CHAPTER I

INTRODUCTION

Rationale

In vitro maturation (IVM) of human oocytes is a promising technique to reduce the costs and avert the side-effects of gonadotropin stimulation and hence, would be an attractive alternative to employ the technique for *in vitro* fertilization (IVF) or intracytoplasmic sperm injection (ICSI) programme. However, the efficiency of the currently available IVM technique is suboptimal in terms of the number of mature oocytes per cycle in comparison to gonadotropin stimulated cycles. Also, the quality of maturation appears to be suboptimal, because the fertilization rate resulting from *in vitro* matured oocytes is lower than that of *in vivo* matured oocytes (Eppig et al., 1996 ; Goud et al., 1998). Therefore, in order to make the technique acceptable and clinically applicable, improvement is needed. The strategy for increasing the efficiency of IVM/IVF ought to focus primarily on optimizing the immature oocytes preparation by gonadotropin stimulation and developing an optimal culture system for IVM.

Fairly successful culture conditions have been devised for IVM/IVF in the human, bovine, mouse, and other species. However, interspecies differences in maturation and fertilization physiology, except for the mouse model, do not allow their direct extrapolation to the human system. Many investigations suggested that results obtained from IVF studies in mice could be applied to IVF studies in the human system (Eppig and Downs, 1984 ; Albertini, 1992 ; Eppig et al., 1996 ; Goud et al., 1998). Therefore, appropriate culture conditions for IVM/IVF studies in mice need to be developed into an optimal culture system for IVM/IVF studies in humans.

One of the most important factors regulating the number - determined by oocyte nuclear maturation, and quality - determined by oocyte cytoplasmic maturation, of oocytes maturing *in vitro* is the culture system used for IVM. Culture media components (such as oocyte maturation inducers ; gonadotropins, growth factors and pyruvate) and culture conditions (such as oocyte maturation duration) can affect and even modulate the meiotic regulation and fertilization of mammalian oocytes. The most commonly used design employs a culture medium supplemented with gonadotropins and serum (Downs and Mastropolo, 1997). However, little is known of the optimal oocyte maturation duration and the effect of gonadotropins on *in vivo* and *in vitro* maturation in mouse IVM/IVF. In addition, many investigations suggested that cyclic adenosine 3',5'-monophosphate (cAMP) is involved in mediating maintenance of meiotic arrest of mammalian oocytes and that a drop in

intraoocyte cAMP concentration results in resumption of meiosis. This role for cAMP is based upon the well-documented observations that cAMP analogues and cAMP phosphodiesterase inhibitors reversibly inhibit resumption of spontaneous meiotic *in vitro* maturation of mammalian oocytes. Although the results of these experiments are discussed in terms of a possible role of cAMP in the regulation of resumption of meiosis (nuclear maturation) in mammals, they did not show a role of cAMP in cytoplasmic maturation regarding fertilization and preimplantation embryo development of *in vitro* matured oocytes. Therefore, this present study is intended to investigate the effects of oocyte maturation inducers ; pregnant mare's serum gonadotropin (PMSG) and human chorionic gonadotropin (hCG), and oocyte maturation duration (3-24 hours) with respect to nuclear maturation (determined by the number of *in vitro* matured oocytes at the MII stage), cytoplasmic maturation (determined by fertilization rate, zona thickness, zona hardening, and intraoocyte cAMP level), and preimplantation embryo development (determined by the degree of blastocyst development) of IVM/IVF in mouse oocytes in order to develop the optimal conditions for the highest success rate of IVM/IVF in mice.

Literature Review

More than a decade has passed since the demonstration that fully grown mouse oocytes matured and fertilized in culture, after isolation from large antral

follicles, can undergo embryogenesis and development to live offsprings (Schroeder and Eppig, 1984 ; Thibault et al., 1987). Since then, offsprings derived from *in vitro* matured and fertilized oocytes of several mammalian species (Goto et al., 1988 ; Vanderhyden and Armstrong, 1989 ; Yoshida et al., 1993 ; Hasler et al., 1995), including humans (Cha et al., 1991, 1992 ; Trounson et al., 1994), have been born. Moreover, in certain circumstances, maturation of oocytes *in vitro* results in a lower frequency of successful preimplantation embryo development than maturation induced *in vivo* by exogenous gonadotropins. For example, development from the two-cell to the blastocyst stage occurs less frequently after *in vitro* maturation of oocytes than after *in vivo* maturation (Eppig and Wigglesworth, 1994 ; Eppig and O'Brien, 1995). These results suggest that under some conditions the culture system supporting oocyte *in vitro* maturation fails to adequately support the development processes of oocytes for successful preimplantation development and that *in vivo* maturation circumvents this problem (Hampl and Eppig, 1995 ; Eppig et al., 1996).

Correlation of oocyte and follicular development

Oocyte growth and differentiation are usually coordinated with the development of the somatic components of the ovarian follicle. The primordial follicle is the earliest recognizable form of ovarian follicle and comprises a

primordial oocyte surrounded by a single layer of flattened somatic cells that are presumably the progenitors of follicular granulosa cells. The maturation of oocytes within primordial follicles, hereafter referred to as primordial oocytes, is arrested at the diplotene stage of prophase I (PI) of meiosis or germinal vesicle (GV) stage and does not entered the phase of dramatic growth typified by oocytes in preantral developing follicles (Eppig and Downs, 1984 ; Eppig, 1991 ; Eppig et al., 1996). In addition, Funahashi and colleagues (1997) indicated that the nuclear morphology of oocytes at the GV stage can be differentiated as five categories : **GV-X**, characterized by a nucleolus and filamentous chromatin distributed over the whole area of the GV; **GV-I**, characterized by a nucleolus and chromatin in the form of a ring or horseshoe around the nucleolus; **GV-II**, characterized by a nucleolus and chromatin as a ring or horseshoe around the nucleolus but with some well-stained clumps distributed mainly around the ring- or horseshoe-like chromatin; **GV-III**, characterized by an irregular network of filamentous bivalents in the whole area of the GV and lack of nucleolus; and **GV-IV**, characterized by diakinesis chromatin.

Oocytes in preantral follicles undergo a great increase in mass. The increase in oocyte volume correlates with the increased protein content required for maturation, fertilization, and preimplantation embryo development. For example, the zona pellucida (ZP), a relatively thick acellular sulfated glycoprotein layer surrounding the oocyte, is synthesized and secreted by

oocytes in preantral follicles (Bleil and Wassarman, 1980 ; Philpott et al., 1987 ; Eppig and Schroeder, 1989). The ZP plays an important role in gamete interactions resulting in fertilization and maintaining blastomere contact in early cleavage stages, as well as providing a barrier to cells of the immune system (Garside et al., 1997). The ZP has been studied most extensively in humans and mice and has been shown to contain three major specific glycoproteins, ZP1, ZP2, and ZP3, with the principal function of ZP1 thought to be structural, whereas both ZP2 (secondary sperm receptor) and ZP3 (primary sperm receptor) also participate actively in gamete interaction (Bleil and Wassarman, 1980).

Immediately after fertilization there are two major changes that prevent polyspermy : a rapid electrical depolarization of the egg plasma membrane that blocks additional sperm in the perivitelline space from fusing with the egg, and biochemical modifications of the ZP. These latter changes occur secondarily to the fusion of cytoplasmic cortical granules with the egg plasma membrane, and the subsequent discharge of the granules' enzymatic contents into the perivitelline space. The release of proteinases and glycosidases modifies the ZP (zona reaction), resulting in a block to additional sperm binding and inhibition of zona-bound sperm penetration (Dean, 1992).

Oocytes in early antral follicles have usually undergone an important fundamental change : they have become competent to resume the first meiotic

division because molecules essential for the resumption of meiosis, such as cyclin B, maturation promoting factor, and mitogen-activated protein kinases, have been synthesized during this stage of oocyte development by follicle - stimulating hormone (FSH) stimulation at the puberty stage (Wickramasinghe et al., 1991 ; Christmann et al., 1994 ; Harrouk and Clarke, 1995 ; Taieb et al., 1997). Dramatic changes occur in both the oocyte and the somatic compartments of the follicle after the preovulatory surge of gonadotropins. In most, but not all mammalian species, the oocytes resume meiosis and undergo GV breakdown (GVBD ; the disappearance of the nuclear envelope and the nucleoli), the chromosomes condense, meiosis progresses to the metaphase I (MI) stage, and the metaphase II (MII) stage before ovulation (Downs et al., 1988 ; Buccione et al., 1990 ; Das et al., 1992 ; Eppig, 1991 ; Albertini, 1992).

Before birth : primordial germ cells (2n)

(oogenesis) ↓ **mitosis**

oogonia (2n) epithelial cells

↓ **mitosis** ↓

primary oocytes (2n / PI / GV) primary follicles

↓

Birth : primary oocytes (2n / PI / GV) primary follicles

↓ **OMI** ← granulosa cells

Puberty : **FSH** → ↓

(oocyte maturation) ↓ secondary follicles

↓

Graafian follicle

↓ **E2** ← granulosa cells

LH → and

↓ **OMS** ← cumulus cells

first meiotic division

primary oocyte (2n / MI / GVBD)

↓ **second meiotic division**

secondary oocyte (n / MII)

↓

ovulation (mature oocyte, MII stage)

↓

fertilization

Figure 1.1 Mammalian oocyte maturation

Meanwhile, the cumulus cells (the granulosa cells adjacent to the oocyte) secrete hyaluronic acid, with the consequence that the space between the cumulus cells becomes enlarged, and the mature oocyte and the cumulus cells become embedded in a sticky, mucinous matrix. This process is called cumulus expansion, or mucification. The remaining follicular somatic cells, meanwhile, undergo remarkable changes that, after ovulation, result in the formation of the corpus luteum (Jinno et al., 1990 ; Hirshfield, 1991 ; Kranen et al., 1993 ; Eppig et al., 1996).

The designation of oocyte maturity is important as it permits accurate determination of the appropriate preinsemination incubation interval for each oocyte. The timing of hCG injection might be altered if a high proportion of immature or postmature oocytes is obtained. Oocyte grading is performed with a dissecting or inverted microscope. This classification is based upon characteristics of the oocyte-corona-cumulus complex, including the extent of mucification and dispersal of the corona radiata and cumulus and the presence or absence of a nuclear membrane (germinal vesicle) or polar body (Trounson and Gardner, 1993).

A mature, preovulatory oocyte displays extensive dispersal of the surrounding granulosa cells, with an expanded cumulus and corona radiata. The ZP is readily visualized and the ooplasm is clear. The presence of the first polar body in the perivitelline space indicates that an oocyte is in the MII stage. An

oocyte of intermediate maturity is marked by a slightly dense corona with a dispersed cumulus. Neither a GV nor a polar body can be identified. An oocyte at the stage of GVBD is in the MI stage. An immature oocyte has a compact corona, with a cumulus composed of only a few cell layers. The GV may be easily visualized in a grossly immature oocyte at the GV stage (Table 1.1) (Figure 1.2-1.4) (Trounson and Gardner, 1993).

Oocyte maturation inhibitor and stimulator

In most mammals, meiotic maturation occurs in the oocytes of a selected group of antral follicles as a result of the endogenous preovulatory gonadotropin surge or the administration of exogenous gonadotropins to a suitably prepared female. The meiotic process of the oocytes in nonatretic follicles is arrested in PI prior to stimulation with gonadotropins (Eppig and Downs, 1984).

Table 1.1 Morphological parameters used for assessment of oocyte maturity

Oocyte status	Description
immature	poorly expanded, dense compact cumulus, compact and adherent not radiating corona, aggregated granulosa cells, oocyte obscured, GV observed, cytoplasm may be dark with clumped organelles
premature	expanded cumulus and slightly compact corona, well-dispersed granulosa, oocyte may be visible
mature	very expanded cumulus and well-dispersed radiating corona, evenly distributed around oocyte, loosely aggregated granulosa, clear zona and ooplasm, polar body visible
postmature	expanded cumulus with clumps of cells, radiant corona but often clumped, visible zona, ooplasm may be granular or dark
atretic	absent cumulus or present in small amounts, aggregated corona, misshapen ooplasm, visible zona

(from : Trounson and Gardner, 1993)

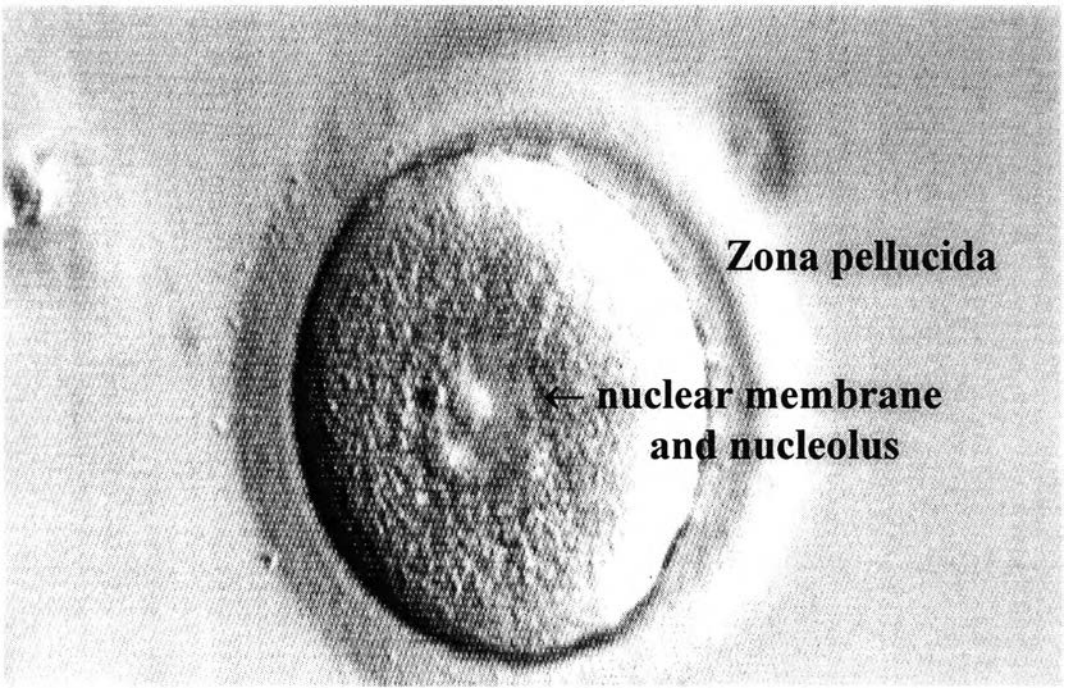

Figure 1.2 Morphology of a mouse oocyte at the germinal vesicle stage. Nuclear membrane and nucleolus (arrow) are present, cytoplasm may be dark with clumped organelles.

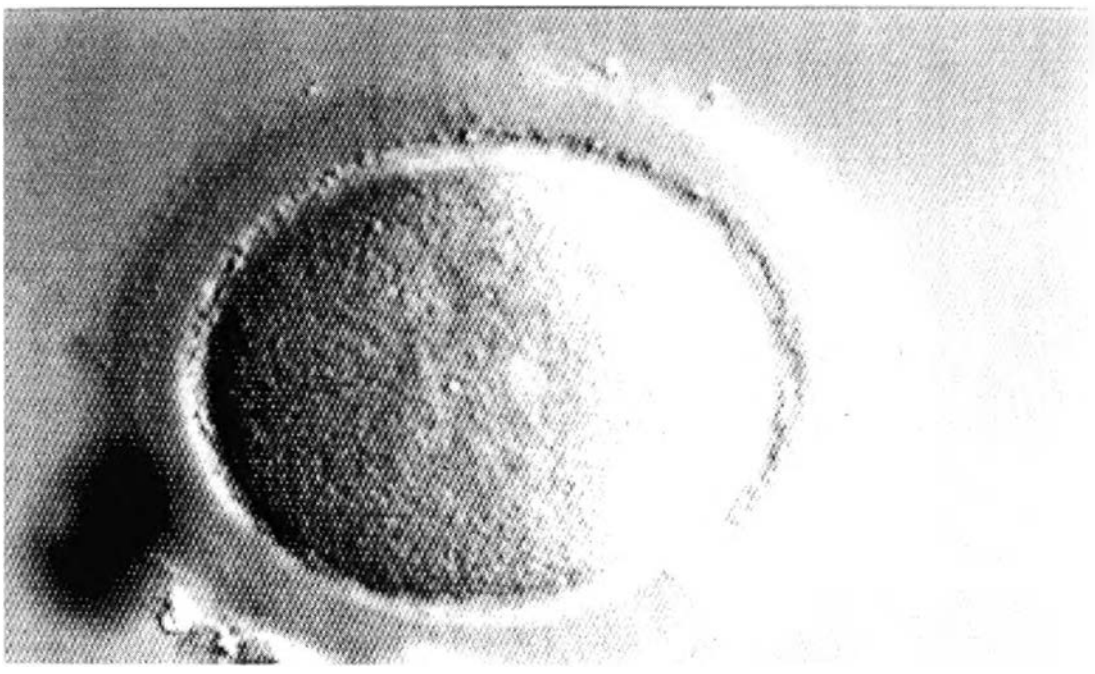

Figure 1.3 Morphology of a mouse oocyte at the GVBD or metaphase I stage. Nuclear membrane and nucleolus are disappear. The first polar body is not yet formed.

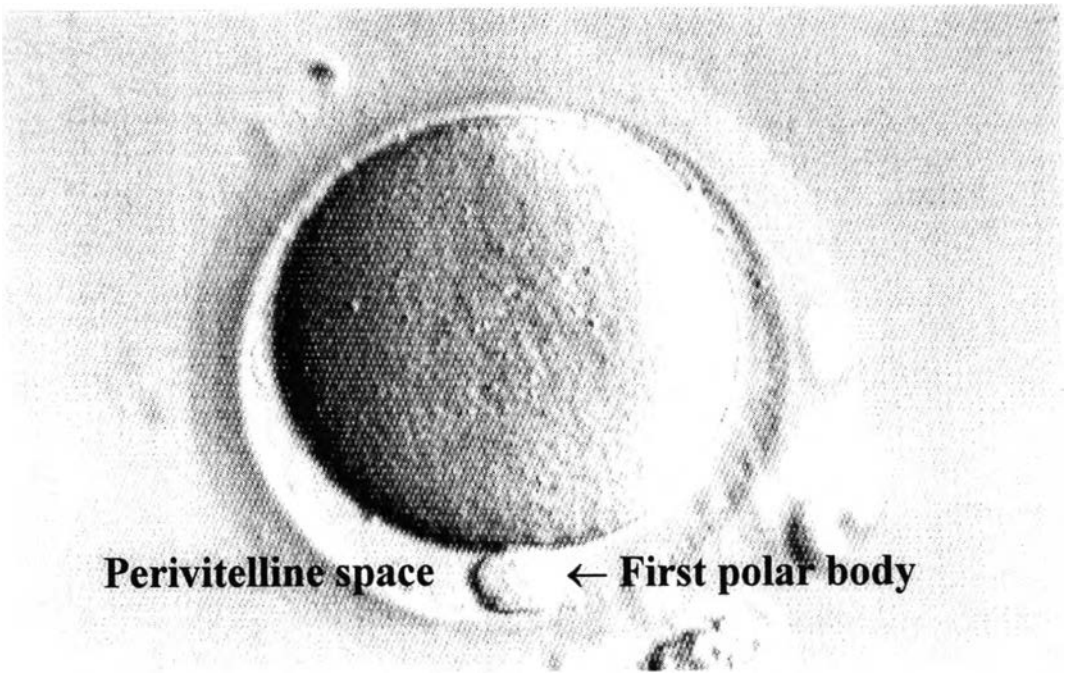

Figure 1.4 Morphology of a mouse oocyte at the metaphase II stage. First polar body (arrow) is clearly seen in the perivitelline space.

In 1935, Pincus and Enzmann showed that when GV stage oocytes were removed from the environment of the antral follicle, they underwent a spontaneous meiotic *in vitro* maturation. They suggested that some follicular component (s) may serve to maintain the oocyte in a stage of meiotic arrest. This observation has provided the framework for proposing another type of chemical signal for the regulation of oocyte maturation, a maturation-inhibiting factor or oocyte maturation inhibitor (OMI) (Eppig and Downs, 1984).

The oocytes in preantral follicles do not undergo spontaneous meiotic maturation in culture or mature in response to gonadotropins *in vivo*. The ability to mature spontaneously *in vitro* is not acquired until about 15 days of age in mice (Sorensen and Wassarman, 1976). Primary follicular development in mice comprises 10 stages, with stages 1-4 representing small preantral follicles. At this stage the follicle has FSH and prolactin receptors on the follicular membrane. Stages 5-7 represent large preantral follicles with FSH, luteinizing hormone (LH), and prolactin receptors. Finally, stages 8-10 constitute antral follicles with the same receptors as at stages 5-7 (Greenwald, 1994). Thus, LH fails to affect maturation in the small preantral follicle. Eppig and his colleagues (1996) suggested that the antral follicle was presented upon 14 days of age in mice. Therefore, in the present study we have investigated the effects of hCG on the stimulation of immature female mice to obtain immature oocytes for the IVM/IVF study, in comparison with PMSG-primed mice. *In vivo*, the LH surge induces maturation of the oocyte within the previously inhibitory

follicular environment by stimulating oocyte maturation stimulator (OMS) or oocyte maturation inducer secretion from the cumulus cells of Graafian follicles and it is associated with a change in follicular fluid (FF) components of both steroid and nonsteroid regulators of oocyte maturation in small antral follicles (Tsafriri, 1988). These regulators include estradiol, progesterone, prolactin, inhibin, hypoxanthine (HX), cAMP, and Mullerian inhibiting substance which are called oocyte maturation inhibitors (Sato and Ishibashi, 1977 ; Tsafriri et al., 1982 ; Chari et al., 1983 ; Downs and Eppig, 1987 ; Das et al., 1992 ; Eppig et al., 1993). The OMI was detected in the FF of many mammalian species, and this inhibition by OMI was not species specific (Hillensjo et al., 1988 ; Schroeder et al., 1988). Thus, in terms of effects on oocyte maturation, the FF surrounding the mature preovulatory oocyte (high OMS concentration) has a composition very different from that of small antral follicles (high OMI concentration).

For example, OMI and HX maintain both cumulus cell - enclosed and denuded oocytes in meiotic arrest when the oocytes are incubated in culture medium containing HX at concentrations within the range estimated for FF. The concentration of HX in mouse FF has been estimated to be 2 - 4 mM. However, the concentration of HX in mouse FF does not decrease before gonadotropin - induced GVBD, therefore the mechanism for inducing GVBD *in vivo* does not involve a decrease in the concentration of this purine in FF. Accordingly, gonadotropins induce the generation of an oocyte maturation

promoting action by granulosa cells that supercedes or negates the presence of HX in the FF (Eppig and Downs, 1987).

A number of growth factors (GF), such as epidermal growth factor, insulin-like growth factor I, transforming growth factor-β, gonadotropins, and serum are present in mature FF in concentrations that stimulate oocyte maturation both *in vivo* and *in vitro* (Dekel and Sherizly, 1985 ; Feng et al., 1988 ; Vanderhyden and Armstrong, 1990 ; Das et al., 1991). These substances have been known as OMS and may act in a synergistic manner to stimulate oocyte maturation *in vivo*. Many reports have indicated high *in vitro* maturation and fertilization rates of mammalian oocytes when mature FF was used as the culture medium (Vanderhyden and Armstrong, 1990 ; Cha et al., 1991 ; Richards, 1994). However, high GF concentrations have been reported in small antral follicles that may be necessary for granulosa cell proliferation and follicular development. In these follicles, oocyte maturation is inhibited despite high concentrations of GF probably because of high concentrations of OMI. In the preovulatory follicle, due to a decrease in FF concentrations of OMI and the resulting LH activity, GF may be allowed to act on the oocyte to stimulate the onset of meiosis (Das et al., 1992 ; Harper and Brackett, 1993).

Other investigators have reported that GF and LH reverse the inhibitory effect of OMI on oocyte maturation in *in vitro* cultures. It is possible that GF at concentrations present in FF may be able to stimulate meiosis only once

concentrations of OMI are below a certain threshold level (Das et al., 1992). It may be that other than an increase in the GF receptor content of granulosa cells occuring with gonadotropin stimulation, the important event occurring after the LH surge is a decrease in FF concentrations of OMI, allowing GF to interact with its receptors to stimulate oocyte maturation (Shalgi et al., 1979 ; Shoham et al., 1993).

The oocytes at the GV stage isolated from the early antral follicles and cultured in hormone-free medium, spontaneously resume meiosis but since they are only "partially competent" to complete nuclear maturation, the processes in oocytes associated with the resumption of the first meiotic division, become arrested at MI rather than MII (Sorensen and Wassarman, 1976 ; Wickramasinghe et al., 1991 ; De Smedt et al., 1994). In contrast, GV oocytes from large antral follicles complete nuclear maturation and progress to MII *in vitro* (Eppig et al., 1994 ; Crozet et al., 1995). In addition, oocytes isolated from preantral follicles and cultured denuded of granulosa cells do not undergo significant growth, but oocytes cultured with intact gap junctions between the oocytes and the granulosa cells grow and become competent to undergo nuclear maturation (Hirao et al., 1990 ; 1994). Hence, oocyte growth, but not the acquisition of competence to resume meiosis, depends upon gap junction-mediated communication between the oocyte and its neighbouring granulosa cells. In fact, the rate of oocyte growth is directly related to the number of granulosa cells coupled to it. The granulosa cells increase the surface area of

the oocyte and thereby increase the surface to volume ratio of the oocyte. This increases the rate at which small molecules having nutritional or regulatory functions enter the oocyte (Haghighat and Van Winkle, 1990 ; Sherr, 1996). Moreover, mammalian oocytes are apparently deficient in the transport systems required for the entry of some molecules. Gap junction-mediated transport of these molecules initially taken up by granulosa cells is essential for metabolic processes crucial for oocyte growth and development. In addition to interactions that occur through gap junctions, granulosa cells appear to regulate mammalian oocyte development via paracrine signals. Denuded oocytes cocultured with somatic cells acquire competence to resume meiosis, thus showing that the role of granulosa cells in this process does not depend entirely upon gap junctional communication and appears to involve a paracrine factor (s) secreted by the somatic cells (Canipari et al., 1984 ; Chesnel et al., 1994).

Nuclear and cytoplasmic maturation of oocytes

Nuclear maturation includes the processes in oocytes associated with the resumption of the first meiotic division and the progression of meiosis to MII. Initial failures in efforts to fertilize oocytes matured *in vitro* were, however, attributed to incomplete or aberrant cytoplasmic maturation despite the apparent completion of nuclear maturation. Normal cytoplasmic maturation encompasses the processes occurring in oocytes and preparing them for fertilization,

activation, and preimplantation embryo development (Goodrowe et al., 1991 ; Eppig et al., 1996).

The broad definition of cytoplasmic maturation includes processes or events that may not coincide with nuclear maturation. For example, the synthesis of the ZP, which occurs mostly during the oocyte growth phase in preantral follicles (Bleil and Wassarman, 1980 ; Philpott et al., 1987), is essential for normal fertilization, and thus the broad definition of cytoplasmic maturation could include the synthesis and secretion of the ZP. It would be very difficult to establish exactly when, during oocyte growth or nuclear maturation, some processes of cytoplasmic maturation occur unless specific molecules associated with the respective process are identified. Nevertheless, some processes of cytoplasmic maturation are coordinated with, and in some cases might actually depend upon, the initial events of nuclear maturation (Fujiwara et al., 1993).

It is clear that the completion of nuclear maturation is not necessary for the completion of many aspects of cytoplasmic maturation. It does not assure the completion of normal cytoplasmic maturation. Oocytes from small antral follicles that complete nuclear maturation, as evident by progression to MII and the emission of the first polar body, are rarely competent to develop to the blastocyst stage. In contrast, oocytes from large antral follicles that complete nuclear maturation are often competent to develop to the blastocyst stage

(Eppig et al., 1994 ; Crozet et al., 1995). Thus, oocytes at the GV stage undergo further differentiation during the time of development from small to large antral follicles that directly relates to oocyte competence to complete preimplantation development (Eppig et al., 1994 ; Blondin and Sirard, 1995 ; Crozet et al., 1995). The resolution of the specific molecular events of this differentiation of oocytes, and how they are regulated, will be a major contribution of oocyte culture technology to the understanding of the mechanisms governing early mammalian embryogenesis.

In vitro oocyte maturation

Many reports have suggested that the acquisition of competence to undergo nuclear maturation by oocytes *in vitro* does not require gonadotropin stimulation (Daniel et al., 1989 ; Hirao et al., 1993). Accordingly, oocytes developing in hypophysectomized animals or in mutant hypogonadal mice that have undetectable levels of circulating gonadotropins are able to grow, and many of them are competent to resume meiosis (Bar-Ami and Tsafriri, 1986 ; Halpin et al., 1986 ; Schroeder and Eppig, 1989). Nevertheless, a higher percentage of oocytes becomes competent to resume meiosis when they are cultured in the presence of gonadotropins or when hypophysectomized or hypogonadal mice are treated with exogenous gonadotropins (Schroeder and Eppig, 1989 ; Carroll et al., 1991a,b ; Eppig, 1992 ; Wang, 1993). Thus,

gonadotropins are clearly beneficial for the acquisition of competence to resume meiosis. In addition, several reports have indicated that gonadotropin supplementation in maturation medium improves the fertilization rate and the extent of embryonic development in *in vitro* matured oocytes (Shalgi et al, 1979 ; Schroeder et al, 1988 ; Sirard et al., 1988 ; Brackett et al, 1989 ; Buccione et al, 1990 ; Jinno et al, 1990 ; Saeki et al., 1991 ; Eppig et al, 1992), though others have not detected a beneficial effect of gonadotropin supplementation (Goodrowe et al, 1991 ; Allworth and Albertini, 1993 ; Yang et al., 1993 ; Trounson et al, 1994). Thus, the extent to which gonadotropins directly affect meiotic cell cycle progression as well as the cellular basis for gonadotropin-enhanced maturation in mammalian oocytes remains to be established.

Systems supporting oocyte growth and development *in vitro* are valuable experimental tools for studying the mechanisms regulating oogenesis and oocyte maturation (Eppig et al., 1992). Studies on *in vitro* maturation of mouse oocytes have identified the PMSG as the hormone various concentrations of which, either 5, 7.5, or 10 IU, can be used for superovulation (Downs et al., 1986 ; Downs et al., 1988 ; Das et al., 1992 ; Chesnel et al., 1994 ; Goren et al., 1994). Thus, this study has been designed to investigate the best concentration of either PMSG or hCG for the stimulation of immature female mice to obtain immature oocytes for IVM/IVF studies in our laboratory. In addition, a wide variety of maturation media have been used for IVM studies, such as M199 medium, Menezo's B2 and B3 medium, Waymouth's medium, Eagle's

minimum essential medium, Eagle's salts medium, and Ham's F-10 medium. These media were supplemented with many substances and oocyte maturation inducer (s), such as gonadotropins, growth factors, serum, insulin, pyruvate, and antibiotics. Immature oocytes were cultured in these maturation media under a humidified atmosphere of 5% CO_2 in air at 37°C for various durations, such as 24 hours with mouse and 34-38 hours with human oocytes (Eppig and Downs, 1987 ; Downs et al., 1988 ; Das et al., 1992 ; Barnes et al., 1995 ; Goud et al., 1998). However, the oocyte maturation duration of 24 hours for IVM in mice has shown a high complete nuclear maturation rate of *in vitro* matured oocytes but the cytoplasmic maturation is supposed to be incomplete for the fertilization process. Therefore, this study has investigated the effects of oocyte maturation duration on nuclear and cytoplasmic maturation, including *in vitro* oocyte maturation, zona thickness, zona hardness, and intraoocyte cAMP level, in relation to their fertilizing potential and preimplantation embryo development in the mouse model. In addition, successfully *in vitro* matured oocytes can be used for coculture of certain cell types, for example Vero cells (monkey kidney epithelial cells), bovine oviduct epithelial cells, and human Fallopian tube epithelial cells. However, it is most common to use either M199, Menezo's B2 medium or bovine oviduct epithelial cells for IVM (Trounson et al., 1994 ; Janssenswillen et al., 1995).

Many investigators have suggested that oocytes can mature *in vitro* from the GV to the MII stage of about 70-80% in maturation medium, and show 15-

20% of spontaneous maturation in humans and several mammalian species. However, the rate of fertilization with both IVF and/or ICSI of oocytes matured *in vitro* is lower than that of oocytes matured *in vivo*. The oocyte maturation *in vitro* can lead to a premature discharge of cortical granule contents and thereby to zona hardening. This result points at a reduced incidence of fertilization due to inhibited sperm penetration (Downs et al., 1988 ; Ducibella et al., 1990 ; DeMeestere et al., 1997 ; Hinsch et al., 1997). The zona hardening is caused by a precocious release of some oocyte cortical granules (e.g., peroxidase) during maturation and a resultant proteolytic cleavage of one of the ZP glycoproteins, ZP2, to form ZP2 fragment (ZP2f) , identified by electrophoretic analysis, which remain joined by intramolecular disulfide bonds (Moller and Wassarman, 1989 ; Ducibella et al., 1990 ; Schiewe et al., 1995). This change in the structure of ZP2 occurs normally during fertilization and is thought to constitute a prevention of polyspermic fertilization. Some cortical granules are released during oocyte maturation both *in vivo* and *in vitro*, but, when this prefertilization release occurs *in vitro* in the absence of serum, it suppresses fertilization (Ducibella et al., 1990). Serum and FF prevent ZP2 to ZP2f conversion and ZP hardening during oocyte maturation *in vitro*. Fetuin is a glycoprotein component of serum that prevents conversion of ZP2 during oocyte maturation (Schroeder et al., 1990 ; Eppig et al., 1992 ; Eppig et al., 1993 ; George et al., 1993). Many substances within serum are also present in FF, and this is consistent with the finding that FF prevents ZP hardening in culture (see Table 1.2) (Downs et al., 1986).

Table 1.2 Mammalian follicular fluid components

Plasma proteins

Steroid-binding protein

Platelet-activating factor

Follistatin

Growth factors

insulin-like growth factor- I, epidermal growth factor, and transforming growth factor -β

Cholesterol

Enzymes

side-chain cleavage enzyme, 3β-hydroxysteroid dehydrogenase, 17β-hydroxysteroid dehydrogenase, 17α-hydroxylase, aroma - tase, and plasminogen (proteases)

Micropolysaccharides (proteoglycans)

hyaluronic acid, heparin sulfate, and glycosaminoglycans

Steroids

estrogens, androgens, and progestins

Pituitary hormones

FSH, LH, prolactin, oxytoxin, and vasopressin

Nonsteroidal ovarian factors

inhibin, OMI, and OMS

Vitamins

β-carotene and vitamin E

(From : Rouderush et al., 1995)

In previous studies, oocytes having hardened zona pellucidae had very low frequencies of cleavage to the two-cell stage that was mostly due to the decreased ability of sperm to penetrate the ZP and fertilize the oocytes (Downs et al., 1986 ; Schroeder et al., 1990). Therefore, cleavage to the two-cell stage in this study is essentially an indicator for successful sperm penetration and fertilization. In addition, subsequent to oocyte maturation *in vitro* in the absence of cumulus cells and / or serum, decreased fertilizability was associated with an increased ZP digestion time by α-chymotrypsin, which is an index of ZP hardening and reflects the potential penetrability of the ZP by sperm (Downs et al., 1986 ; Schroeder et al., 1990 ; Eppig et al., 1992).

Enzymatic digestion of the ZP has been used in the past to indentify the presence of various substrates and to elucidate the chemical composition of the ZP before and after fertilization. However, conflicting results have occasionally been obtained regarding the potency of different enzymes to digest the ZP, suggesting differences in the purity and properties of individual enzyme preparations. Nevertheless, more recent reports have shown α-chymotrypsin to be effective and applicable as a bioassay for zona hardness (Downs et al., 1986 ; Johnson et al., 1988 ; Matson et al., 1997). The present study has used α-chymotrypsin digestion of the ZP to help determine changes taking place after oocyte maturation *in vitro*, with the aim of formulating and justifying treatment strategies to be introduced in the human IVM/IVF programme.

In addition, the recent development of novel techniques for the culture of primordial follicles or ovarian oocytes raises the possibility of growing human oocytes to maturity *in vitro*. It could be used to test the effect of toxic substances on oocyte maturation. Furthermore, perhaps such a system could one day provide a source of human oocytes for IVF. If this could be achieved, patients would be able to donate large numbers of oocytes, which could be stored by cryopreservation (Carroll and Gosden, 1993), thus avoiding the need for repeated superovulation and allowing oocytes to be obtained from patients unable to be stimulated into Graafian follicles, and hence oocyte production. Oocytes could then be returned directly by autografting or grown and fertilized *in vitro*, after the patient's recovery.

Primordial follicle is the earliest form of ovarian follicle and is composed of a primordial, or quiescent, oocyte surrounded by a single layer of flattened somatic cells that are presumably the progenitors of follicular granulosa cells. The oocytes of primordial follicles, hereafter referred to as primordial oocytes, are arrested in the prophase of meiosis I and have not entered the phase of dramatic growth typifying oocytes in primary (preantral) follicles (Eppig, 1991 ; Roy and Treacy, 1993 ; Spears, 1994). There are thousands of primordial follicles present in the ovaries of neonatal mammals. Primordial follicles can be considered the storage form of the ovarian follicles, though many of them undergo degeneration. In fact, very few primordial follicles ever develop to the Graafian follicle stage, where they can ovulate and

provide an oocyte for fertilization. Thus, the primordial follicles are a large and potentially valuable source of oocytes that could be used for clinical, agricultural, and zoological purposes. Indeed, it has already been shown that primordial follicles harvested by enzymatic digestion can restore fertility when transplanted within plasma clots to the ovarian bursa of sterilized humans and / or animals (Eppig and O'Brien, 1996).

The *in vitro* development of oocytes from the primordial stage to mature oocytes has been achieved for the first time. This was accomplished using a two-step system. The first step was to use an ovarian organ culture for the initial processes of preantral follicle development and oocyte growth. In the second step in this system, oocyte-granulosa cell complexes were isolated from the organ culture and cultured further to complete oocyte development. The development of the oocytes with this two-step culture system is described in terms of both oocyte growth and oocyte competence to undergo maturation, fertilization, and embryogenesis (Eppig and O'Brien, 1996).

Cyclic AMP and oocyte maturation

Mammalian oocytes grow while arrested in the first meiotic prophase. Cyclic AMP has been proposed to be involved in mediating the maintenance of meiotic arrest of mammalian oocytes and that a drop in oocyte cAMP

concentration results in resumption of meiosis. The decrease in oocyte cAMP during the period of commitment to resume meiosis has been proposed to result in dephosphorylation of an inactive phosphoprotein, converting it to an active form that promotes GVBD (Schultz et al., 1983 ; Cicirelli and Smith, 1985). This decrease in cAMP is believed to lead to a decline in the activity of cAMP-dependent protein kinase A (PKA), and this in turn dephosphorylates a putative maturation-inhibiting phosphoprotein (Smith, 1989) (Figure 1.5). This hypothesis is supported by reports that (1) there is a moderate decrease in the intracellular concentration of cAMP in the oocyte upon progesterone exposure (Maller et al., 1979 ; Cork et al., 1990) ; (2) microinjection of the catalytic subunit of PKA inhibits progesterone-induced maturation, whereas injection of the regulatory subunits of PKA induces maturation directly in the absence of progesterone (Maller and Krebs, 1977) ; (3) microinjection of an inhibitor of PKA causes maturation (Sadler and Maller, 1981 ; Daar et al., 1993 ; Matten et al., 1994) ; (4) injection of an antibody against the α-subunit of the G-protein, Gs, induces maturation (Gallo et al., 1995). These results indicate that a decrease in PKA activity is both necessary and sufficient to induce oocyte maturation. In addition, lower levels of the intraoocyte cAMP would preferentially activate type I PKA and suppress oocyte maturation, whereas higher levels, achieved in response to the preovulatory gonadotropin surge, would activate the type II holoenzyme and lead to cumulus expansion and oocyte maturation (Downs and Hunzicker-Dunn, 1995).

maturation inducers (gonadotropins, growth factors, progesterone)

↓

immature oocytes at GV stage

↓

decrease intraoocyte cAMP level

↓

increase protein kinase A type II activity

↓

increase c-mos proto-oncogene activity

↓

increase maturation promoting factor level

↓

increase p34cdc2 kinase activity

↓

increase mitogen-activated protein kinase activity

↓

stimulate the separation of spindle fibers and chromosomes in nucleas

(meiotic resumption)

↓

decrease cyclin B protein level

↓

oocyte maturation to MII stage

Figure 1.5 Mechanism of mammalian oocyte maturation

Supplementing the culture medium with analogues of cAMP, such as dibutyryl cAMP, or cAMP phosphodiesterase inhibitors, such as 3 - isobutyl - 1 - methyl xanthine (IBMX), or agents that stimulate an increase in cAMP production, such as forskolin, cause a greater inhibition of maturation in cumulus cell - enclosed oocytes than in denuded oocytes. An explanation for this result is that the cAMP analogues stimulate the cumulus cells to suppress oocyte maturation, perhaps by generating and / or activating an OMI that is transferred to the oocytes via the gap junctions (Eppig and Downs, 1984 ; Mehlmann et al., 1996).

Results from a previous study have suggested that a maturation inhibitory factor other than cAMP and of follicle cell origin is transmitted to the oocyte via the gap junctions between the oocyte and the cumulus cells (Gilula et al., 1978). It is tempting to speculate that the putative maturation inhibitory factor may be involved in regulating oocyte cAMP levels. While LH elevates follicle cAMP which appears necessary for granulosa cell lutenization and cumulus cell mucification (Eppig, 1979), LH may also result in inactivation of the inhibitory factor. This could result in a decrease in oocyte cAMP and resumption of meiosis.

It is well established that cAMP is inhibitory to mammalian oocyte maturation. FSH produced first an inhibition of oocyte maturation, followed by a stimulation of GVBD when cumulus cell-enclosed oocytes were cultured in

the presence of dibutyl cAMP. This initial inhibitory effect of FSH on oocyte maturation might be the result of elevated intraoocyte cAMP levels and of this gonadotropin having become stimulatory when the amount of cAMP generated positive signals had reached the maturation threshold. However, FSH induced GVBD in the intact cumulus cell-enclosed oocytes, but not in the denuded oocytes. Thus, the GVBD-inducing action of FSH was mediated by the cumulus cells. Moreover, since FSH did not induce GVBD in the denuded oocytes cocultured with cumulus or granulosa cells, it appears that intimate contact of the cumulus cells with oocytes,via gap junctions, is required for the induction of GVBD (Eppig and Downs, 1987).

The objectives of the study

In the present study, the objectives are

(1) to study the effects of oocyte maturation inducers, PMSG and hCG, and oocyte maturation duration , 3-24 hours, on *in vitro* oocyte maturation, fertilization, and preimplantation embryo development,

(2) to study the relationship among intraoocyte cAMP, oocyte maturation, fertilization, preimplantation embryo development, zona thickness and zona hardness,

(3) to develop the optimal condition for the highest success rate of IVM /IVF in mice.

The hypothesis of the study

For the success of the *in vitro* oocyte maturation, fertilization, and preimplantation embryo development in mice, hCG is the better inducer than PMSG and the conventional 24-hour incubation period is not the optimal duration of the maturation process which is indicated by the level of the intraoocyte cAMP, the zona thickness and the zona hardness.