis of EGF by these tissues. The absence of immunoreactive EGF in the presence of its mRNA in theca cells may indicate a controlled translation of the message. Although it is possible to obtain 100% pure preparations for oocytes, cumulus and granulosa cells, due to vascularization, thecal cell preparations often contain some blood cells. Therefore an alternate explanation would

be that the weak mRNA signal observed in theca cells may have resulted from some contaminating blood cells.

The pronounced immunostaining for EGF and EGF-R in the oocytes of primordial and primary follicles suggests a possible role of EGF in the early differentiation of the oocyte. The presence of EGF and EGF-R in the oocyte of primary follicles is consistent with the observations in the human where, in the primary follicle, EGF and EGF-R immunostaining was exclusively localized in the oocyte (Maruo *et al.*, 1993). However, in contrast to the present observations in the pig, EGF and EGF-R immunostaining was absent in the oocyte of primordial follicles in the human. Furthermore, in the human, the intensity of EGF and EGF-R immunostaining in the oocyte increased as the follicle reached the preovulatory stage (Maruo *et al.*, 1993), whereas, in the pig, the intensity of immunostaining in oocyte was maximal in the primordial and primary follicles and decreased as the follicle progressed to the antral stage. These differences in the staining pattern of the oocyte during follicular growth between the pig and the human may be attributed to species differences. The staining of EGF in the oocyte appeared to be cytoplasmic. This is consistent with a previous report in the pig where cytoplasmic localization of EGF was observed in the distal convoluted tubule of the kidney (Vaughan *et al.*, 1991). The receptor immunostaining was present in the periphery, cytoplasm, and nucleus of the oocyte. This suggests that EGF has cell surface as well as intracellular sites of action. Previous studies have shown that EGF can directly regulate target cell nuclear functions (Schindler & Jiang, 1987; Jiang & Schindler, 1990). Since EGF-R gene expression was negligible in the oocyte of antral follicles (Figure 7-2), and the oocytes from primordial and primary follicles were not subjected to RT-PCR, it is difficult to ascertain the source of EGF-R observed in oocytes at the early stages of follicular development. It may have been synthesized in abundance in the oocyte at an earlier stage of development and retained after synthesis, or alternatively, transported into the oocyte from cumulus cells via one of the mechanisms present in the oocyte for macromolecule transport such as active pinocytosis or endocytosis (reviewed by Tsafriri, 1978). In the antral follicle, the presence of EGF mRNA and peptide in the oocyte and its receptor in the surrounding somatic cells may suggest a paracrine role for EGF of

oocyte origin.

Both EGF and EGF-R mRNAs and peptides were localized in the cumulus cells. As mentioned in Chapters 5 and 6, addition of exogenous EGF to the culture media containing isolated porcine OCC results in the induction of cumulus cell expansion and oocyte maturation. It has also been shown in the mouse (Downs *et al.*, 1988) and in the pig (Coskun & Lin, 1992) that exogenous EGF induces oocyte maturation by generating a positive signal in the cumulus cells. This suggests that the receptors present in cumulus cells are functional. However, since the resumption of meiotic maturation and cumulus expansion *in vivo* does not occur before the endogenous preovulatory surge of LH, the levels of EGF, though detectable by such sensitive methods, may be subthreshold for triggering these OCC events.

In the primary and antral follicles, granulosa cells exhibited staining for both EGF and EGF-R, whereas little or no staining was observed for EGF in theca cells. May *et al* (1992) reported a potent mitogenic effect of EGF on porcine granulosa cells, whereas theca cells were much less responsive. Thus, EGF in granulosa cells of primary and early antral follicles may be involved in an autocrine fashion with rapid granulosa cell proliferation associated with follicular growth. In addition, EGF has also been implicated in the regulation of differentiation and steroidogenesis of granulosa cells (Hsueh *et al.*, 1981; Mondschein & Schomberg, 1981; Jones *et al.*, 1982; Knecht & Catt, 1983; Roy & Greenwald, 1991). In contrast to the hamster ovary where EGF peptide was absent in granulosa cells of atretic follicles (Roy & Greenwald, 1990), both EGF and EGF-R staining was observed in cumulus/granulosa cells of atretic follicles in the pig ovary. In the human, however, both EGF and EGF-R were localized predominantly in the theca interna cells of the atretic follicles, whereas the granulosa cells exhibited negligible staining (Maruo *et al.*, 1993). Both EGF and TGF-α have been shown to inhibit spontaneous onset of apoptosis, an indication of follicular atresia, in cultured rat ovarian granulosa cells and intact follicles (Tilly *et al.*, 1992). These observations suggest that EGF of follicular origin may have a role in the prevention of onset of follicular atresia.

The presence of abundant mRNA, and the immunocytochemically detectable EGF-R peptide in theca cells, suggests synthesis of the receptor for EGF in these cells. This is consistent with a previous report in the rat (Chabot *et al.*, 1986) and in the human (Maruo *et al.*, 1993), where EGF-R were detected in theca cells. In the pig, however, EGF binding sites were exclusively localized in granulosa cells, whereas the theca cells were completely negative (Fujinaga *et al.*, 1992). On the other hand, EGF has been shown to influence porcine theca cell proliferation alone and synergistically with PDGF (May *et al.*, 1992), suggesting the presence of functional EGF-R in theca cells. The present study in which theca cells exhibited even more pronounced staining for EGF-R than granulosa cells, indicates the presence of at least immunoreactive receptors in these cells. The presence of EGF-R in the absence of its ligand in theca cells suggests a paracrine role of EGF acquired from other components of the follicle or from the general ovarian circulation in these cells. In addition, several other peptides including TGF-α have been reported to interact with the EGF-R, and trigger a signal transduction cascade similar to EGF (Prigent & Lemoine, 1992). Furthermore, TGF-α mRNA and peptide have been localized in porcine granulosa cells (Chapter 10), and TGF-α protein in bovine theca cells (Skinner & Coffey, 1988; Lobb *et al.*, 1989), suggesting that these other ligands may interact with EGF-R in theca cells and may alter their proliferation, steroidogenesis, or differentiation in an autocrine/paracrine manner.

In summary, studies in this chapter have provided evidence that both EGF and its receptor are synthesized by the oocyte and somatic follicular cells of the porcine ovary. From the relative abundance of the mRNAs and peptides, it appears that, within the follicle, the oocyte is the primary, although not necessarily the exclusive source of EGF and the somatic cell that of EGF-R, thereby suggesting that an EGF paracrine system may be in place in the porcine ovary. Although these localization studies provide further support to implicate EGF in follicular physiology, additional studies are needed to understand the regulation of production and secretion of EGF, and the synthesis of its receptor in the follicle. Previous studies in Chapters 4 and 5 have found EGF to be a potent stimulant of porcine oocyte maturation and cumulus expansion, the events

associated *in vivo* with the preovulatory surge of LH which lead to the release of fertilizable oocytes. A better understanding of the regulation of EGF and EGF-R production and secretion in the oocyte and other somatic follicular cells will help to determine the events leading to the normal controlled proliferation of somatic cells, the development of a healthy follicle, and the release of a developmentally competent oocyte.

CHAPTER 8 INVOLVEMENT OF TYROSINE KINASE AND cAMP-DEPENDENT PKA IN EGF-STIMULATED OOCYTE MATURATION AND CUMULUS EXPANSION

8.1 Introduction

Prevailing opinion holds that the cAMP-dependent PKA is the second messenger pathway for gonadotropin-induced cumulus expansion and oocyte maturation (Dekel & Kraicer 1978; Eppig, 1979a; Downs *et al.*, 1988). On the other hand, cAMP is inhibitory to mammalian oocyte maturation as the cAMP analogs such as dbcAMP, or PDE inhibitors such as IBMX, inhibit the spontaneous maturation of both cumulus cell-enclosed and denuded oocytes (Tsafriri *et al.*, 1982; Eppig & Downs, 1984). The effect of FSH on murine oocyte maturation has also been shown to be at first inhibitory and later stimulatory (Downs & Eppig, 1985). These authors proposed that the initial inhibitory action of FSH on oocyte maturation may be the result of elevated cAMP levels and that once the amount of cAMP reaches the maturation threshold, a positive signal is generated in cumulus cells which make this gonadotropin stimulatory. Consistent with this idea was the induction of GVBD by a brief exposure of OCC to a high concentration (1 mM) of the dbcAMP (Downs *et al.*, 1988). Evidence therefore suggests that oocyte maturation is modulated according to the duration and magnitude of elevated cAMP levels.

The mechanisms of EGF-induced cumulus expansion and oocyte maturation, however, are unknown. EGF stimulation of the intrinsic tyrosine kinase (EGF-TK) activity of its receptor is considered the most likely event in the generation of second messengers (Oberg *et al.*, 1990). EGF stimulation of the tyrosine kinase has been shown to result in hydrolysis of phosphatidyl inositol-4, 5-bisphosphate to form inositol-1, 4, 5-trisphosphate and DAG in A-431 cells (Wahl & Carpenter, 1988). These products in turn liberate calcium from endoplasmic reticulum and activate calcium-dependent PKC respectively (Abdel-Latif, 1986). However, tyrosine kinase stimulation is not always an exclusive event in the generation of second messengers by EGF. Bovine luteal cells possess an aberrant form of EGF-R which is devoid of intrinsic TK activity (Chakravorty

et al., 1993). The ligand activates other signalling systems, such as MAP kinase by modulating critical post-receptor phosphorylation/dephosphorylation events in the luteal cells. Furthermore, EGF stimulated cAMP accumulation in mouse OCC, indicating that EGF-induced oocyte maturation may, in fact, be mediated through a cAMP-dependent PKA pathway (Downs *et al.*, 1988). This cAMP stimulation in OCC is consistent with observations in A-431 cells where activation of the EGF-R resulted in increased intracellular cAMP levels (Ball *et al.*, 1990). On the other hand, EGF actions in follicle cells are often inconsistent with those of cAMP. For instance, EGF failed to stimulate cAMP synthesis in rat granulosa cells and antagonized FSH-stimulated cAMP accumulation (Knecht & Catt, 1983). In addition, EGF attenuated cAMP-mediated LH receptor induction in rat and pig granulosa cells (Schomberg *et al.*, 1983) and inhibited FSH-, dbcAMP- or cholera toxin-stimulated steroidogenesis in rat granulosa cells (Hsueh *et al.*, 1981). To date no study looking at the signal transduction pathways of EGF-induced cumulus expansion and oocyte maturation has been reported for any mammalian species. The present study examines the involvement of EGF-TK and PKA pathways in the mediation of EGF-induced porcine cumulus expansion and oocyte maturation.

8.2 Materials and Methods

8.2.1 Isolation and culture of OCC

Intact OCC surrounded by 4-5 layers of unexpanded cumulus oophorus cells and with a homogeneous cytoplasm were isolated and cultured as described in Chapter 4. For determining effects on cumulus expansion and oocyte maturation, the culture media was supplemented with EGF (1 ng/ml) or FSH (1.5 μg/ml) in the absence or presence of various inhibitors. These levels of EGF and FSH are based on the studies described in the previous Chapters. Incubations were carried out at 39°C for different time periods and treatment conditions depending upon the experiments. At the end of cultures, cumulus expansion was scored on a scale of 0-4 and the oocytes examined for GVBD as previously described in Chapter 4.

8.2.2 Intracellular cAMP measurements

To test the involvement of cAMP, freshly isolated OCC were cultured for 3 hours in medium supplemented with IBMX (100 μM) in the absence/presence of 1 ng/ml EGF or 1.5 μg/ml FSH. To determine the effects of tyrphostin (a putative EGF-R tyrosine kinase inhibitor from Gibco) on EGF- and FSH-stimulated cAMP production, OCC were cultured for 17 hours with 100 μM tyrphostin and then exposed to the above hormones for 3 hours. These cultures were carried out in the absence of IBMX to eliminate any interaction of this chemical particularly with EGF in the stimulation of cAMP production in OCC. The overnight pre-incubations with tyrphostin were done based on the results from previous studies suggesting that initial overnight incubations with tyrphostins are necessary to allow for their inhibitory effects on EGF actions (Lyall *et al.*, 1989; Dardik & Schultz, 1991). At the end of the culture period, OCC were washed in medium and D-PBS containing IBMX to minimize cAMP hydrolysis, transferred immediately to 20 μl of 0.5% sodium dodecyl sulphate for cell lysis (5-10 min with vortexing), and then the samples were stored at -70°C. Before extraction of cAMP with 100 μl of 6% trichloroacetic acid at 4°C, 10 μl BSA (10 μg/ml) was added to each tube to act as a carrier protein to facilitate the precipitation of cellular proteins. After centrifugation at 2000 X g for 5 min, supernatants were removed and lyophilized. The extracted samples were diluted and assayed after acetylation. Cyclic AMP was measured by a previously validated radioimmunoassay (Reddoch & Armstrong, 1984).

8.2.3 Statistical analysis

Each experiment was replicated at least three times to confirm consistency of results. Statistical analysis using the Chi-square (X^2) test was carried out on pooled data for oocyte maturation and cumulus expansion studies; P values <0.05 were considered significant. The effects of EGF, FSH, and tyrphostin upon cAMP contents in OCC were assessed by analysis of variance (ANOVA). When required, data were logarithmically transformed prior to statistical analysis to reduce heterogeneity of variance (Snedecor, 1956). Differences between means for multiple comparisons were assessed by Duncan's New Multiple Range test (Steel & Torrie, 1960).

8.3 Results

8.3.1 Involvement of EGF-TK in EGF-induced porcine cumulus expansion and oocyte maturation

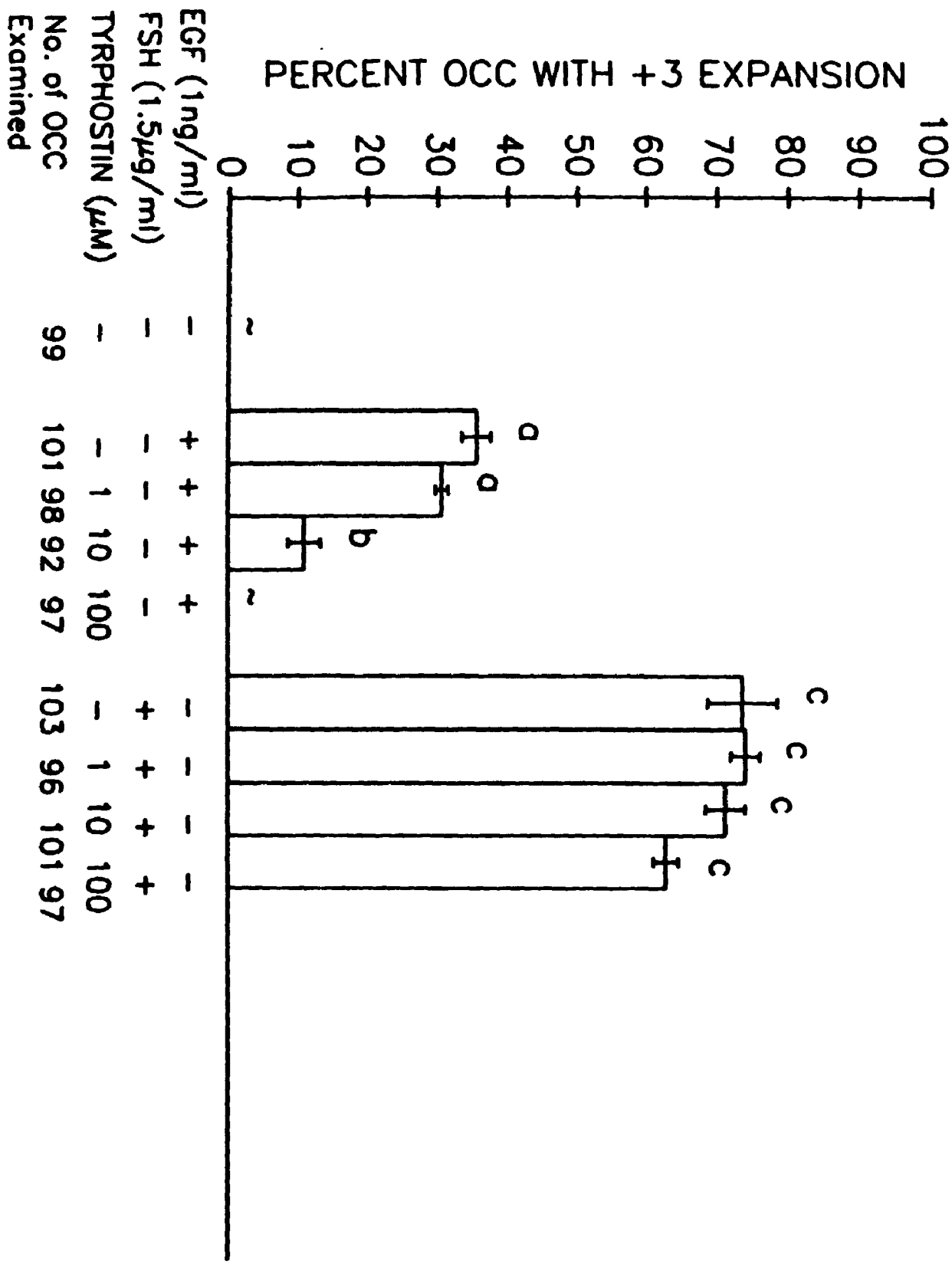
To determine involvement of EGF-TK, the OCC were cultured overnight (17 hours) with varying concentrations of tyrphostin (1-100 μM), and then exposed to EGF (1 ng/ml) or FSH (1.5 μg/ml) for another 12 hours for oocyte maturation studies, and 24 hours for cumulus expansion studies. Based on studies in Chapter 5, the total culture periods for oocyte maturation studies were kept as close to 24 hours as possible to keep the control levels of GVBD low. Both EGF and FSH significantly stimulated cumulus expansion over the control (Text Figure 8-1), although the proportion of expanded OCC particularly in response to EGF was lower compared to the previous studies in Chapters 5 and 6. The maximal degree of cumulus expansion observed at the end of culture periods was +3 and, due to the low number of complexes exhibiting +1 and +2 degrees of cumulus expansion, only the complexes with +3 expansion were used for comparisons among various groups. Tyrphostin concentration-dependently inhibited EGF-induced cumulus expansion with complete inhibition at 100 μM, but had no significant effect on FSH-induced cumulus expansion.

EGF, but not FSH, significantly increased the incidence of GVBD in the absence of tyrphostin (Text Figure 8-2). Tyrphostin failed to inhibit EGF-induced GVBD, and in fact by itself significantly increased the proportion of oocytes undergoing GVBD when added at a concentration of 100 μM (Text Figure 8-2). FSH alone did not stimulate GVBD over the control after 12 hours of culture, but significantly inhibited the incidence of GVBD observed at each tyrphostin concentration (Text Figure 8-2).

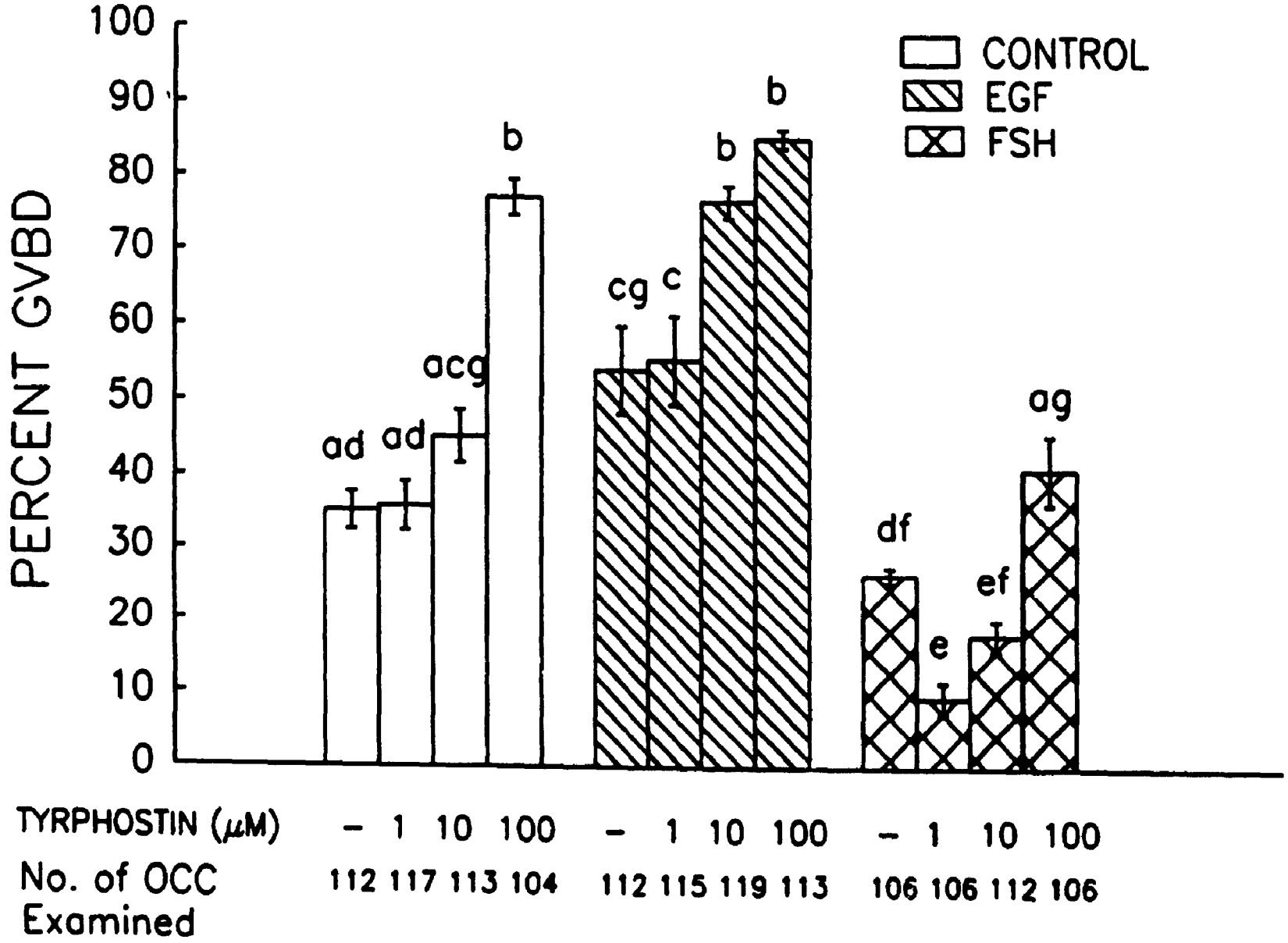
8.3.2 Effects of EGF and FSH on cAMP contents of OCC

As shown in Text Figure 8-3, EGF caused a small but significant, 2-fold stimulation of cAMP production over the control (7.1 $\pm$ 0.9 vs 3.6 $\pm$ 0.5 fmol/OCC); In contrast, FSH caused a much greater, 60-fold stimulation (210 $\pm$ 23 vs 3.6 $\pm$ 0.5 fmol/OCC in the control).

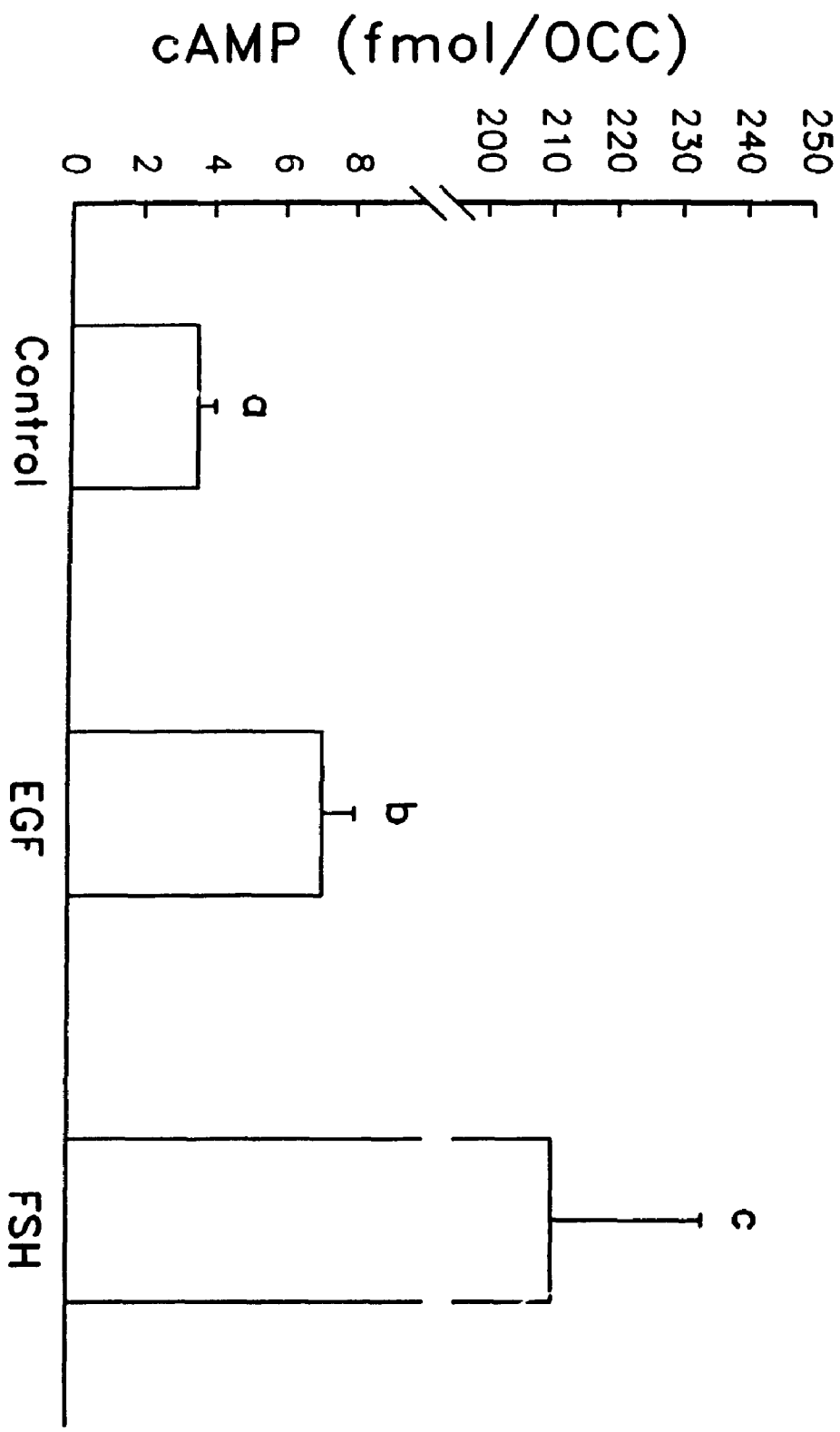
Text Figure 8-1. Effects of tyrphostin on EGF- and FSH-induced porcine cumulus expansion. Oocyte-cumulus cell complexes were cultured in the medium containing varying concentrations of the tyrphostin for 17 hours and then exposed to EGF (1 ng/ml) or FSH (1.5 μg/ml) for another 24 hours before assessment of cumulus cell expansion. Data represent percent mean $\pm$SEM of three independent experiments. Bars with different superscripts indicate significant difference ($P < 0.05$).


PERCENT OCC WITH +3 EXPANSION
0
10
20
30
40
50
60
70
80
90
100
EGF (1ng/ml)
FSH (1.5μg/ml)
TYRPHOSTIN (μM)
No. of OCC Examined
- - - 99
+ - - 101
+ - 1 98
+ - 10 92
+ - 100 97
- + - 103
+ + 1 96
+ + 10 101
+ + 100 97
a
a
b
a
c
c
c
c

Text Figure 8-2. Dose-dependent effects of tyrphostin on EGF- and FSH-induced GVBD. Oocyte-cumulus cell complexes were cultured in the medium containing different concentrations of the tyrphostin for 17 hours prior to EGF (1 ng/ml) or FSH (1.5 μg/ml) addition and GVBD assessed after 12 hours of hormonal stimulation. Data represent percent mean $\pm$SEM of three independent experiments. Bars with different superscripts indicate significant difference ($P < 0.05$).


PERCENT GVBD
100
90
80
70
60
50
40
30
20
10
0
CONTROL
EGF
FSH
ad
ad
acg
b
cg
c
b
b
df
e
ef
ag
TYRPHOSTIN (μM) – 1 10 100 – 1 10 100 – 1 10 100
No. of OCC Examined 112 117 113 104 112 115 119 113 106 106 112 106

Text Figure 8-3. The effects of EGF and FSH on intracellular cAMP accumulation. Oocyte-cumulus cell complexes were cultured for 3 hours in the presence of EGF (1 ng/ml) or FSH (1.5 μg/ml) and cAMP levels measured by RIA. Data are presented as the mean $\pm$SEM of duplicate observations from three independent experiments (n=6). Bars with different superscripts indicate significant difference ($P < 0.05$).


cAMP (fmol/OCC)
0
2
4
6
8
200
210
220
230
240
250
Control
EGF
FSH
a
b
c

8.3.3 Effects of tyrphostin on EGF- and FSH-stimulated cAMP production

The effects of tyrphostin on EGF- and FSH-stimulated cAMP production are depicted in Text Figure 8-4. Similar to that in fresh OCC, both EGF and FSH significantly stimulated cAMP production over the control (2.4 $\pm$ 0.3 and 24.5 $\pm$ 3.7 fmol/OCC respectively vs 1.3 $\pm$ 0.02 fmol/OCC in the control) in the preincubated OCC. However, the levels of cAMP production were lower in all groups of these precultured OCC compared to those in freshly cultured OCC (Text Figure 8-3 vs Text Figure 8-4). Tyrphostin had no effect on cAMP production by itself, but completely inhibited EGF-stimulated cAMP production. In contrast, tyrphostin did not inhibit FSH-stimulated cAMP production, but rather there was a trend towards stimulation of cAMP by tyrphostin in the presence of FSH.

8.3.4 Involvement of PKA in EGF- and FSH-stimulated porcine cumulus expansion and oocyte maturation

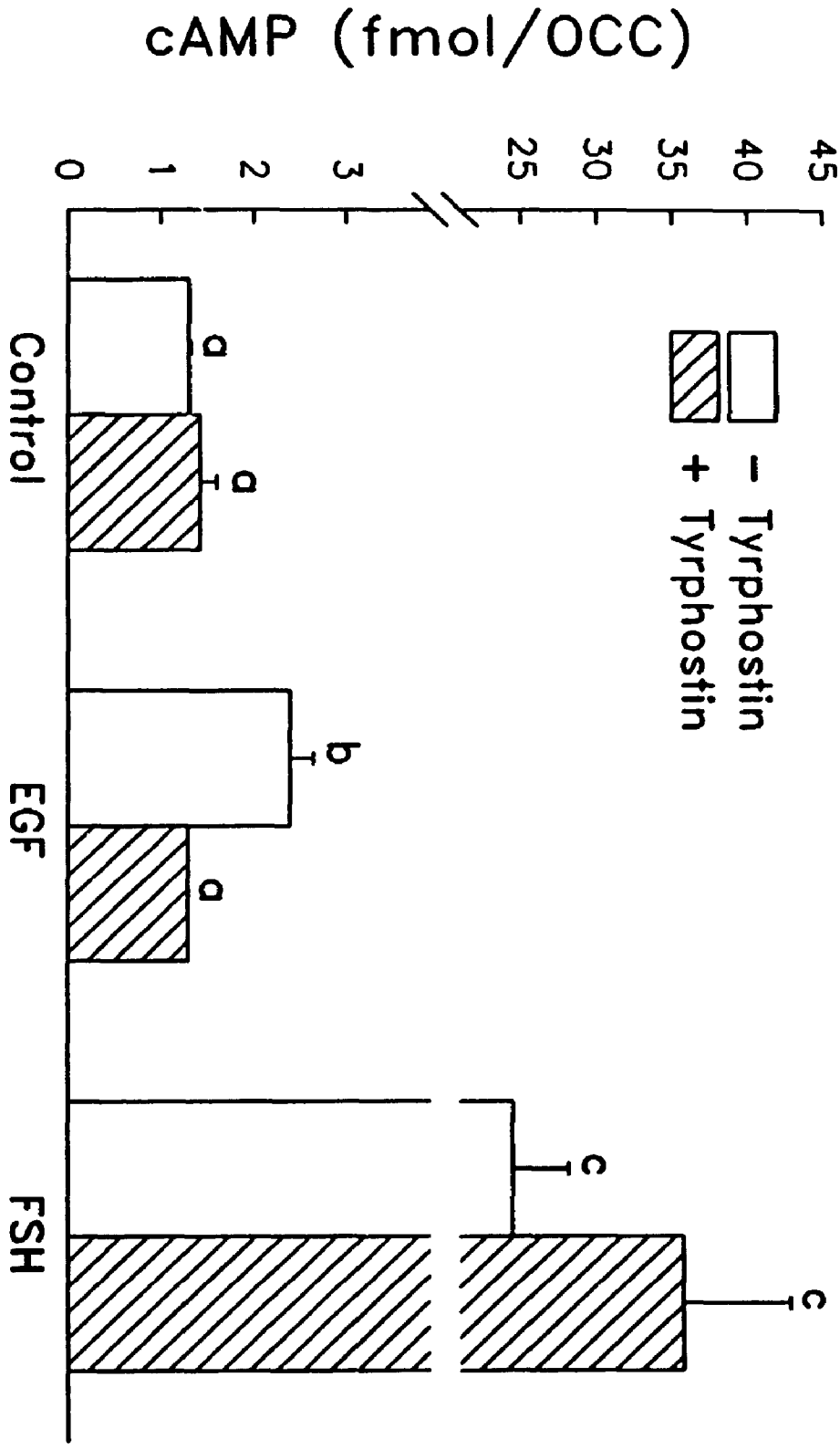
Since H-89 (a specific PKA inhibitor, purchased from Calbiochem, La Jolla, CA) penetrates live cells rapidly (Chijiwa *et al.*, 1990), the OCC were precultured for only 2-3 hours in the presence of 0.5-10 μM and 10 μM H-89 before the addition of EGF and FSH respectively, and cultures were continued for another 22 hours. The OCC were then observed for cumulus cell expansion and the oocytes for GVBD. The proportions of complexes undergoing expansion in response to EGF were significantly reduced by 5 and 10 μM H-89 and to FSH by 10 μM H-89 (Text Figure 8-5). However, 10 μM H-89 failed to completely inhibit cumulus expansion in response to either EGF or FSH.

The proportion of oocytes undergoing GVBD in response to EGF was decreased significantly by H-89, with complete inhibition occurring at 10 μM (Text Figure 8-6). Concentrations of H-89 as low as 0.5 μM significantly decreased EGF-induced porcine oocyte maturation (Text Figure 8-6). At the single concentration used (10 μM), H-89 also completely inhibited the oocyte maturation in response to FSH (Text Figure 8-6).

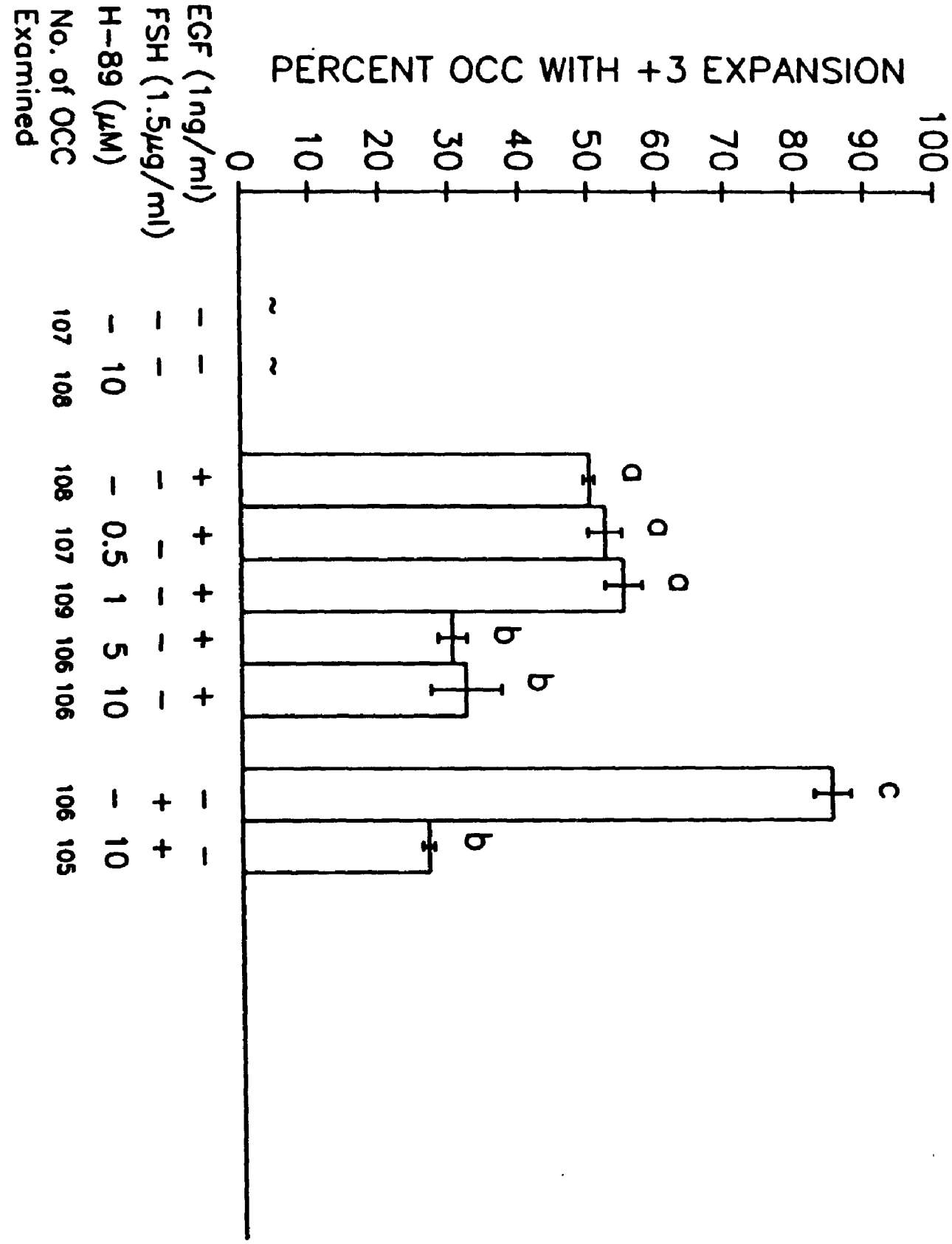
8.4 Discussion

Tyrphostins are membrane-permeable inhibitors of EGF-TK and are believed to

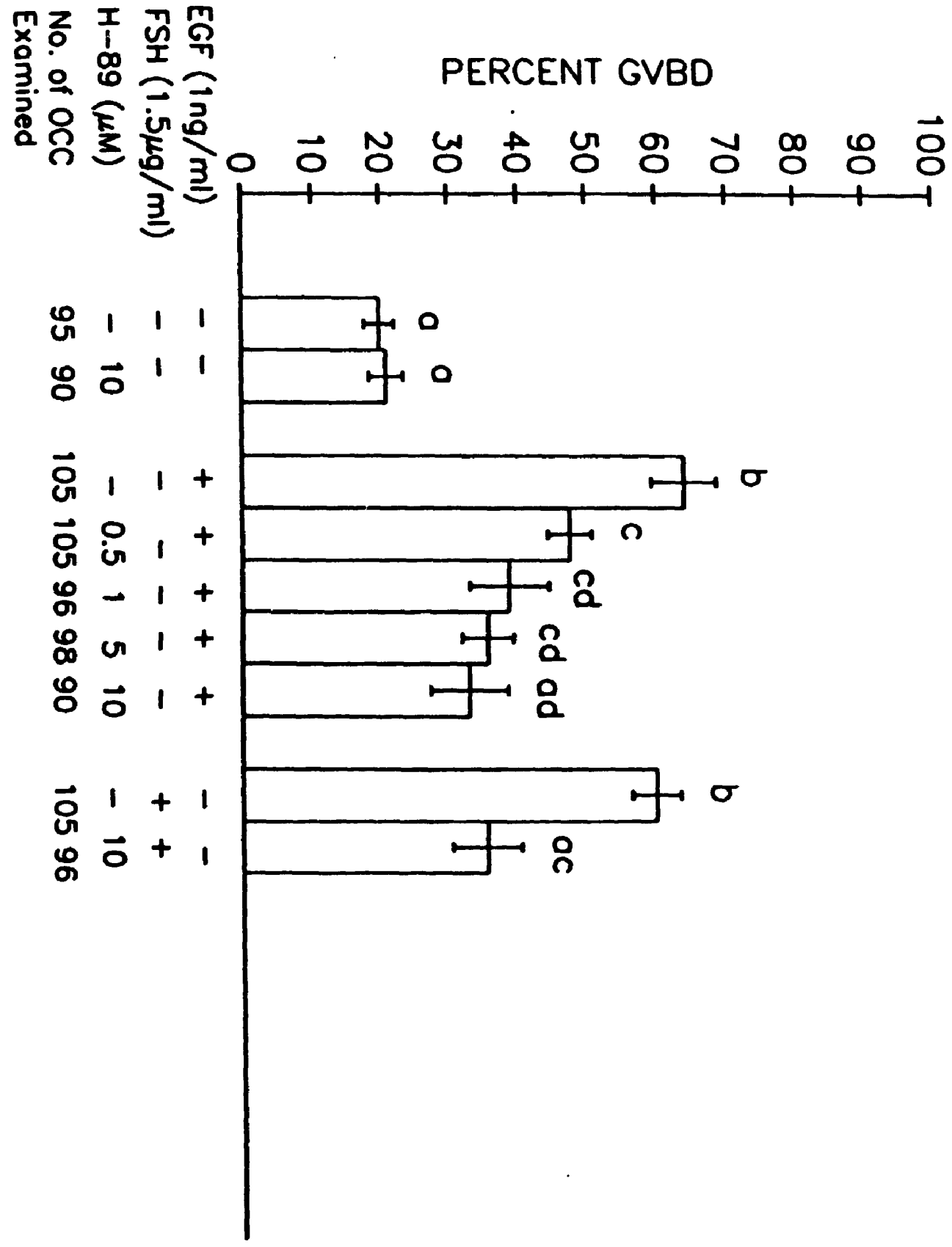
Text Figure 8-4. The effects of tyrphostin on EGF- and FSH-stimulated intracellular cAMP accumulation. Intact oocyte-cumulus cell complexes were cultured overnight for 17 hours with 100 μM tyrphostin prior to EGF (1 ng/ml) or FSH (1.5 μg/ml) exposure for 3 hours and cAMP levels measured by RIA. Data are presented as the mean $\pm$SEM of triplicate observations confirmed in three independent experiments (n=9). Bars with different superscripts indicate significant difference ($P < 0.05$).


cAMP (fmol/OCC)
0
1
2
3
25
30
35
40
45
- Tyrphostin
+ Tyrphostin
Control
EGF
FSH
a
a
b
a
c
c

Text Figure 8-5. The effects of H-89 on EGF- and FSH-induced cumulus expansion. Oocyte-cumulus cell complexes were cultured for 2-3 hours in the presence of different concentrations of H-89 and then exposed to EGF (1 ng/ml) or FSH (1.5 μg/ml) for another 22 hours before assessment of cumulus cell expansion. Data represent percent mean $\pm$SEM of three independent experiments. Bars with different superscripts indicate significant difference ($P < 0.05$).


PERCENT OCC WITH +3 EXPANSION
0
10
20
30
40
50
60
70
80
90
100
EGF (1ng/ml)
FSH (1.5μg/ml)
H-89 (μM)
No. of OCC Examined
- - - 107
- - 10 108
+ - - 108
+ - 0.5 107
+ - 1 109
+ - 5 106
+ - 10 106
- + - 106
+ + 10 105
a
a
a
b
b
c
b

Text Figure 8-6. The effects of H-89 on EGF- and FSH-induced GVBD. Oocyte-cumulus cell complexes were cultured in the presence of different concentrations of H-89 for 2-3 hours prior to EGF (1 ng/ml) or FSH (1.5 μg/ml) exposure for another 22 hours and then observed for GVBD. Data represent percent mean $\pm$SEM of three independent experiments. Bars with different superscripts indicate significant difference ($P < 0.05$).


PERCENT GVBD
0
10
20
30
40
50
60
70
80
90
100
EGF (1ng/ml)
FSH (1.5μg/ml)
H-89 (μM)
No. of OCC Examined
a
a
b
c
cd
cd
ad
b
ac
95 90 105 105 96 98 90 105 96

be relatively specific for EGF-R (Yaish *et al.*, 1988; Lyall *et al.*, 1989; Lamb & Shubhada, 1993). The Gibco tyrphostin used in this study dose-dependently inhibited EGF-induced cumulus expansion with complete inhibition at 100 μM, a level which is consistent with that found effective in other cell types (Lyall *et al.*, 1989; Margolis *et al.*, 1989). The tyrphostin had no significant effect on FSH-induced cumulus expansion suggesting that the inhibition of EGF-induced expansion was not due to a non-specific cytotoxic effect. Tyrphostins do not alter cell surface display of EGF-R, EGF binding or EGF-induced internalization, degradation and down-regulation of EGF-R (Lyall *et al.*, 1989). Thus, the inhibition of EGF-induced porcine cumulus expansion is most likely due to specific inhibition of the tyrosine kinase activity of the EGF-R. The proportion of OCC undergoing cumulus expansion particularly in response to EGF was lower in this experiment compared to the H-89 experiment and previous studies (Chapters 5 and 6). This may be due the fact that OCC became less responsive to EGF due to 17 hours preincubation in the absence of ligand in this experiment.

In a totally unexpected observation, however, this tyrphostin not only failed to inhibit EGF-induced porcine oocyte maturation, but by itself significantly increased the proportion of oocytes undergoing GVBD. The specificity of tyrphostins for EGF-TK is consistent with observations that 1000-fold higher concentrations of tyrphostins were required to inhibit other tyrosine kinases (TKs) including the insulin receptor kinase (Gazit *et al.*, 1989; Lamb & Shubhada, 1993). Therefore, the stimulation of GVBD by this tyrphostin is unlikely to be due to non-specific interactions with other TKs which may mediate the maintenance of the porcine oocyte in meiotic arrest. The stimulation of GVBD by this tyrphostin may be due to a direct effect on the oocyte or an indirect effect via uncoupling of junctional complexes between cumulus cells and the oocyte which are involved in maintaining the meiotic arrest in the oocyte. This is further supported by the fact that removal of cumulus cells from the oocyte enhances the resumption of nuclear maturation in the oocyte (Downs *et al.*, 1988; Singh & Armstrong, unpublished). That the tyrphostin inhibited EGF-stimulated expansion while stimulating GVBD by itself further support the previous concept that the two physiological events

of oocyte maturation and cumulus expansion are not causally related (Downs, 1989).

In this same experiment, FSH failed to stimulate oocyte maturation over the control and significantly inhibited tyrphostin-stimulated GVBD. This may be attributed to the shorter exposure time (12 hours) of OCC to FSH compared to 24 hours in other experiments due to a prior 17 hours exposure to tyrphostin, and because the effect of FSH on oocyte maturation is first inhibitory and later stimulatory (Downs *et al.*, 1988). It has been proposed that the initial inhibitory action of FSH on oocyte maturation may be due to elevated cAMP levels and once the amount of cAMP-generated positive signal reaches the maturation threshold, the effect of FSH becomes stimulatory (Downs & Eppig, 1985). EGF, on the other hand, significantly stimulated GVBD over the control even after 12 hours exposure. This further supports the previous observations in the mouse where EGF was found to stimulate GVBD at all time points (Downs *et al.*, 1988).

In the present study, EGF produced a small but significant, 2-fold stimulation of cAMP production compared to a much greater (60-fold) stimulation by FSH. This difference may be explained on the basis that FSH is a more potent stimulant of cumulus cell expansion than EGF in the pig (Chapters 5 & 6). However, in the mouse where EGF was a more effective stimulant of cumulus cell expansion than FSH (Downs, 1989), EGF produced only a 4-fold increase in cAMP compared to 15-fold increase by FSH (Downs *et al.*, 1988). Therefore, the difference in cAMP levels between EGF and FSH groups may be due to differences in kinetics of stimulation of cAMP production by EGF compared to FSH.

Similar to that in fresh OCC, both EGF and FSH significantly stimulated cAMP production over the control in the preincubated OCC (Text Figure 8-4). However, in general the levels of cAMP produced (fmol/OCC) were lower in all groups of these precultured OCC compared to that in freshly cultured OCC. This may partially be due to the absence of IBMX in these precultured OCC, and partially due to some loss of responsiveness of OCC to the hormones due to 17 hours preculture. Tyrphostin had no effect on cAMP production by itself, but completely inhibited EGF-stimulated cAMP production. This further supports our proposed hypothesis that the stimulatory effect of tyrphostin on GVBD is not due to alterations in cAMP content of OCC, but may in fact

be due to direct effects of this chemical on OCC. Complete inhibition of EGF-stimulated cAMP production by tyrphostin indicates that cAMP is involved downstream of EGF-TK. Tyrphostin did not inhibit FSH-stimulated cAMP production, but rather there was a trend towards stimulation of cAMP by tyrphostin in the presence of FSH. Although this increase in cAMP levels was not statistically significant, this slight elevation by tyrphostin of already high FSH-stimulated cAMP content may partially explain FSH inhibition of tyrphostin-stimulated GVBD. Since EGF has been shown to inhibit FSH-stimulated cAMP production in granulosa cells (Knecht & Catt, 1983), this stimulatory trend in cAMP production in response to tyrphostin when added with FSH may be due to inhibition of actions of either endogenous EGF produced by OCC or the EGF present in serum.

The ability of H-89 to block both EGF- and FSH-stimulated cumulus expansion and GVBD confirmed the involvement of cAMP-dependent PKA in the regulation of porcine cumulus expansion and oocyte maturation. H-89 is a selective, non-cytotoxic and potent inhibitor of PKA with an inhibition constant of 0.048 ± 0.008 μM, which inhibits PKA by competing with ATP for PKA substrate phosphorylation and exhibits very weak inhibitory actions against other kinases (Chijiwa *et al.*, 1990). There was a significant inhibition of EGF-induced cumulus expansion by H-89 suggesting the involvement of PKA in this response. However, H-89 did not completely inhibit EGF-induced cumulus expansion when used up to 10 μM. H-89 at 10 μM also failed to completely inhibit FSH-induced cumulus expansion, which is considered to be primarily mediated through cAMP-dependent PKA pathway (Dekel & Kraicer, 1978; Eppig, 1979a; Downs *et al.*, 1988). Thus the failure of H-89 to completely inhibit EGF- and FSH-induced porcine cumulus expansion may be due, in part, to inadequate permeability of H-89 in intact OCC *in vitro*. H-89 is considered to penetrate live cells rapidly and in the original study (Chijiwa *et al.*, 1990), PC 12D cells were exposed to H-89 only for 30 minutes prior to hormonal stimulation compared to 2-3 hours in the present study. Hence, an alternate explanation for the incomplete inhibition of EGF- and FSH-induced cumulus expansion by H-89 may be high initial content of ATP in fresh OCC. Since H-89 completely

inhibited EGF- and FSH-induced oocyte maturation in the same OCC, the incomplete inhibition of cumulus expansion may in fact be due to an involvement of some other second messenger pathway(s) in the induction of cumulus expansion .

In summary, the present study has examined the mechanisms of EGF-induced porcine cumulus expansion and oocyte maturation. Evidence was obtained that EGF-induced cumulus expansion may be mediated through EGF-TK-cAMP-PKA pathway and oocyte maturation through cAMP-PKA pathway. The results from this study support the previous concept that the two events of oocyte maturation and cumulus expansion are not causally related and that they are mediated via different pathways. Although the maturation studies failed to answer whether or not EGF-TK is involved in EGF-induced oocyte maturation, they provided evidence that the tyrphostin is a potent stimulant of porcine oocyte maturation and that its effect is not mediated through cAMP. Further studies are required to test the participation of some other kinases including DAG-calcium-dependent protein kinase C and cGMP-dependent PKG in the mediation of these EGF-induced OCC processes. The mechanism(s) of tyrphostin-stimulated oocyte maturation also remains to be elucidated.

CHAPTER 9 EFFECTS OF EGF AND FSH ON CYTOPLASMIC MATURATION OF THE OOCYTE

9.1 Introduction

The dominant feature of oocyte maturation is that it represents that period during which the female gamete changes from a developmentally incompetent cell to one with the capacity to direct and support the events of fertilization and early embryonic development. As reviewed in Chapter 2, although spontaneous nuclear maturation of competent oocytes *in vitro* upon liberation from follicles results in morphologically normal secondary oocytes, the functional normality of these oocytes is not assured. These deficiencies are attributed to abnormalities in cytoplasmic maturation of these oocytes even though apparently normal nuclear maturation is observed (Thibault, 1977; Leibfried-Rutledge *et al.*, 1987).

In spite of continuous efforts, *in vitro* maturation and fertilization in the pig has met with very limited success. Polyspermy is a major problem encountered in pig oocytes matured and fertilized *in vitro*, and as high as 80% of the oocytes have been reported to be polyspermic in some studies (Cran & Cheng, 1986; Mattioli *et al.*, 1988b; Mattioli *et al.*, 1989). Furthermore, only a very low proportion of these *in vitro* matured and fertilized oocytes are capable of transforming sperm into the male pronucleus (Nagai *et al.*, 1984; Mattioli *et al.*, 1988b; Yoshida *et al.*, 1990). These problems may result from deficiencies in the culture media used for oocyte maturation.

Studies described in the previous Chapters implicate EGF as a potent stimulant of porcine oocyte maturation and cumulus expansion *in vitro*, the events associated *in vivo* with the endogenous preovulatory surge of gonadotropins. These effects of EGF appear to be highly specific as the other known growth factors failed to stimulate these preovulatory processes in isolated mouse OCC (Downs, 1989). Furthermore, localization of the mRNAs and the peptides for EGF and its receptor in porcine ovarian follicles (Chapter 7) indicates local ovarian production, and therefore suggests a possible physiological role for this growth factor in the process of oocyte maturation.

In vitro studies of gonadotropin-induced maturation of rodent oocytes have shown that FSH is able to induce GVBD in the presence of various meiotic inhibitors and that its effect is first inhibitory and later stimulatory (Downs & Eppig, 1985; Downs *et al.*, 1988). Studies in Chapter 5 indicate that FSH is a potent stimulant of resumption of oocyte maturation and cumulus cell expansion in the pig, and that it enhances the effect of EGF on oocyte maturation.

The effects of EGF and FSH on cytoplasmic maturation of porcine oocytes, however, are not known. Some preliminary reports in other species suggest that EGF may influence oocyte cytoplasmic maturation. EGF improved the developmental potential of *in vitro* matured bovine oocytes to the eight-cell stage (Coskun *et al.*, 1991) and to the blastocyst stage (Harper & Brackett, 1993). The present study aimed to investigate the influences of EGF and FSH during IVM on cytoplasmic maturation of porcine oocytes as indicated by their ability to induce sperm decondensation and pronucleus formation, and by the changes in the pattern of protein synthesis in oocytes and cumulus cells. The cumulus cells were included in protein studies because of their well known role in nuclear and cytoplasmic maturation of oocytes (Biggers *et al.*, 1967; Crosby *et al.*, 1981; Osborn & Moor, 1982; Staigmiller & Moor, 1984; Downs *et al.*, 1988).

9.2 Materials and Methods

9.2.1 *In Vitro* maturation and fertilization (IVM-IVF)

The OCC were isolated and cultured in the presence of 1 ng/ml human recombinant EGF, 1.5 μg/ml FSH or a combination of both for 44 hours as described in Chapter 4. The concentration of EGF used in these studies represented the concentration observed to elicit maximal cumulus expansion and oocyte maturation in porcine OCC (Chapter 5), and that of FSH was similar to the concentration used in previous studies of IVM of pig oocytes (Mattioli *et al.*, 1988b). For IVF, boar sperm were capacitated using a modified version of the standing procedure described by Cheng *et al* (1986). Briefly, an aliquot of the whole ejaculate after dilution with commercial

extender (Thames Bend Farms Ltd, Tavistock, Ontario) was incubated at 20°C for 24 hours. The highly motile sperm fraction was selected using a swim-up procedure; 2-4 ml aliquots of the diluted semen were centrifuged at 1000 g for 4 min in 15 ml sterile centrifuge tubes. A swim-up time of 30 min was given at room temperature prior to the collection of the supernatant containing motile sperm. The motile fractions were pooled and further washed by centrifugation (3 x 3 min) with incubation medium consisting of Medium 199 with Earle's salts supplemented with 3.05 mM D-glucose (BDH Chemicals, Toronto, Ontario), 2.92 mM calcium lactate (BDH), 0.91 mM sodium pyruvate and 12% (v/v) fetal calf serum, pH 7.4. Aliquots of the resuspended pellet were added to the matured oocytes in the fertilization medium [incubation medium + 2 mM caffeine (BDH), pH 7.4] to give the desired sperm concentration (5 x 10^5 sperm/ml). Fertilization was accomplished after culturing 20 OCC/ml of the fertilization medium in 4 well plates at 39°C for 6-8 hours. After this period, the OCC were removed from the fertilization wells, washed by gentle pipetting with incubation medium and further cultured in 100 μl drops of this medium in 35 mm dishes for additional 14 hours before examination for pronuclei formation.

9.2.2 Assessment of maturation and pronuclei formation

After IVM-IVF, the oocytes were fixed, stained and examined as whole mounts using the technique previously described (Chapter 4). The oocytes were examined for completion of nuclear maturation and sperm penetration, and based on the stage of sperm head chromatin decondensation, spermatozoa found in the ooplasm were classified as unswollen sperm heads or forming male pronucleus. Distinction was made between unswollen sperm that had entered the ooplasm and those remaining on the oolemma, based on the fact that the former had more intense staining than the latter. Furthermore, the majority of spermatozoa which remained unswollen in the ooplasm were found close to the female pronucleus and were on the same plane of focus.

9.2.3 Radiolabelling of oocyte and cumulus cell proteins

The OCC were cultured in the presence of EGF (1 ng/ml), FSH (1.5 μg/ml) or

both in Medium 199 for 6, 24 and 44 hours as described earlier. During the last 3 hours of culture, the OCC were incubated in methionine deficient MEM medium (prepared using MEM Select-Amine Kit from Gibco) containing 0.5 mCi/ml L-[^{35}S]methionine (New England Nuclear, Mississauga, Ontario). For this purpose, 10 OCC were cultured in each 100 μl drop of MEM containing radiolabelled methionine and a total of 40 OCC was included for each treatment group. At the end of culture, the OCC were washed free of radiolabelled precursor by three transfers through unlabelled MEM, followed by separation of oocytes and cumulus cells by repeated pipetting through a fine-bore pipette ($\sim$100 μm). The denuded oocytes and cumulus cells were further washed in 10 mM Tris-HCL pH 7.4 and frozen at -70°C until required for electrophoresis.

9.2.4 One-dimensional polyacrylamide gel electrophoresis (PAGE)

The separated oocytes and cumulus cells from 40 OCC in each group were lysed by adding 40 μl of sodium-dodecyl sulphate (SDS)-sample buffer (Laemmli, 1970), followed by heating at 100°C in a boiling water bath for 2 min. Duplicate aliquots of 2 μl were used for the determination of incorporation of radioactivity into trichloroacetic acid-precipitable proteins. Denatured radiolabelled polypeptides were separated by one-dimensional SDS-PAGE according to the procedure of Laemmli (1970). For oocytes, 10 μl of the sample (equivalent to 10 oocytes) was loaded on each lane, whereas for cumulus cells equal number of counts (5,000 cpm) were used in each lane for gel electrophoresis. For molecular weight determinations, a mixture of proteins of known molecular weights (14.4-205 kDa) were run simultaneously as standards. The gels were dried onto filter paper under vacuum at 80°C and exposed to X-ray film at -70°C for 1-2 days. Films were scanned with an LKB laser densitometer to obtain relative quantifications of radiolabelled bands.

9.2.5 Statistical analysis

Fertilization experiments were replicated three times and statistical analysis using X^2 test was performed on the pooled data; P values <0.05 were considered significant. Protein synthesis studies were carried out twice and a similar pattern of synthesis was

observed both times.

9.3 Results

9.3.1 Effects of EGF and FSH on IVM-IVF

The different developmental stages of *in vitro* matured oocytes before and after IVF are represented by the photomicrographic plates in Figure 9-1. Effects of EGF and FSH during IVM on subsequent IVF of porcine oocytes are summarised in Table 9-1. Addition of EGF, FSH and EGF+FSH significantly ($P<0.05$; X^2 test) increased the proportion of oocytes reaching MII stage. Although, there was no significant difference in the percentage of oocytes penetrated by spermatozoa between various treatment groups and the control, the addition of EGF alone significantly decreased the percentage of polyspermic oocytes (25% vs 62% in the control). Furthermore, the addition of EGF alone significantly increased the proportion of monospermic oocytes forming two pronuclei (54% vs 16% in the control and 24% with FSH) and, therefore, significantly decreased the proportion of polyspermic oocytes forming two and more than two pronuclei. FSH abolished these effects of EGF and significantly increased the percentage of polyspermic oocytes forming more than two pronuclei when added alone or with EGF.

9.3.2 Effects on the pattern of protein synthesis

The changes induced by EGF and FSH in the pattern of protein synthesis in the oocytes at different time periods during IVM are depicted in Figure 9-2. Addition of EGF and FSH alone stimulated the synthesis of proteins corresponding to molecular weights of 34, 45 and 97 kDa (peptides B, C and E) after 6 hours of culture; however, the addition of EGF and FSH together was without effect. After 24 hours, EGF alone inhibited the synthesis of these peptides, whereas FSH alone and with EGF maintained the stimulation of synthesis of 34 and 45 kDa proteins (peptides B and C). Two additional peptides corresponding to 66 and 200 kDa (peptides D and F) appeared at this time as a result of exposure to FSH alone or with EGF. After 44 hours of culture, these two new peptides were observed in all groups and the stimulatory effect of FSH and FSH+EGF was still evident. An additional peptide of 26 kDa (peptide A)

Figure 9-1. Photomicrographs of oocytes prepared as whole mounts and stained with haematoxylin in different stages of development. **A:** An uncultured oocyte in dictyate stage showing intact germinal vesicle with a nucleolus. **B:** A 44 hours cultured unfertilized secondary oocyte with first polar body (arrow) and MII chromosomes arranged on the metaphase plate. **C:** A fertilized monospermic oocyte showing two normal pronuclei. **D:** A fertilized polyspermic oocyte with two normal looking pronuclei and an extra sperm head (arrow). **E:** A polyspermic oocyte showing three pronuclei. **A-E,** X 250.

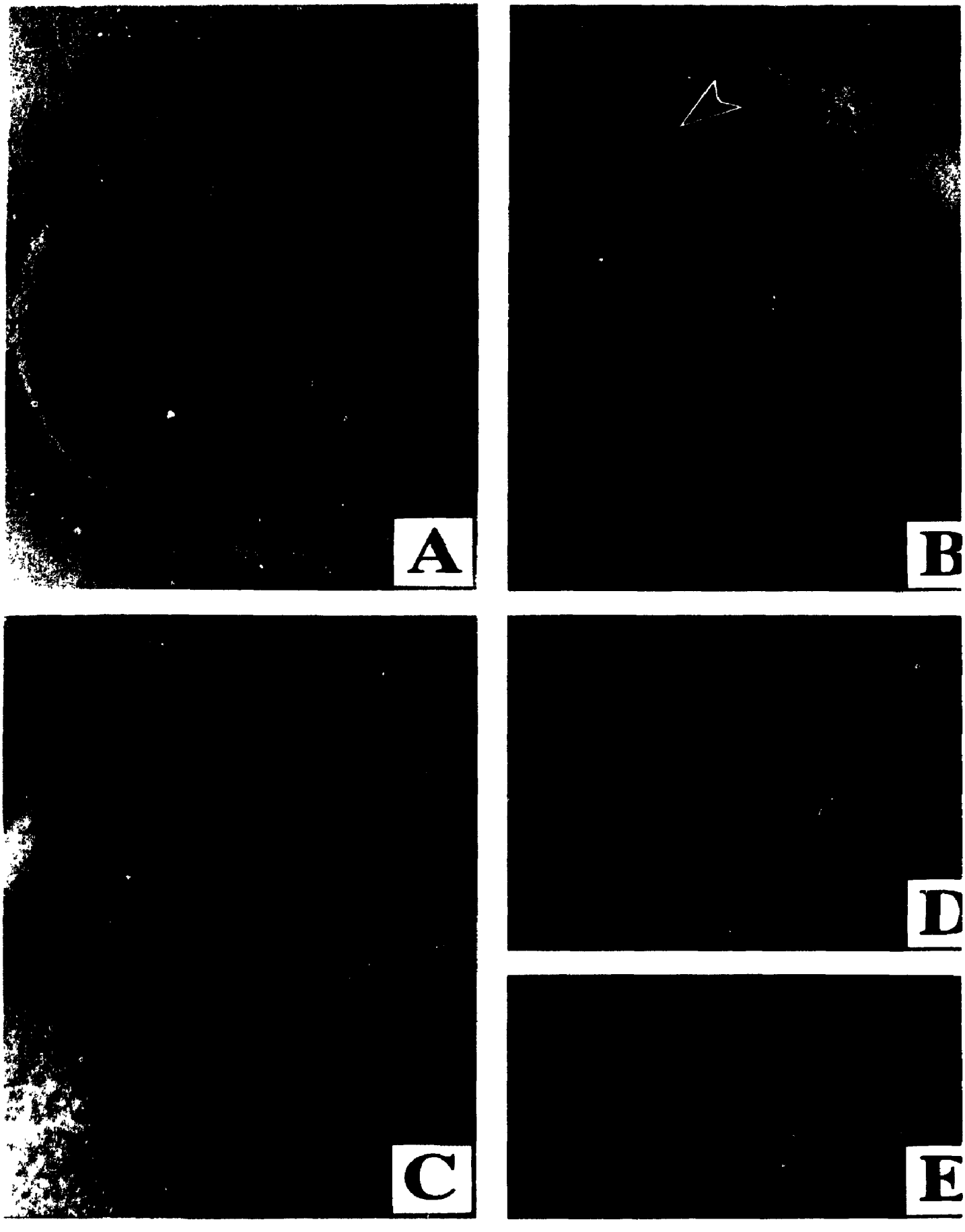

A
B
C
D
E

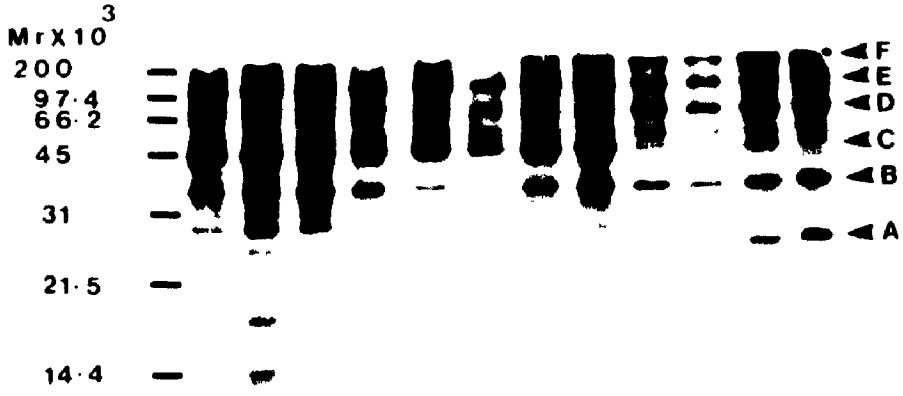
Table 9-1. Effects of EGF and FSH during IVM on IVF of porcine oocytes.

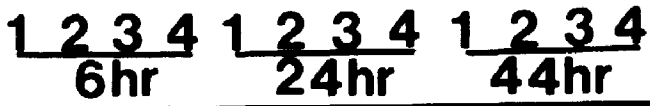
Group	No. of oocytes	No. reaching MII (%)	No. penetrated (% of MII)	No. (% of penetrated oocytes)			
				Poly-spermic	Mono-spermic with 2 Pn	Poly-spermic with 2 Pn	More than 2 Pn
Control	119	63(53)[a]	61(97)[ab]	38(62)	10(16)	17(28)	5(8)[ab]
EGF	95	81(85)[b]	71(88)[a]	18(25)[a]	38(54)[a]	4(6)[a]	1(1)[a]
FSH	95	88(93)[bc]	82(93)[ab]	52(63)	20(24)	13(16)	11(13)[bc]
EGF +FSH	107	105(98)[c]	102(97)[b]	75(74)	16(16)	19(19)	20(20)[c]

Data pooled from three independent experiments.
[a-c] In each column, values with different superscripts are significantly ($P < 0.05$) different.
Pn: Pronuclei

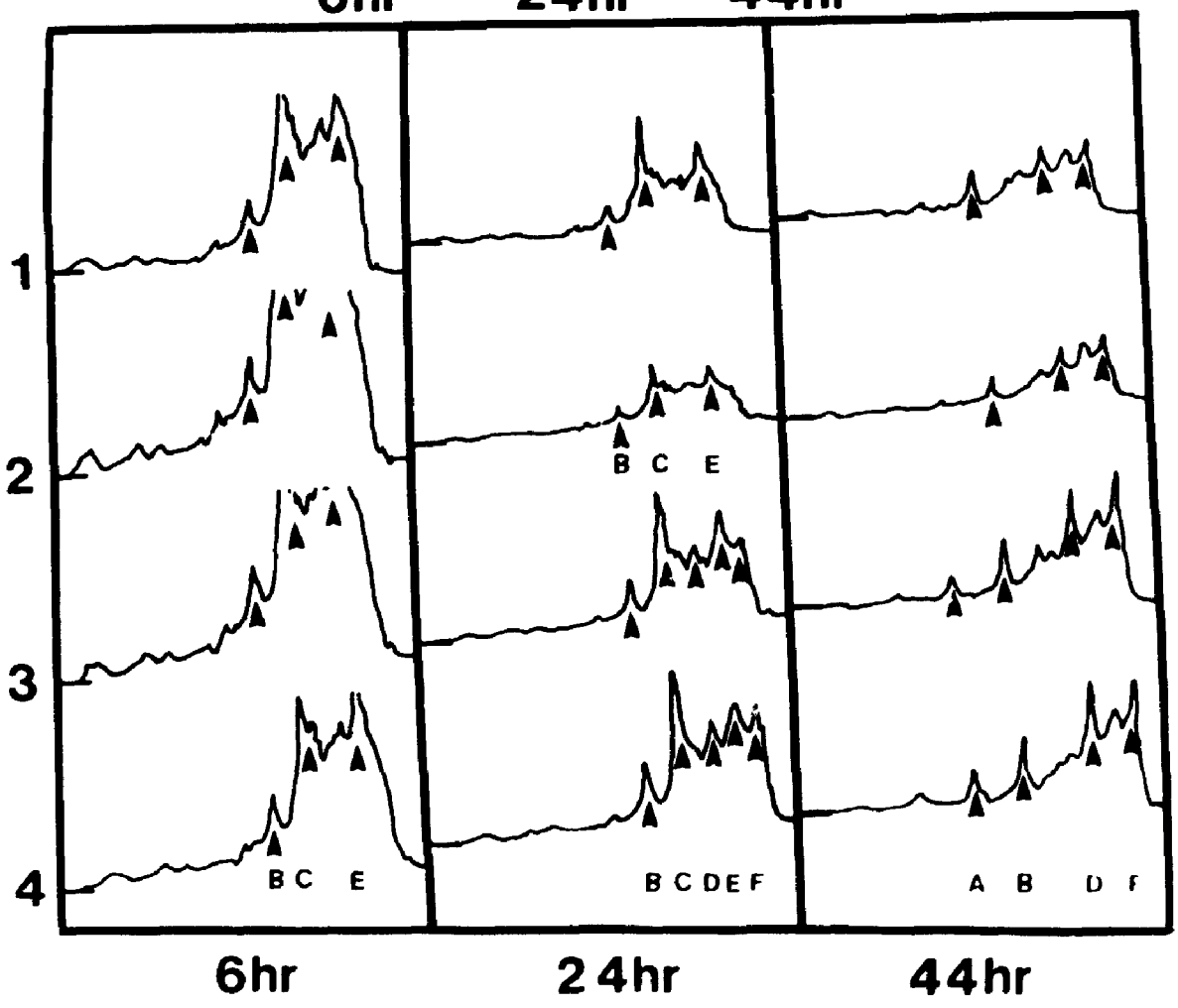
Figure 9-2. Synthesis profile of [^{35}S]methionine-labelled polypeptides from oocytes cultured *in vitro* as intact oocyte-cumulus cell complexes (OCC) for 6, 24 and 44 hours in the presence of control (1), EGF (2), FSH (3) and EGF+FSH (4). The letters with arrowheads indicate the peptides whose synthesis was either changed or induced by the hormonal treatments. **A.** Fluorograph obtained by one-dimensional SDS-PAGE; the OCC were exposed to 0.5 mCi/ml L-[^{35}S]methionine during the last 3 hours of culture, prior to separation of oocytes and cumulus cells by repeated pipetting. Samples equivalent to 10 oocytes were loaded onto each slot of the gel, and fluorographs developed after 30 hours. **B.** Densitometric tracers of fluorographs of [^{35}S]methionine-labelled polypeptides in **A**. The baselines for various treatment groups are indicated by tick marks on left hand side and start at the same level for all treatment groups in each time column.

A.


Mr X 10 3
200
97·4
66·2
45
31
21·5
14·4
F
E
D
C
B
A


1 2 3 4 1 2 3 4 1 2 3 4
6hr
24hr
44hr

B.


1
2
3
4
B C E
B C E
B C D E F
A B D F
6hr
24hr
44hr

appeared at this time as a result of FSH and EGF+FSH treatments.

The changes induced by EGF and FSH in the pattern of protein synthesis in cumulus cells during IVM are shown in Figure 9-3. Each hormone alone induced the synthesis of a peptide of 26 kDa (peptide A) after 6 hours of culture. FSH, when added alone or with EGF, induced the synthesis of an additional peptide corresponding to 29 kDa (peptide B) and its synthesis remained unchanged at 24 and 44 hours. After 24 hours, FSH alone and in combination with EGF induced the synthesis of an additional protein of 38 kDa (peptide C) and its synthesis was still maintained at 44 hours. EGF alone had no effect on protein synthesis in cumulus cells at 24 and 44 hours.

9.4 Discussion

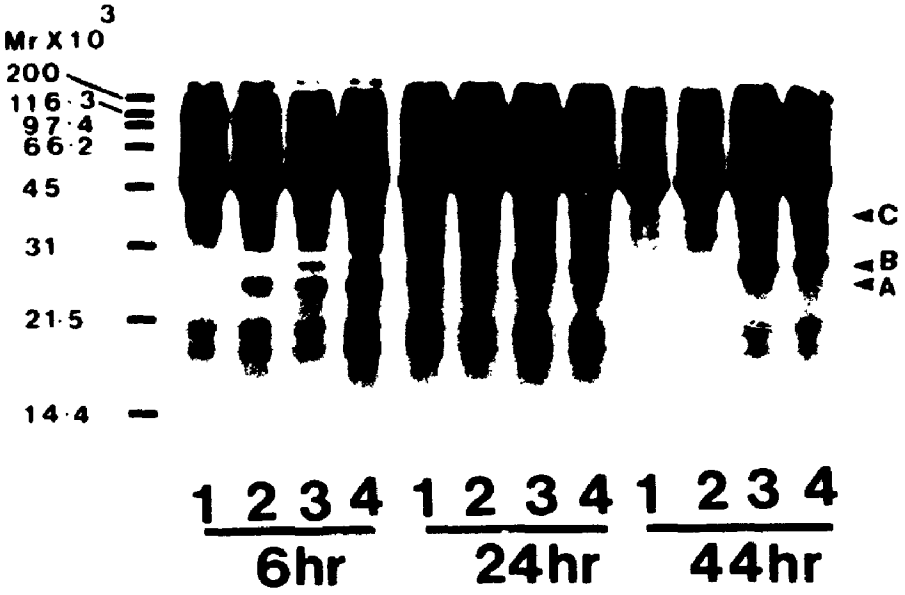
9.4.1 IVF studies

The results in the present study have demonstrated that both EGF and FSH, when added during IVM, influence cytoplasmic maturation of porcine oocytes as revealed by the success of fertilization and by the changes in the pattern of protein synthesis. Previous studies in Chapter 5 have shown that both EGF and FSH are potent stimulants of resumption of meiotic maturation in the porcine oocyte and in the induction of expansion in the cumulus cells. In the present study, EGF, FSH and EGF+FSH significantly increased the percentage of oocytes completing nuclear maturation as revealed by the extrusion of the first polar body. This indicates that, in addition to their role in the initiation of the meiotic process, these hormones are also effective in the completion of nuclear maturation.

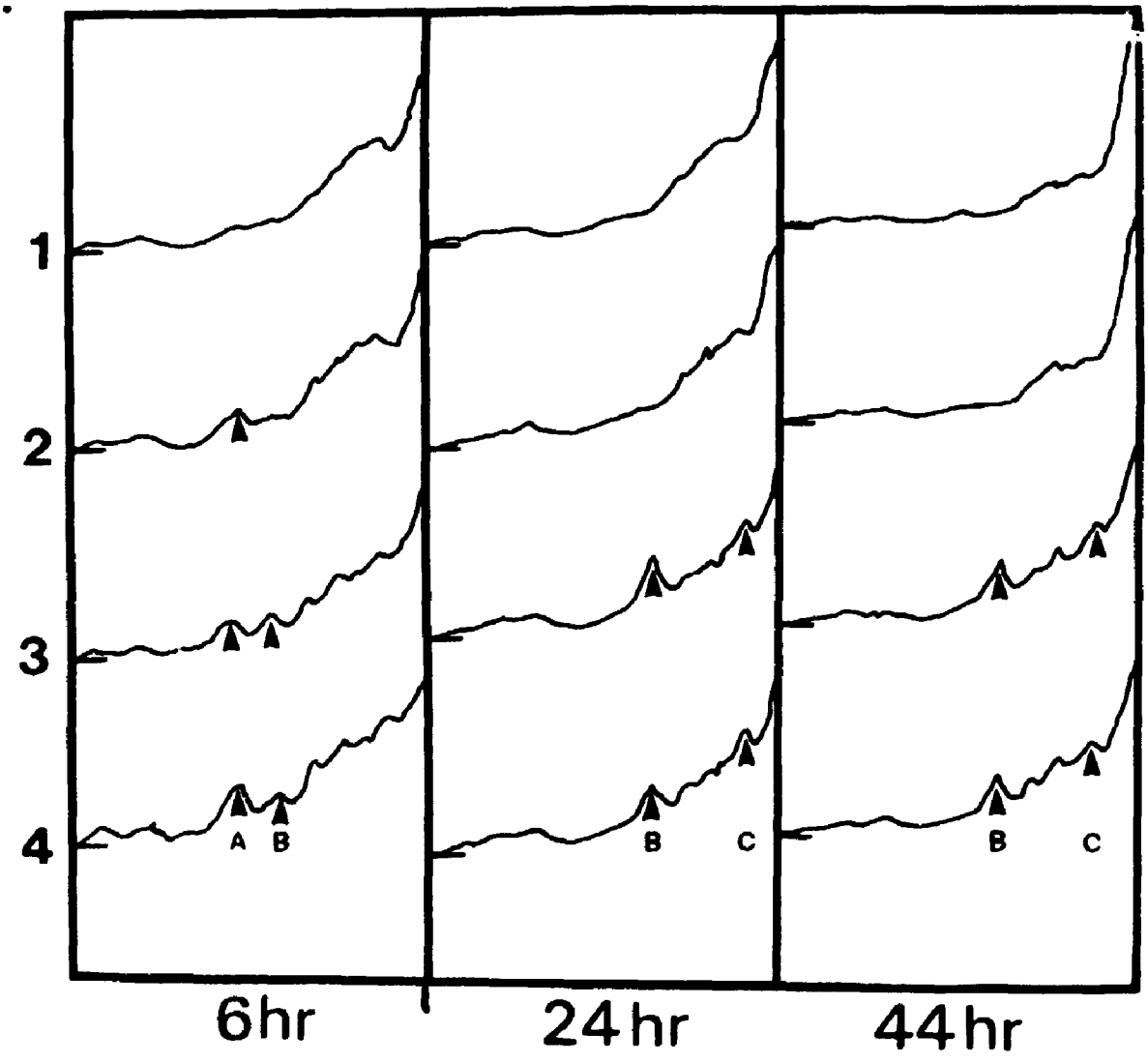
Polyspermy is a frequently encountered problem in pig oocytes matured and fertilized *in vitro* and as high as 80% of the oocytes have been reported to be polyspermic (Cran & Cheng, 1986; Mattioli *et al.*, 1988b; Mattioli *et al.*, 1989). Although the precise mechanism involved in the block to polyspermy remains incompletely understood, a variety of proposals implicating the cortical granules for the occurrence of polyspermy have been suggested. Polyspermy may be due to delayed cortical reaction or may be due to its taking place at a rate insufficient to block repeated penetration by the fertilizing spermatozoa (Sathananthan & Trounson, 1982). Another

Figure 9-3. Synthesis profile of [^{35}S]methionine-labelled polypeptides from cumulus cells cultured *in vitro* as intact oocyte-cumulus cell complexes (OCC) for 6, 24 and 44 hours in the presence of control (1), EGF (2), FSH (3) and EGF+FSH (4). The letters with arrowheads indicate the peptides whose synthesis was induced by the hormonal treatments. **A.** Fluorograph obtained by one-dimensional SDS-PAGE; the OCC were exposed to 0.5 mCi/ml L-[^{35}S]methionine during the last 3 hours of cultures, prior to separation of oocytes and cumulus cells by repeated pipetting. Equal number of trichloroacetic acid-precipitable counts (5,000 cpm) were loaded onto each slot of the gel, and fluorographs developed after 24 hours. **B.** Densitometric tracers of fluorographs of [^{35}S]methionine-labelled polypeptides in **A**. The baselines for various treatment groups are indicated by tick marks on left hand side and start at the same level for all treatment groups in each time column.

A.


Mr X10 3
200
116·3
97·4
66·2
45
31
21·5
14·4
C
B
A
1 2 3 4 1 2 3 4 1 2 3 4
6hr 24hr 44hr

B.


1
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A B
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B C
6hr
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possibility is that the cortical granule exudate becomes diluted in the culture media, such that a blocking threshold can not be achieved (Gulyas, 1980). Cran and Cheng (1986) proposed that following IVF in the pig, vital constituents of the cortical granules remain unavailable, probably in a zymogen form, for interaction with the zona pellucida. In the present study, addition of EGF alone during IVM significantly reduced the proportion of polyspermic oocytes and FSH abolished this effect of EGF. Whether these effects of EGF and FSH are associated with the difference in release of cortical granules remains to be determined.

In the present study, EGF significantly increased the proportion of monospermic oocytes forming male pronuclei. This is not totally consistent with a very recent report in the pig (Ding & Foxcroft, 1994) in which addition of EGF alone during IVM had no effect on male pronuclear formation. However, when EGF added with gonadotropins, it significantly increased the proportion of oocytes forming male pronuclei. It is difficult to compare the present results with those in that study due to the differences in the composition of the culture media used. The culture media used by Ding and Foxcroft (1994) contained high levels of insulin (35 μg/ml) and glutamine (70 μg/ml), both of which may mask the effects of EGF.

The decondensation of the sperm and its transformation into a male pronucleus is considered to be dependent on the presence of a cytoplasmic factor called MPGF, the synthesis of which is induced during normal oocyte maturation (Moor *et al.*, 1980a). The significant increase in monospermic oocytes forming two pronuclei in response to EGF alone may, therefore, be due to induction of MPGF synthesis by EGF. Although the proportion of monospermic oocytes forming two pronuclei in the presence of FSH is significantly lower compared to EGF alone, the total proportion of oocytes forming male pronuclei, including polyspermic with two and more than two pronuclei, is not significantly different. This indicates that there may be sufficient synthesis of MPGF in response to FSH; however, due to a very high incidence of polyspermic oocytes, the proportion of monospermic oocytes forming two pronuclei is significantly lower. With the help of electron microscopy, additional sperms were observed in 1-4 cell stage porcine embryos produced *in vivo* and there were some indications of phagocytosis of

these additional sperms (Xia & Qin, 1992). It was therefore speculated that the penetration of accessory spermatozoa may be normal phenomena during the process of fertilization in the pig. Therefore, the high proportion of polyspermic oocytes forming two pronuclei observed in the FSH group may cleave normally. However, the fate of polyspermic oocytes forming more than two pronuclei in this group is uncertain. Also, there is considerable evidence that follicular steroids act as mediators of the action of gonadotropins on maturing oocytes including the regulation of MPGF synthesis (Thibault *et al.*, 1975; Moor *et al.*, 1980a). Thus the effect of FSH on MPGF synthesis may be mediated by steroids produced by cumulus cells in response to this gonadotropin. However, the mechanisms by which EGF and FSH regulate MPGF synthesis remains to be determined.

9.4.2 Protein synthesis studies

Experiments conducted on mouse oocytes have consistently shown that neither new transcription nor new protein synthesis is required for the condensation of chromosomes and the breakdown of the germinal vesicle (Stern *et al.*, 1972; Golbus & Stein, 1976). Protein synthesis, however, is required for the progression of meiosis after GVBD since continuous protein inhibition during maturation of mouse oocytes blocked the chromosomes at the circular bivalent stage (Wassarman *et al.*, 1976). On the other hand, the ovine and porcine oocytes require synthesis of new proteins for the resumption of meiosis (Moor & Crosby, 1986). Studies in Chapter 5 indicated that both EGF and FSH stimulate the resumption of meiosis in porcine oocytes after 24 hours of culture. In the present study, both EGF and FSH significantly increased the proportion of oocytes completing nuclear maturation. The maturation-promoting factor (MPF), which brings about GVBD and the subsequent maturational changes in the oocyte, has been shown to be formed of two major proteins of 34 and 45 kDa in *Xenopus* oocyte (Rime & Ozon, 1990 and other references cited there). The 34 kDa component corresponds to the product of the cdc2$^+$ gene in yeast while the 45 kDa component has been identified as being homologous to 'cyclin'. In the present study, both EGF and FSH stimulated the synthesis of peptides corresponding to 34 and 45 kDa in the oocyte (peptides B and C

in Figure 9-2). Since the precise identification of these peptides, other than electrophoretic mobility, was not made in the present study, it is difficult to correlate these peptides with absolute certainty with MPF components. However, due to their similar molecular size, it is possible that any or both of these peptides form a part of the MPF. Therefore the stimulation of their synthesis in the oocyte by EGF and FSH may be responsible for the augmentation of meiosis and for the higher proportion of oocytes reaching MII in the presence of these hormones.

The effects of EGF, FSH and their combination on fertilization are also consistent with the induced changes in the pattern of protein synthesis in the oocyte and cumulus cells by these peptides. For instance, the abolishment by FSH of EGF effects on fertilization is in agreement with the different patterns of protein synthesis observed in response to EGF and EGF+FSH. Likewise, the similar effects of FSH and FSH+EGF on fertilization coincide with their similar effects on the pattern of protein synthesis in the oocyte and cumulus cells. It has been proposed that of the proteins synthesized during oocyte maturation, some may be required for normal maturation processes, while others may be required for fertilization and/or post fertilization development (McGaughey & Van Blerkom, 1977 and other references cited there). In other words, the developmental program which functions during the preimplantation stage of pregnancy, may in fact be initiated during oocyte maturation. These experiments, where different patterns of protein synthesis in the oocyte and cumulus cells in response to EGF and FSH resulted in different rates of success after fertilization, support such a concept. Although with the present data, it is difficult to correlate the synthesis of any specific peptide in the oocyte and/or cumulus cells with the fertilization response, the molecular size of some of the peptides whose synthesis was stimulated by hormonal treatments, match with some known peptides previously studied in porcine oocytes. For instance, both EGF and FSH stimulated the synthesis of a 45 kDa protein in the oocyte after 6 hours of culture which, in addition to 'cyclin' also corresponds to 'actin', a cytoskeletal peptide whose synthesis in the porcine oocyte was shown to be dependent on cumulus cells (Osborn & Moor, 1982). The components of cytoskeleton including actin may play a vital role in the release of cortical granules which prevents polyspermy. Similarly, some other

peptides whose synthesis was stimulated by EGF and/or FSH may form a part of the MPGF, the exact nature of which is still not clear.

The mammalian oocyte, like that of amphibians, is actively synthesizing RNA from early in follicular growth to within an hour or so of GVBD and some of the newly synthesized RNA is released into cytoplasm before GVBD (Warnes *et al.*, 1977). Furthermore, there is evidence that poly(A)-containing RNA synthesis continues in fully grown oocytes (Brower *et al.*, 1981). Therefore, it is not clear whether control of protein synthesis in the maturing oocyte-cumulus cell complexes by these hormones, is exerted at the transcriptional or translational level.

In conclusion, the results from this study have provided evidence for a close relationship between the pattern of protein synthesis in the oocyte and cumulus cells with the events of maturation and subsequent fertilization. Although, this study is unable to correlate the synthesis of any specific peptide in the oocyte and/or cumulus cells with the observed maturation and/or fertilization response, it has provided insight into the significance of autocrine/paracrine regulators during the process of oocyte maturation. Results indicate that the addition of EGF during IVM may help to reduce the incidence of polyspermy frequently encountered in porcine oocytes matured and fertilized *in vitro*. It may also help to improve the usual low success of IVM-IVF in this species by enhancing subsequent developmental potential of fertilized oocytes. *In vitro* studies of this nature will facilitate the development of a more defined maturation medium for pig oocytes and will contribute to the knowledge of intrafollicular regulation of oocyte development.

CHAPTER 10 TRANSFORMING GROWTH FACTOR-ALPHA GENE EXPRESSION AND PEPTIDE LOCALIZATION IN PORCINE OVARIAN FOLLICLES

10.1 Introduction

As discussed in Chapter 2, TGF-α is a small polypeptide that shares 30-40 % structural homology with EGF, binds to the same receptor, and has similar, although not entirely identical, biological activities as EGF (Derynck, 1986, 1990). It is a unique gene product that is produced as a large transmembrane precursor which is later processed into a soluble extracellular protein (Bringman *et al.*, 1987). Initially, it was shown to be produced only by virally transfected (DeLarco & Todaro, 1978) and neoplastic (Derynck *et al.*, 1987) cells. However, in the last few years, TGF-α production has been demonstrated in nontransformed cells from postnatal tissues (Kobrin *et al.*, 1986; Coffey *et al.*, 1987; Madtes *et al.*, 1988; Wilcox & Derynck, 1988), suggesting its role as a regulator in normal adult tissues.

Although the EGF-related family of growth factors has grown considerably and now includes a number of members including EGF, TGF-α, heparin-binding EGF, amphiregulin, cripto, and heregulin for a common receptor, the EGF receptor (Prigent & Lemoine, 1992), only EGF and TGF-α have been implicated in ovarian follicular physiology. TGF-α is probably the most extensively studied for its potential roles in the regulation of follicular functions and has been shown to influence a variety of somatic cell functions. It stimulates the proliferation of isolated theca and granulosa cells from bovine ovarian follicles (Lobb *et al.*, 1988; Skinner & Coffey, 1988). In the rat, TGF-α attenuated FSH-stimulated basal aromatase activity and inhibited estradiol production by cultured granulosa cells in a dose-dependent manner (Adashi & Resnik, 1986; Mason *et al.*, 1986; Dorrington *et al.*, 1987). Infusion of TGF-α into the ovarian artery of the sheep inhibited estradiol secretion, increased peripheral LH pulse amplitude and FSH concentration, and delayed the preovulatory surge of LH (Murray *et al.*, 1990). Such observations lead to the formulation of a general view that TGF-α/EGF are negative

regulators of FSH-induced differentiated functions in granulosa cells. However, more recently, in a contrasting observation, TGF-α was found to induce aromatase activity in granulosa and theca cells obtained from prepubertal gilts (Gangrade *et al.*, 1991). In addition, TGF-α/EGF stimulated progesterone production by cultured rat granulosa cells synergistically with FSH (Yeh *et al.*, 1993). Apart from actions on somatic follicular cells, TGF-α also augmented meiotic maturation of cumulus-enclosed mouse oocytes *in vitro*, similar to that by EGF (Brucker *et al.*, 1991).

Since previous studies in Chapter 7 have demonstrated the production of EGF in the porcine ovarian follicle, predominantly by the oocyte and granulosa cells, it was considered important to determine the localization pattern of TGF-α in the follicle. To date, the reports concerning the production of TGF-α in the ovarian follicle are controversial. Kudlow *et al* (1987) found a significant increase in TGF-α mRNA expression in the whole ovary after stimulation of prepubertal rat ovaries with FSH for 3 days and localized TGF-α peptide only in theca cells by immunocytochemistry. Yeh *et al* (1993), however, were able to detect TGF-α mRNA transcript in both fresh and cultured rat granulosa cells using the more sensitive technique of RT-PCR. Their immunocytochemical studies revealed the presence of TGF-α only in granulosa, but not in theca cells of rat ovaries (Yeh *et al.*, 1991). In the human, immunoreactive TGF-α was localized in both granulosa and theca cells, although the staining was more intense in granulosa cells (Chegini & Williams, 1992). On the other hand, Skinner and Coffey (1988), using Northern blot analysis, detected TGF-α mRNA in bovine thecal cells, but not in granulosa cells. Lobb *et al* (1989) localized the peptide exclusively in bovine theca cells by using a specific monoclonal antibody. To date, no study looking at the localization of both the mRNA and the peptide for TGF-α in all the different components of ovarian follicles, including the oocyte and cumulus cells, has been reported for any mammalian species. The present study aimed to determine the expression of the gene for TGF-α in various components of medium-sized ovarian follicles by RT-PCR and to immunolocalize the peptide during folliculogenesis by immunocytochemistry in the pig. The results indicate TGF-α to be a cumulus-granulosa cell product in this species.

10.2 Materials and Methods

10.2.1 RT-PCR

The various follicular components were separated, and the total RNA was extracted from pools of 100 oocytes, cumulus cells from 100 OCC, 1 million granulosa, and 1 million theca cells, and reverse transcribed to cDNA as previously described in Chapter 7. PCR was carried out in a total volume of 50 μl using a Perkin Elmer-Cetus DNA Thermal Cycler. Each reaction mixture contained 1-2 μl cDNA solution, 50 pmol of each primer, 5 μl of 10X PCR buffer (Promega), 2 μl of 5 mM dNTPs and water to 50 μl. The mixture was overlaid with 75 μl of paraffin oil and was heated to 94°C for 5 min prior to the addition of 1-2 units of Taq polymerase. The amplification profile consisted of 35 cycles: at 94°C for 0.3 min (dissociation); 55°C for 0.5 min (annealing); 72°C for 1.0 min (extension). The final cycle included a further 5 min at 72°C for complete strand extension. All RT-PCR reactions were carried out at least twice for each individual RNA sample.

10.2.2 PCR primers

The primers for TGF-α were designed according to conserved regions of the rat and human cDNA sequences with few modifications to suit porcine system as previously described by Vaughan *et al* (1992b). Briefly, the upstream primer (5' - TGCCCAGATTCCCACACT- 3') is identical to nucleotides 280-297 of the rat cDNA and the downstream primer (5'- TG(G/T)ATCAGCACACA(G/T)GTG- 3') represents the reverse complement of nucleotides 489-506. This primer pair spans known introns which help to distinguish amplifications of mRNA reverse transcribed to cDNA from amplifications of any contaminating genomic DNA, and predicts a DNA fragment of 243 bp. To test the specificity of the primers for the pig, rat kidney cDNA was subjected to PCR amplification using these TGF-α primers. As described in Chapter 7, a primer pair for β-actin was included as an internal control, since transcripts for cytoplasmic β-actin are expected to be present in all cell types. The identity of PCR products was confirmed by cutting the DNA bands with a diagnostic restriction enzyme *RsaI* (Gibco BRL) according to the supplier's instructions.

10.2.3 Immunocytochemistry

Anti-TGFα, a purified mouse monoclonal neutralizing antibody (TAb-1), was kindly provided by Dr. P.K. Lala, Department of Anatomy, University of Western Ontario, who received it as a generous gift from Dr. B. Langton, Berlex Biosciences, Alamada, CA. This antibody has been shown to be highly specific for TGF-α and has previously been used for neutralization studies (Lysiak *et al.*, 1993). Immunocytochemistry was carried out on 5-7 μm sections using 1:100 (70 μg/ml) anti-TGF-α (TAb-1) in D-PBS, essentially as outlined in Chapter 7. For negative controls, the primary antibody was either omitted from the procedure or pre-absorbed with the same mass of TGF-α peptide.

10.3 Results

10.3.1 TGF-α mRNA

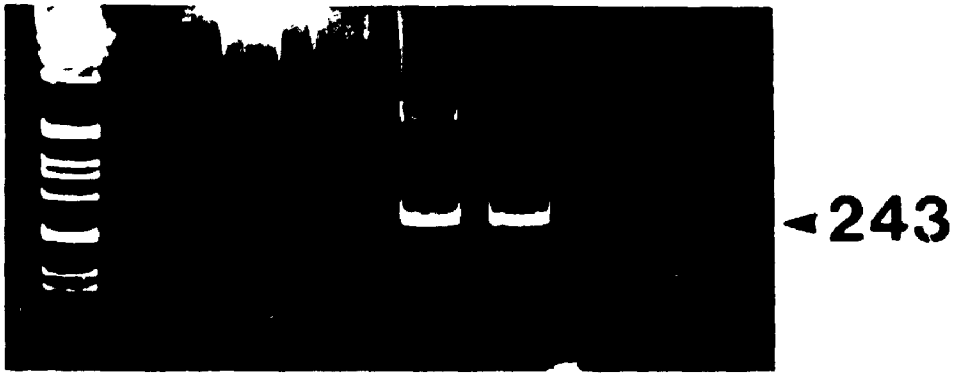
A strong signal for TGF-α transcript was detected in cumulus and granulosa cells, whereas the signal in theca cells was very weak but detectable (lanes C and G vs lane T in Figure 10-1A). No TGF-α message was detected in the oocyte (lane O, Figure 10-1A). The identity of the amplified product was confirmed by cutting the band with the diagnostic restriction enzyme *RsaI* which yielded the expected fragments of 146 and 97 bp (Figure 10-1B). No amplified product was detected when the somatic cells were absent from the rinse medium (lane R, Figure 10-1A) or when the cDNA was replaced with water (lane W, Figure 10-1A) demonstrating the specificity of the amplification procedure. Also, no TGF-α transcript was detected in the cDNA obtained from rat kidney (lane K, Figure 10-1A) suggesting the specificity of the primers for the pig.

10.3.2 TGF-α peptide

The immunolocalization of TGF-α in porcine ovarian follicles is shown in Figure 10-2A-E. Intense TGF-α staining was observed in cumulus and granulosa cells of all stages of follicular development (Figure 10-2C-E). TGF-α immunostaining was also observed in the oocytes of primordial and primary follicles (Figure 10-2B, C). In antral follicles, immunostaining was observed only in cumulus and mural granulosa cells,

Figure 10-1. Expression of TGF-α mRNA in the porcine ovarian follicle. **A:** Total RNA was isolated from oocytes (O), cumulus (C), granulosa (G) and theca (T) cells, submitted to RT-PCR using primers specific for TGF-α, and the amplified cDNA fragments analyzed by PAGE. For the negative controls, oocytes or somatic cells were absent from the rinse medium (R), or the cDNA was replaced with water (W), or rat kidney cDNA was used (K). **B:** The PCR amplified product in cumulus cells was confirmed after digestion with the restriction enzyme *RsaI*, which yielded the expected 97 and 146 bp fragments. U, uncut; C, cut. Molecular weight markers (L) are a 1 kb ladder containing vector DNA fragments that range from 75 to 1,636 bp (Gibco, BRL). The size (bp) of predicted amplified products is indicated.

A.



B.

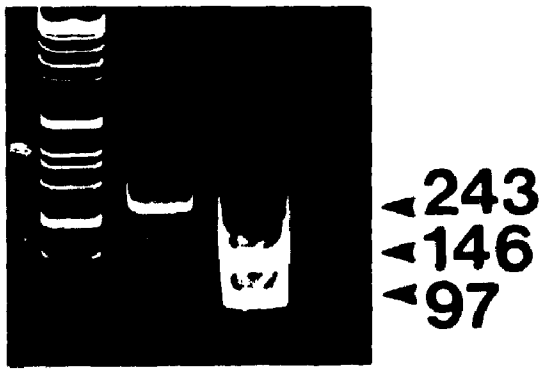
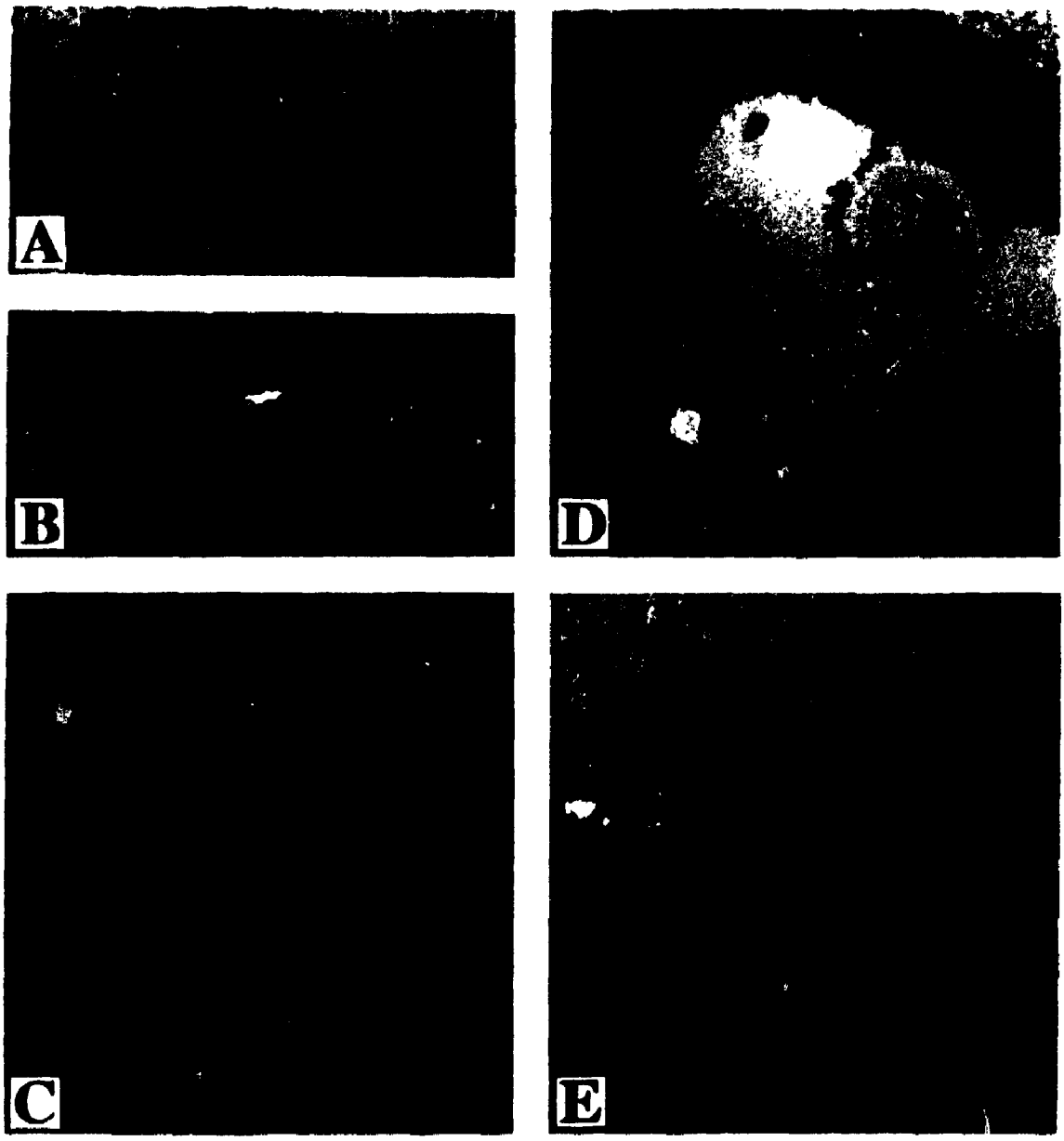


Figure 10-2. Immunocytochemical localization of TGF-α in the porcine ovarian follicle. **A:** Reduced immunostaining in primordial (PL) follicles when the primary antibody was preabsorbed with an equal amount (w/w) of TGF-α peptide. **B:** Similar section of ovary as in **A**, incubated with the TGF-α antibody showing pronouced staining in primordial (PL) follicles. **C:** A primary follicle showing TGF-α staining in the oocyte (O) and granulosa cells (G). **D:** An early antral follicle showing staining in cumulus (C) and granulosa (G) cells; the oocyte (O) and theca cells (T) show very little or no staining. **E:** Part of a large antral follicle showing TGF-α staining in granulosa cells (G), whereas theca interna (TI) and theca externa (TE) show no staining. **A, B** and **D**, X160; **C** and **E**, X250.


A
B
C
D
E

whereas theca cells and oocytes exhibited very little or no detectable staining (Figure 10-2D, E). TGF-α immunostaining was absent in follicles undergoing atresia (not shown). No TGF-α immunostaining was observed when the antibody was omitted from the procedure and the staining was substantially reduced when the primary antibody was pre-absorbed with the same mass of TGF-α peptide (Figure 10-2A).

10.4 Discussion

This study reports the first demonstration of TGF-α gene expression in all the different components of the ovarian follicle for any mammalian species. A very strong signal for TGF-α transcript was detected in cumulus and mural granulosa cells whereas the signal in the theca cells was very weak but detectable. The expression of TGF-α gene in granulosa cells is consistent with a previous report in the rat (Yeh *et al.*, 1993) where TGF-α mRNA was detected both in fresh and cultured granulosa cells by RT-PCR. However, Skinner and Coffey (1988), using Northern blot analysis, failed to detect TGF-α mRNA transcripts in bovine granulosa cells but were able to detect it in theca cells. This difference in TGF-α gene expression in granulosa cells was attributed to the better sensitivity of RT-PCR over Northern analysis (Yeh *et al.*, 1993). Although the PCR method used in this study was not a quantitative one, the signal for TGF-α mRNA transcript was much stronger in the cumulus and granulosa cells compared to theca cells. This may suggest quantitative differences in TGF-α mRNA abundance between the cumulus-granulosa and theca cell compartments in the pig. It is further supported by the fact that the number of granulosa cells used for isolating total RNA was similar to that of theca cells (see Materials and Methods). Although quantitative comparison of TGF-α mRNA abundance among various components of the porcine ovarian follicle remains a subject for future studies, consistency of a stronger TGF-α gene expression in granulosa cells than in theca cells in two separate cell preparations support such a notion. Therefore, the difference in somatic cell TGF-α gene expression between cows, rats and pigs may in fact be due to species differences.

In the present study, no TGF-α mRNA was detected in the oocyte which is in agreement with a previous report of Vaughan *et al* (1992) who were unable to detect

TGF-α mRNA in denuded porcine oocytes by RT-PCR. However, TGF-α transcript was detected in the unfertilized mouse oocyte (Werb, 1990) which further supports the fact that the distribution of various peptides may vary substantially among species.

Based on the balance of the evidence to date, TGF-α was considered predominantly to be a theca cell product (Kudlow *et al.*, 1987; Skinner & Coffey, 1988; Lobb *et al.*, 1989). However, the present study in which TGF-α peptide was localized only in cumulus and granulosa cells proves to the contrary and indicates that in the pig cumulus and mural granulosa cells are the chief source of TGF-α. The presence of both TGF-α mRNA and the peptide in cumulus and granulosa cells further supports the synthesis of TGF-α by these cells. TGF-α immunostaining was also observed in the oocytes of primordial and primary follicles, whereas the oocytes in antral follicles exhibited very little or no detectable staining. Since oocytes from primordial and primary follicles were not included in RT-PCR studies and no TGF-α mRNA was detected in oocytes from antral follicles, it cannot be concluded whether the peptide is locally synthesized by the oocytes or it is sequestered from the surrounding cumulus and/or granulosa cells. The absence of immunoreactive TGF-α in the presence of its mRNA in theca cells may indicate a controlled translation of the message. Although it is possible to obtain 100% pure preparations for oocytes, cumulus and granulosa cells, due to vascularization, thecal cell preparations often contain some blood cells. Therefore an alternate explanation would be that due to a very high sensitivity of the RT-PCR technique, the weak cDNA signal observed in theca cells may in fact have resulted from few contaminating blood cells.

The presence of immunoreactive TGF-α in the oocytes of primordial and primary follicles is consistent with a previous study in the rat (Yeh *et al.*, 1991) and suggests that this peptide may play a role in the early differentiation of the oocyte. However, in the cow (Lobb *et al.*, 1989), TGF-α was not detected in the primordial and primary follicles further suggesting species specificity for the distribution of this peptide.

Previous studies in Chapter 7 have shown the presence of EGF-R, a common receptor shared by several ligands including TGF-α (Prigent & Lemoine, 1992) in

porcine cumulus cells. Brucker *et al* (1991) have shown that the addition of exogenous TGF-α to the culture media containing isolated mouse OCC stimulates the resumption of meiotic maturation in the oocyte and that a positive signal is generated in the cumulus cells to augment this maturation. Studies in the preceding Chapters have shown EGF to be a potent stimulant of the resumption of oocyte maturation and cumulus cell expansion in the pig *in vitro* (Chapters 5 and 6). Therefore, TGF-α of cumulus cell origin may have a physiological role in the regulation of oocyte maturation and cumulus cell expansion in this species. Since EGF was also localized in porcine cumulus cells and oocytes in previous studies (Chapter 7), the presence of two ligands which trigger similar signal transduction cascade after binding to the same receptor, may indicate the presence of a redundant mechanism in the porcine system.

Granulosa cells expressed TGF-α gene and exhibited pronounced staining for TGF-α in both preantral and antral follicles whereas no staining was observed in theca cells. This is consistent with previous reports in the rat where TGF-α mRNA was detected in fresh and cultured granulosa cells by RT-PCR (Yeh *et al.*, 1993) and the peptide was localized only in granulosa cells but not in theca cells by immunocytochemistry (Yeh *et al.*, 1991). In the cow, however, both the mRNA (Skinner & Coffey, 1988) and the peptide (Lobb *et al.*, 1989) for TGF-α were localized only in theca cells and the granulosa cells were negative. Furthermore, in another rat study involving immunolocalization of TGF-α in the ovary (Kudlow *et al.*, 1987), this peptide was exclusively localized in theca cells and the granulosa cells exhibited no staining. The difference in TGF-α expression between cows and pigs may be explained on the basis of species differences. However, the ability to detect immunoreactive TGF-α in granulosa but not in theca cells in one study (Yeh *et al.*, 1991) and *vice versa* in an another study (Kudlow *et al.*, 1987) in the rat is difficult to explain. Our results in the pig are in total agreement with the rat studies of Yeh *et al* (1991, 1993), and support the presence of both the mRNA and the peptide in granulosa cells. May *et al* (1992) reported potent mitogenic effect of EGF on porcine granulosa cells whereas theca cells were much less responsive. TGF-α has been shown to stimulate the proliferation of

isolated granulosa cells from bovine ovarian follicles (Lobb *et al.*, 1988; Skinner & Coffey, 1988). Thus TGF-α in granulosa cells may be involved in an autocrine fashion with rapid granulosa cell proliferation associated with follicular growth. In addition, TGF-α has also been implicated in the regulation of differentiation and steroidogenesis of granulosa cells *in vitro* (Adashi & Resnik, 1986; Mason *et al.*, 1986; Dorrington *et al.*, 1987; Gangrade *et al.*, 1991; Yeh *et al.*, 1993), indicating that TGF-α of granulosa cell origin may also have a role in the differentiation and steroidogenesis of these cells.

Similar to granulosa cells, TGF-α stimulated the proliferation of isolated theca cells from bovine ovarian follicles (Skinner & Coffey, 1988) and induced aromatase activity in theca cells obtained from prepubertal gilts (Gangrade *et al.*, 1991). However, no staining for TGF-α was observed in the theca cells in the present study. Studies in Chapter 7 have shown the presence of EGF-R and the absence of EGF in porcine thecal cells. The presence of EGF-R in the absence of EGF and TGF-α in theca cells supports a paracrine role of EGF/TGF-α acquired from other components of the follicle or an endocrine role of circulating EGF/TGF-α. Furthermore, several other peptides including amphiregulin and cripto, have been reported to interact with the EGF-R and trigger a signal transduction cascade similar to TGF-α and EGF (Prigent & Lemoine, 1992). Future localization studies may reveal the presence of some of these other EGF-R ligands in theca cells. Thus these ligands may interact with EGF-R in theca cells in an autocrine fashion and may alter their proliferation, steroidogenesis and/or differentiation.

Previous studies (Chapter 7) have shown the presence of EGF and EGF-R in follicles undergoing atresia. In addition, both EGF and TGF-α have been shown to inhibit spontaneous onset of apoptosis, an indicator of follicular atresia, in cultured rat ovarian granulosa cells and intact follicles (Tilly *et al.*, 1992). These observations suggested that EGF/TGF-α may have a role in the prevention of onset of follicular atresia. However, in the present study, no TGF-α staining was observed in atretic follicles. This suggests that TGF-α may be present only during follicular growth and may have no role in the prevention of follicular atresia in the pig.

In summary, studies in this chapter have provided evidence that TGF-α is

synthesized by the cumulus and granulosa cells of the porcine ovary. Since, TGF-α was considered largely to be a theca cell product based on bovine studies, the present results in the pig support the idea that the distribution of peptides in ovarian follicles may vary substantially among species. Previous studies (Chapter 7) have shown the presence of EGF in the same cell types and has implicated it as a potent stimulant of oocyte maturation and cumulus expansion (Chapters 5 and 6), the events associated *in vivo* with preovulatory surge of LH which lead to the release of fertilizable oocytes. The localization of two ligands for the same receptor in the same cell types may indicate the presence of redundant mechanisms that regulate follicular functions. A better understanding of the regulation of production and secretion of these autocrine/paracrine factors will help to understand the complex mechanisms that determine the controlled growth of ovarian follicles and the release of developmentally competent oocytes.

CHAPTER 11 SUMMARY AND CONCLUSIONS WITH GENERAL DISCUSSION

As outlined in Chapters 1 and 3, the overall objective of the study presented in this thesis was to investigate the role of EGF in the regulation of both nuclear and cytoplasmic maturation of the porcine oocyte, and in the expansion of cumulus cells using an *in vitro* model system. Investigations were also extended to study EGF-interactions with gonadotropins and ovarian steroids that regulate these preovulatory processes, signal transduction pathways of EGF-stimulated GVBD and cumulus expansion, and local EGF production in the ovarian follicle. Additional studies included the elucidation of the role of the porcine oocyte in EGF-induced cumulus expansion, and the local follicular production of TGF-α, an additional ligand for EGF receptor, extensively studied for its roles in follicular functions.

The results obtained from the studies in this thesis can be summarized as follows:

1) EGF is a potent stimulant of the resumption of meiosis in the porcine oocyte *in vitro*, and the addition of FSH, but not LH, to EGF further enhances its effect on GVBD. Among the gonadotropins, at the concentrations used, only FSH stimulates the resumption of meiosis. Both E_2 and A_4 inhibit FSH-stimulated GVBD, but have no effect on EGF-stimulated GVBD.

2) Although FSH is much more effective in inducing cumulus expansion in porcine OCC, EGF can stimulate cumulus expansion in about 50% of OCC. Unlike mouse OCC, LH can induce cumulus expansion in porcine OCC.

3) Like FSH, EGF-induced expansion of porcine cumuli oophori is not dependent on the oocyte. Furthermore, the maintenance of the original structure and orientation of the cumulus cells around the zona pellucida is not required for their expansion.

4) Although the porcine cumulus expansion *in vitro* is not dependent on the oocyte,

the porcine oocyte is capable of secreting the putative cumulus expansion-enabling factor required for mouse cumulus cell expansion, and this factor is not species specific.

5) EGF is locally produced and its receptors are present in the porcine ovarian follicle. EGF is localized in the oocyte and cumulus and granulosa cells, but not in the theca cells. In antral follicles, EGF-R is present only in somatic follicular cells of cumulus, granulosa and theca, but not in the oocyte.

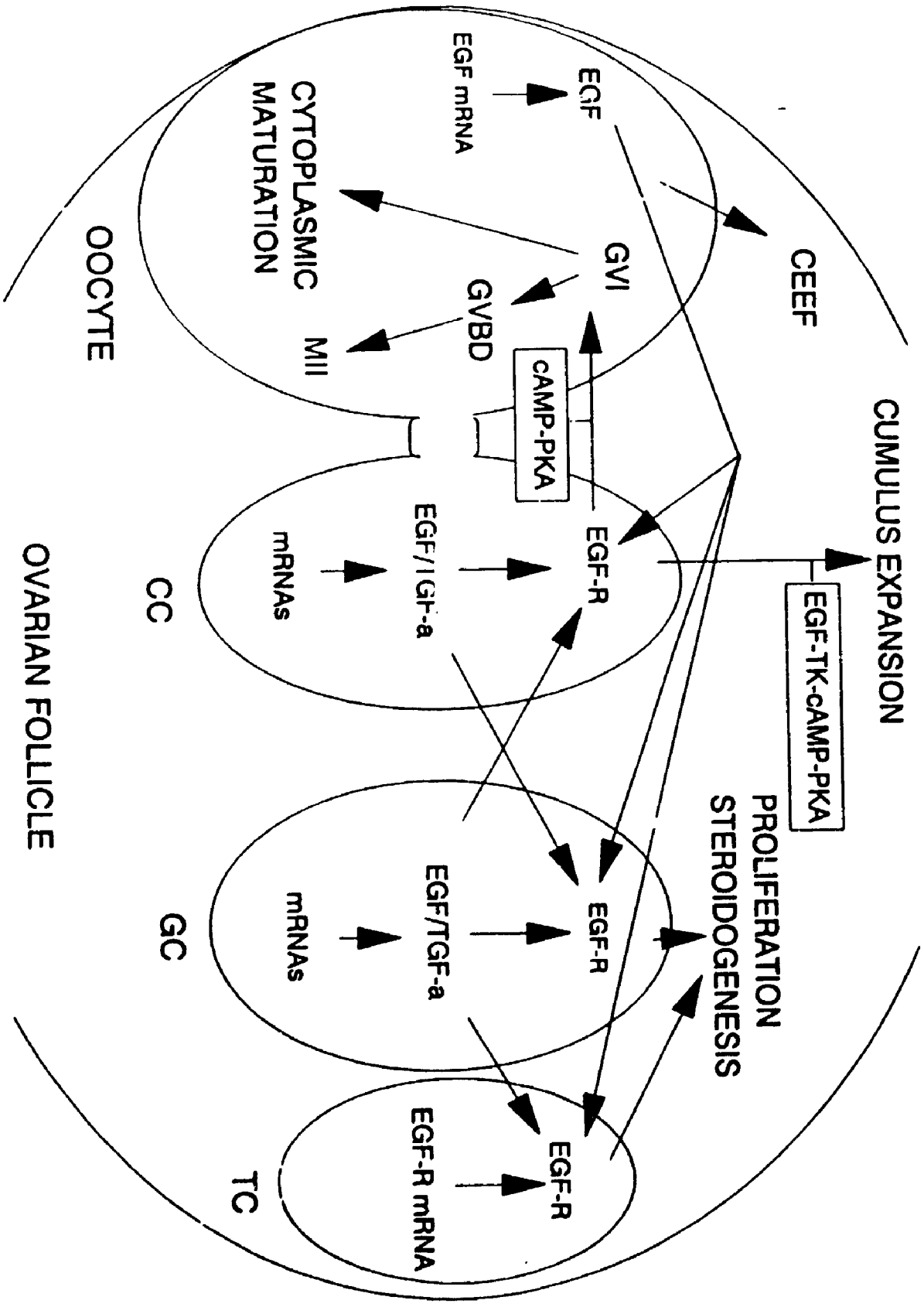
6) EGF-induced cumulus expansion is mediated through the EGF-TK-cAMP-PKA pathway and oocyte maturation through the cAMP-PKA pathway. The tyrphostin from Gibco BRL is a potent stimulant of porcine oocyte maturation and its effect is not mediated through cAMP.

7) Like FSH, the presence of EGF in the IVM media increases the percentage of porcine oocytes completing nuclear maturation. Furthermore, the addition of EGF alone to the culture media decreases the proportion of polyspermic oocytes and increases the percentage of monospermic oocytes forming the male pronucleus. On the other hand, FSH abolishes these effects of EGF and increases the percentage of polyspermic oocytes forming more than two pronuclei when added alone or with EGF.

8) The effect of EGF on protein synthesis in the porcine oocyte is stimulatory after 6 hours, and inhibitory after 24 hours, with no effect after 44 hours of culture. In the cumulus cells, EGF stimulates protein synthesis after 6 hours, but has no effect after 24 and 44 hours of culture. FSH, on the other hand, stimulates protein synthesis both in the oocyte and cumulus cells at each time interval.

9) In antral porcine ovarian follicles, TGF-α is produced only by the cumulus and granulosa cells. Immunoreactive peptide, however, is also present in the oocytes of primordial and primary follicles. The results presented in this thesis are summarized in a schematic diagram in Figure 11-1.

Figure 11-1 Schematic representation summarizing the results presented in this thesis. In the antral follicle, EGF and TGF-α of oocytic and/or cumulus-granulosa cell origin can bind the receptor in cumulus (CC), granulosa (GC) and theca (TC) cells in autocrine and/or paracrine fashion. EGF from the oocyte and TGF-α from cumulus cells can bind the receptor in cumulus cells in paracrine and autocrine manners respectively to generate a positive signal in cumulus cells that can stimulate GVBD in the oocyte. EGF/TGF-α can also influence cytoplasmic maturation in the oocyte in a similar fashion. The stimulation of GVBD in the oocyte by EGF appears to be mediated through the activation of cAMP-dependent PKA. The binding of EGF/TGF-α to the receptor in cumulus cells can also trigger the expansion of these cells, which is mediated through EGF-TK-cAMP-PKA pathway. In the pig, the oocyte has no role in enabling the cumulus cells to expand in response to EGF, although the porcine oocyte is capable of secreting the cumulus expansion-enabling factor (CEEF) required for mouse cumulus cell expansion. Results from other laboratories and from my own studies which were not included in the thesis indicate that in the granulosa and theca cells, EGF/TGF-α can stimulate proliferation and can alter steroidogenesis.


CUMULUS EXPANSION
CEEF
EGF-TK-cAMP-PKA
PROLIFERATION
STEROIDOGENESIS
EGF
EGF mRNA
GVI
GVBD
MII
CYTOPLASMIC
MATURATION
OOCYTE
cAMP-PKA
EGF-R
EGF/TGF-a
mRNAs
CC
GC
TC
EGF-R mRNA
OVARIAN FOLLICLE

The findings that EGF stimulates the spontaneous onset of meiotic maturation in the oocyte and expansion of surrounding cumulus cells in the pig are in agreement with previous observations in laboratory animal species (Dekel & Sherizly, 1985; Downs *et al.*, 1988; Ueno *et al.*, 1988; Downs 1989). This along with the fact that both EGF and its receptor are present in the ovarian follicle (Chapter 7) suggest that EGF may have a physiological role in the regulation of these periovulatory processes *in vivo*. That E_2 and A_4 inhibited only FSH-, but not EGF-stimulated GVBD, suggests that EGF and FSH act through different mechanisms to initiate meiosis. Similarly, since FSH was much more effective in inducing cumulus expansion than EGF, it appears that the induction of cumulus expansion by the two peptides also involves different mechanisms. Indeed, studies in Chapter 8 provided more concrete evidence to indicate that EGF and FSH do not converge on a common pathway to stimulate both oocyte maturation and cumulus expansion. Thus it appears that EGF does not play an intermediary role in FSH-stimulated GVBD and cumulus expansion. However, it is reasonable to speculate that *in vivo* local factors such as EGF are controlled by systemic hormones, in particular gonadotropins, to regulate these preovulatory processes. Thus in order to implicate EGF more precisely in these preovulatory events, further studies are needed to understand the regulation of production and secretion of EGF, and the synthesis of its receptor in the follicle by circulating hormones.

Although EGF and FSH stimulate both oocyte maturation and cumulus expansion, the two preovulatory processes appear not to be causally related. For instance, tyrphostin completely inhibited EGF-stimulated cumulus expansion, yet failed to inhibit EGF-stimulated GVBD (Text Figures 8-1 & 8-2). Furthermore, although tyrphostin had no effect on cumulus expansion by itself, it was able to stimulate significantly spontaneous oocyte maturation (Chapter 8). Similarly, H-89 completely inhibited EGF- and FSH-stimulated GVBD, but was unable to totally inhibit cumulus expansion (Text Figures 8-5 vs 8-6). In addition, LH stimulated cumulus expansion, but had no significant effect on GVBD. Previous observations in the mouse where sulfated GAGs completely suppressed EGF-induced cumulus expansion, yet failed to inhibit EGF-stimulated GVBD (Eppig, 1981b, c; Downs, 1989), further support such concept.

It has been shown in the mouse (Downs *et al.*, 1988) and the pig (Coskun & Lin, 1992) that EGF stimulates GVBD in the oocyte derived from antral follicles by generating a positive signal in the cumulus cells. The results in Chapter 7 of this thesis, where EGF-R was localized in cumulus cells but not in the oocyte of antral follicles, support such a concept. This would also suggest that EGF does not break down the junctional communications between the oocyte and cumulus cells to stimulate GVBD, as has been reviewed in Section 2.3.1C. However, it is possible that, in addition to generating a positive signal in the cumulus cells, EGF can inhibit transfer of any suppressive factor(s) from cumulus cells to the oocyte to initiate meiosis. In the mouse, the effect of EGF on GVBD was shown to be stimulatory at all times observed, whereas the effect of FSH was at first inhibitory and later stimulatory (Downs *et al.*, 1988). Although an independent time-course experiment for EGF and FSH effects on GVBD was not done, in the tyrphostin experiment, EGF stimulated GVBD after 12 hours of culture, whereas FSH had either no effect or the effect was inhibitory (Text Figure 8-2). However, both the hormones stimulated GVBD after 24 hours of culture (Chapter 5). These observations, therefore, support a similar pattern of stimulation by EGF and FSH of oocyte maturation in the pig, and provide further support to the concept that the two peptides stimulate GVBD via different mechanisms.

The preponderance of the evidence to date suggests that FSH stimulates GVBD in the oocyte by activating the cAMP-dependent PKA pathway (Dekel & Kraicer, 1978; Eppig, 1979a; Downs *et al.*, 1988; results Chapter 8). Studies in Chapter 8 indicated that, like FSH, EGF also stimulates meiotic maturation through the cAMP-PKA pathway. However, as reviewed in Chapter 2, cAMP has also been implicated with the maintenance of meiotic arrest in the oocyte. Membrane-permeable analogs of cAMP, the inhibitors of PDE, or agents that increase cAMP levels by activation of adenylate cyclase, inhibit spontaneous maturation in the oocyte (reviewed by Eppig & Downs, 1984; Schultz, 1986; Wassarman & Albertini, 1994). Furthermore, microinjection of an inhibitor of cAMP-dependent PKA initiates meiosis in oocytes maintained in GV stage with dbcAMP or IBMX (Bornslaeger *et al.*, 1986). In addition, cAMP antagonists can reverse the meiotic inhibition maintained either by exogenous inhibitors (Downs, 1993),

or by granulosa cells (Eppig, 1991a), resulting in GVBD. Thus it appears that cAMP is involved with the maintenance of meiotic arrest rather than with the stimulation of GVBD. Indeed, a decrease in oocyte cAMP has been shown to precede GVBD in the rat (Racowsky, 1984) and the mouse (Schultz *et al.*, 1983a). A further confusion to the cAMP concept is added by my own observations (Chapter 8) that although both EGF and FSH stimulated GVBD to a similar extent after 24 hours (Chapter 5), the stimulation of cAMP by FSH was much greater than by EGF (60-fold vs 2-fold). It was proposed that the status of nuclear maturation in the oocyte (GVI or GBVD) is determined by the duration and magnitude of elevated cAMP levels (Downs *et al.*, 1988). The observations that acute, but not chronic, exposure of explanted follicles in culture to dbcAMP stimulates spontaneous oocyte maturation (Hillensjo *et al.*, 1978; Dekel *et al.*, 1981), and that the effect of FSH on GVBD is at first inhibitory and later stimulatory (Downs *et al.*, 1988), support such an hypothesis. Thus it is possible that EGF and FSH may use different cAMP stimulatory thresholds to stimulate GVBD.

As alluded to earlier, EGF may be a physiological trigger *in vivo* that induces cumulus cell expansion during the periovulatory period. The expanded cumulus may play a vital role in the detachment of OCC from the membrana granulosa and normal ovulation, oocyte pick-up and transport by the fimbria of the oviduct, fertilization, and subsequent embryonic development, as discussed in details in Section 2.4.4. During the discussion above regarding the central control of local regulators, it was suggested that EGF may not act as an intermediary for FSH in the stimulation of cumulus expansion. However, *in vivo* cumulus cells expand only in response to the preovulatory surge of gonadotropins. Indeed, studies in Chapter 5 indicate very similar cumulus expansion responses to EGF and LH, and the effect of EGF was not further enhanced by LH. Thus it is possible that EGF may act as an intermediary for LH to induce cumulus expansion during the periovulatory period *in vivo*. However, further studies are needed to test such a possibility. Unlike mouse cumulus cells which do not expand in the absence of the oocyte (Buccione *et al.*, 1990; Salustri *et al.*, 1990; studies herein in Chapter 6), the

expansion of porcine cumuli oophori is not dependent on the oocyte (Chapter 6). However, the porcine oocyte is capable of secreting the putative cumulus expansion-enabling factor required by the mouse cumulus cells for expansion. This indicates that there are subtle species differences and that there is no universal model to study this preovulatory process.

Signal transduction results (Chapter 8) indicated that EGF involves activation of EGF-R tyrosine kinase to induce cumulus expansion, suggesting the mediation through functional EGF receptors in the cumulus cells. The involvement of EGF-TK in the induction of cumulus expansion is in agreement with overwhelming evidence in many other cell types in which tyrosine kinase stimulation was observed due to EGF activity (reviewed by Carpenter, 1983; Carpenter & Cohen, 1990; Oberg *et al.*, 1990). However, tyrosine kinase stimulation is not always an exclusive event in the generation of second messengers. As mentioned elsewhere, bovine luteal cells possess an aberrant form of EGF-R which is devoid of intrinsic TK activity (Chakravorty *et al.*, 1993). The ligand activates other signalling systems, such as MAP kinase, by modulating critical post-receptor phosphorylation or dephosphorylation events in the luteal cells. Evidence gathered from studies in other cell types indicates that EGF stimulation of the tyrosine kinase results in the activation of inositol-1, 4, 5-trisphosphate-DAG-calcium-PKC pathway (Abdel-Latif, 1986; Wahl & Carpenter, 1988). However, studies in Chapter 8 have clearly established that during EGF stimulation of cumulus expansion, tyrosine kinase phosphorylation is followed by the activation of the cAMP-PKA pathway. Since EGF-induced GVBD also involves cAMP-PKA activation, it remains to be determined if oocyte maturation and cumulus expansion are mediated through separate transduction signals downstream from the cAMP generation or if those two events reflect different thresholds for the same signal.

As discussed previously at several places, it is possible to obtain morphologically normal secondary oocytes with expanded cumuli oophori on a large scale *in vitro* from slaughter house ovaries. However, such oocytes possess limited potential to support subsequent embryonic development following fertilization, due to inadequacies in their

cytoplasmic maturation. The pig may prove to be an excellent model to produce embryos *in vitro* both for research and commercial purposes due to the reasons discussed in Chapter 3. However, despite continuous efforts, IVM-IVF has met with a very limited success in this species. Polyspermy is a very common problem in pig oocytes matured and fertilized *in vitro*, and in some studies as high as 80% of the oocytes have been reported to be polyspermic (Cran & Cheng, 1986; Mattioli *et al.*, 1988b, 1989). Furthermore, only a very low proportion of these oocytes are capable of transforming sperm into the male pronucleus (Nagai *et al.*, 1984; Mattioli *et al.*, 1988b; Yoshida *et al.*, 1990; Nagai, 1994). These problems appear to result from the deficiencies in the culture media used for IVM leading to inadequate maturation of the oocyte. Indeed reports using media with different compositions indicate that the selection of IVM media influences the outcome of porcine IVF (Wang *et al.*, 1992; Yoshida *et al.*, 1992a; Nagai, 1994). The importance of an adequate culture system is further exemplified by the observations that the coculture of oocytes with follicular cells improves fertilization and subsequent developmental capacity in this species (Mattioli *et al.*, 1988b, 1989; Nagai *et al.*, 1993; Singh & Armstrong, unpublished). Deficiencies in oocyte maturation *in vitro* are further revealed by the facts that the *in vivo* matured porcine oocytes exhibit less incidence of chromosomal anomalies and polyspermy, and higher rates of development following IVF than those matured *in vitro* (Yoshida *et al.*, 1990). Thus it would appear that the somatic cells secrete undefined factor(s) into the culture medium which facilitate oocyte maturation and subsequent embryonic development.

In order to optimize the *in vitro* culture system, it is essential to identify the somatic cell products that influence cytoplasmic maturation of the oocyte. The results derived from studies in Chapter 9 have clearly demonstrated that EGF, when added during IVM, influences cytoplasmic maturation of porcine oocytes as revealed by the success of fertilization and by the changes in the pattern of protein synthesis. Fertilization studies indicated that the effect of EGF is beneficial only when it is added alone to the IVM media. Addition of EGF alone significantly decreased the proportion of polyspermic oocytes and increased the percentage of monospermic oocytes forming

male pronucleus (Table 9-1). Studies in Chapters 7 and 10 have revealed the production of EGF and TGF-α predominantly by the oocyte (EGF) and granulosa cells (TGF-α & EGF). Mural granulosa cells usually form the major component of the somatic follicular material used for coculturing oocytes during IVM (Mattioli *et al.*, 1988b, 1989; Nagai *et al.*, 1993; Singh & Armstrong, unpublished). Thus, EGF and/or TGF-α may be one of the vital components of granulosa cell secretions that improve fertilization and subsequent embryonic development following coculture.

FSH abolished the beneficial effects of EGF and significantly increased the percentage of polyspermic oocytes forming more than two pronuclei when added alone or with EGF. Almost in all porcine IVM-IVF studies (Mattioli *et al.*, 1988b, 1989; Yoshida *et al.*, 1992a), a cocktail of FSH, LH and E_2 is added to the culture media during oocyte maturation. These hormones are added simply based on the facts that they are present in the follicle during oocyte maturation *in situ*, and that they stimulate the preovulatory events of initiation of meiosis and cumulus expansion that are considered to play a beneficial role during fertilization. Since FSH was found to influence fertilization adversely (Chapter 9), another reason for the low success of porcine IVM-IVF may be the indiscriminate supplementation of the IVM media with hormones. Since EGF can replace gonadotropins to stimulate GVBD and cumulus expansion, the supplementation of culture media with exogenous EGF alone during IVM may help to improve the overall success of IVM-IVF in the pig. However, further studies are needed to determine EGF interactions with other follicular hormones that may influence the outcome of IVM-IVF.

The RT-PCR and immunolocalization studies (Chapter 7) revealed local follicular production of EGF and the presence of its receptors in the ovarian follicle, suggesting that the observed effects of this growth factor on oocyte maturation and cumulus expansion are physiological. From the relative abundance of the mRNAs and immunoreactive peptides, it appeared that, within the follicle, the oocyte is the primary, although not necessarily the exclusive, source of EGF, and the somatic cells that of EGF-

R. This may suggest that an EGF paracrine system is in place in the porcine ovarian follicle which regulates the oocyte and somatic cell functions. However, immunoreactive EGF was also detected in the cumulus-granulosa compartment, although the theca cells were completely negative. Given that EGF-R are present in these cells, EGF may also regulate cumulus-granulosa cell functions in an autocrine manner. Although EGF has been shown to stimulate porcine theca cell proliferation *in vitro* (May *et al.*, 1992), Fujinaga and co-workers (1992) failed to detect EGF binding sites in porcine theca cells. In the present study, theca cells expressed a very strong signal for EGF-R mRNA and exhibited a more pronouced staining for EGF-R peptide than cumulus-granulosa cells in antral follicles. All this indicates the presence of at least immunoreactive receptors in these cells and suggests a role for EGF in theca cell functions.

As described in previous Chapters, TGF-α binds to the EGF-R, triggers responses similar to EGF, and has extensively been studied for its role in follicular functions. Based on the balance of evidence to date, TGF-α was considered to be a theca cell product (Kudlow *et al.*, 1987; Skinner & Coffey, 1988; Lobb *et al.*, 1989). However, studies in Chapter 10 indicate that in the pig, cumulus and mural granulosa cells are the chief source of TGF-α. The presence of both TGF-α mRNA and the peptide in cumulus and granulosa cells further supports the synthesis of TGF-α by these cells. Since EGF was also localized in porcine cumulus and granulosa cells (Chapter 7), the presence of two ligands which trigger a similar signal transduction cascade after binding to the same receptor may indicate the presence of a redundant mechanism. As in many other biological systems, the presence of more than one ligands for the EGF-R in the ovarian follicle provides a cautionary note for any future gene knockout strategy aimed at a single ligand.

In conclusion, evidence is provided that EGF of follicular origin may play a vital role in bringing a complete physiological maturation to the porcine oocyte, including expansion of the surrounding cumulus cells. The signal transduction results further confirm the previous concept that oocyte maturation and cumulus expansion are not

causally related events and that they are mediated via separate mechanisms. The oocyte has no role in EGF-induced expansion of porcine cumuli oophori, although it is capable of producing the expansion-enabling factor required by the mouse cumulus cells for their expansion. In addition, porcine ovarian follicles possess ligand redundancy to activate the EGF-R.

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