ABSTRACT

Infertility affects approximately 15% of couples globally. The most effective treatment today is in vitro fertilization. However, not all couples can be helped with the current techniques. To achieve better success, techniques of storage and in vitro maturation of ovarian follicles have to be improved. Meanwhile, understanding of the molecular mechanisms of follicle/oocyte maturation and embryo development would doubtlessly facilitate technique development. Nonetheless, little work has been done in these areas due to the limited availability of human research material. Therefore, we performed five studies on the collection, cryopreservation, culture and gene expression of human ovarian follicles/oocytes respectively.

In **Article I,** we examined if follicular aspirates obtained during oocyte retrieval for IVF were a good source of human ovarian follicles. The presence of follicles in the aspirates was examined by mechanical/enzymatic isolation, culturing and histological analysis. We found only 14 follicles in 86 aspirates. The results indicate that follicular aspirates are not a reliable source of human ovarian follicles.

In **Article II,** we evaluated whether serum-free cryoprotectants could be used for cryopreservation of human ovarian cortical tissue. Biopsies of ovarian cortical tissue donated by healthy women were frozen and thawed using two kinds of cryoprotectants containing either human serum or human serum albumin. Light microscopy, transmission electron microscopy and live/dead fluorescence assay were performed to evaluate the structure and viability of the follicles. The results showed that the majority of the follicles retained normal structure and viability after thawing. Cryoprotectants containing human serum albumin were equally effective as those containing human serum. Therefore, serum free cryoprotectants are suitable for the cryopreservation of human ovarian cortical tissue.

In **Articles III** and **Article IV,** we tested the effect of two secondary messengers, cGMP and cAMP, on human ovarian follicles cultured in ovarian cortical slices. Donated ovarian cortical biopsies from healthy women were cut into slices and cultured in parallel in the presence and absence of 8-br-cGMP or 8-br-cAMP for 1-3 weeks. Oestrdiol production, developmental stage, size and viability of the follicles were recorded. The results showed that both 8-br-cGMP and 8-br-cAMP enhance the survival and development of human early follicles cultured in ovarian cortical tissue.

In **Article V,** to expose the gene expression profile of human germinal vesicle oocytes (hGVO) and reveal different gene expression patterns between hGVO, embryonic stem cells and foreskin fibroblasts, we performed microarray (Affymetrix U133 plus 2.0) analysis and RT-PCR. In total, 11,191 unigenes were expressed in normal human GV oocytes. Forty-nine percent of these genes are as yet unclassified by biological function. A few oocyte specific genes that are obligatory for oocyte maturation/early embryo development in animals were found expressed in hGVO for the first time. Furthermore, known components of MOS-MPF, TGF-beta superfamily, and WNT pathway were identified in hGVO. Last, twelve gene expression patterns were found between hGVO, embryonic stem cells and fibroblasts, suggesting potential candidate genes involved in oocyte maturation and embryonic development.

Our findings will help improve the technique of cryopreservation and culture of human ovarian follicles. Further, our last study provides a rich source for continued research in elucidating the molecular mechanism of oocyte maturation and embryo development in humans.

CONTENTS

1 LIST OF ABBREVIATIONS

AMH	Anti-müllerian hormone, also known as müllerian inhibiting substance (MIS)
AMHRII	AMH type II receptor
bFGF	Basic fibroblast growth factor
BMP	Bone morphogenetic protein
cAMP	Cyclic adenosine 3', 5'-monophosphate
CDC 2	Cell division cycle 2, G1 to S and G2 to M
cGMP	Cyclic guanosine 3', 5'-monophosphate
CPA	Cryoprotective agent
DHT	Dihydrotestosterone
DMSO	Dimethyl sulphoxide
EG	Ethylene glycol
FIGLA	Factor in the germline alpha
FSH	Follicle stimulating hormone
GDF-9	Growth differentiation factor-9
GDF-9B	Growth differentiation factor-9b, also named Bone morphogenetic protein 15 (BMP15)
GV	Germinal vesicle
GVBD	Germinal vesicle breakdown
hESC	Human embryonic stem cells
hFIB	Human foreskin fibroblasts
hGVO	Human germinal vesicle oocytes
HSA	Human serum albumin
IGF	Insulin-like growth factors
IP3	Inositol 1,4,5-trisphosphate
ITS	Insulin/transferrin/selenium
IVF	In vitro fertilization
KL	Kit ligand, also termed stem cell factor-SCF, mast cell factor or steel factor
LH	Luteinising hormone

LIF	Leukemia inhibitory factor
MAPK	Mitogen-activated protein kinase
MATER	Maternal antigen that embryos require
MOS	v-mos Moloney murine sarcoma viral oncogene homolog , also referred as cytostatic factor (CSF)
MPF	M-phase promoting factor or maturation promoting factor
MYT 1	Myelin transcription factor 1
MZT	Maternal embryonic transition
MII	Metaphase II
NAC	N-acetylcysteine
Nobox	Newborn ovary homeobox, also named Og2
NPM 2	Nucleoplasmin 2
p90rsk	Ribosomal S6 protein kinase
PKA	Protein kinase A
Plk 1	Polo-like kinase (Drosophila) 1
PrOH	1,2 propanediol/propylene glycol
SPY1	Speedy
TEM	Transmission electron microscopy
TGF	Transforming growth factor
XIAP	X-linked inhibitor of apoptosis protein
ZAR1	Zygote arrest 1
ZFP	Zinc finger protein
ZGA	Zygotic gene activation
ZP1, 2 and 3	Zona pellucida glycoprotein 1, 2 and 3

2 INTRODUCTION

The ovarian follicle is the basic functional unit of reproduction in the woman. It is located in the outer part of the ovary called the ovarian cortex. The follicle contains an oocyte, surrounded by granulosa cells and outer layers of thecal cells. Follicles develop through primordial, primary, secondary and preantral stages before acquiring an antral cavity. At the antral stage, most follicles undergo atretic degeneration, whereas a few of them, under the cyclic gonadotrophin stimulation that occurs after puberty, reach the preovulatory stage. In response to preovulatory gonadotrophin surges during each reproductive cycle, the dominant preovulatory follicle ovulates to release the mature oocyte for fertilization (Gougeon, 1996; McGee, EA *et al.*, 2000) (**Fig. 1**). In addition to providing mature oocytes, the ovarian follicles also contribute to the production of hormones and growth factors that are essential for initiating and maintaining female sexual characteristics and early embryonic development.

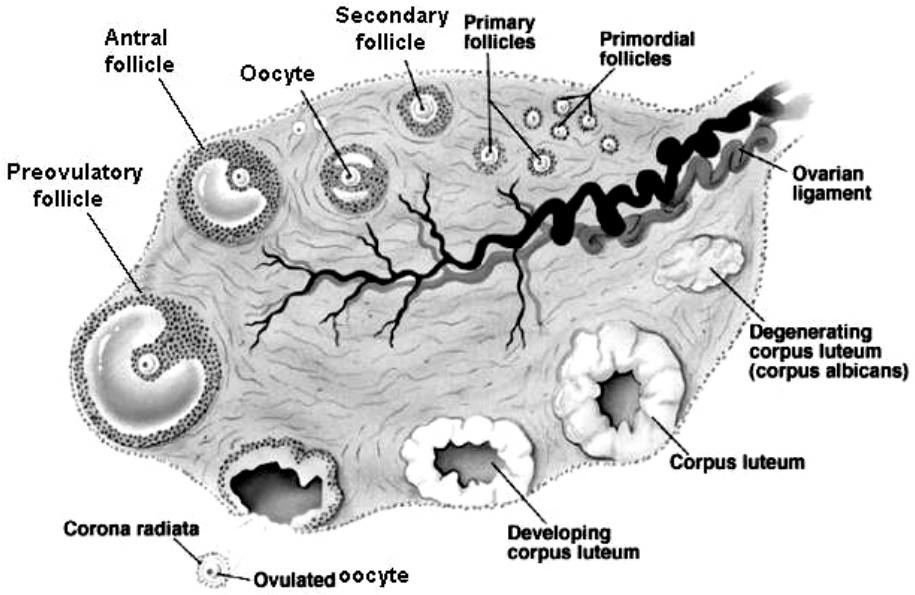

Figure 1. Location and development of ovarian follicles. Modified from "Structure of an ovary" produced by the McGraw-Hill Companies, Inc.

It has been believed for 50 years that a fixed reserve of ovarian follicles is endowed at birth in most mammals. However, a recent study suggests that germline stem cells exist in the postnatal mouse ovary and generate 77 new primordial follicles per ovary per day (Johnson *et al.*, 2004). Even with this new information, the number of ovarian follicles decreases with time, but the rate of decrease is slower according to the new discovery. In humans, the pool of follicles is approximately 7 million in fetal ovaries at 20 weeks gestation and the number decreases to 1 - 2 million at birth. The number steadily declines with age and there are approximately 400,000 follicles remaining at the onset of puberty. From the age of approximately 37 - 40 years, the follicle number declines abruptly and results in 1,000 follicles left at menopause (Faddy, 2000). The follicles are depleted from the ovary by repeated cycles of recruitment, atresia or ovulation. This depletion process is irreversible and is more rapid in women who undergo cytotoxic treatment or have genetic disorders.

Women lose their ovarian function and become infertile when the follicle stock is lower than 1,000. To date there are no treatments available to stop the follicle lose. However, ovarian function can be preserved by cryopreservation of ovarian follicles in cortical tissue (Hovatta, 2000). In cases where in vivo maturation is not possible, culturing of follicles in tissue slices could mature oocytes from cryopreserved tissue. A combination of cryopreservation and tissue culture could therefore solve infertility problems in many patients. However, to date only one livebirth is achieved from cryopreserved ovarian tissue (Donnez *et al.*, 2004) while complete in vitro development of primordial follicles into healthy ovulatory follicles has never been achieved in humans. Techniques of storage and in vitro maturation of ovarian follicles need to be optimized. Meanwhile, understanding of the molecular mechanisms of follicle/oocyte maturation and embryo development would doubtlessly facilitate the technique development. Nonetheless, little work has been done in these areas due to the limited availability of human research

material. Therefore, we performed five studies on the collection, cryopreservation, culture and gene expression of human ovarian follicles/oocytes respectively. The following sections will give a brief review on these four respects.

2.1 RESEARCH SOURCE OF HUMAN OVARIAN FOLLICLES AND OOCYTES

The lack of ovarian follicles/oocytes is always a problem in the research field of human reproduction.

Ovarian cortical tissue could be one source. First, adult ovarian tissue can be obtained from individuals undergoing sterilization or caesarean section (Hovatta *et al.*, 1997), infertile women at diagnostic laparoscopies, or patients having an ovaryectomy due to an ovarian benign tumor. Second, fetal ovaries from abortion is another potential sources (Zhang, J *et al.*, 1995). In addition, ovarian tissue can also be obtained from patients undergoing gender reassignment (Van Den Broecke *et al.*, 2001). Although there are a number of ways to get ovarian tissue, the chance is rare and there is no guarantee that an ovarian biopsy will contain follicles. Moreover, some samples have limited application due to special treatment before the tissue is taken. Therefore, ovarian cortical tissue is not a sufficient source to fulfill the needs in reproduction research.

It has been shown that follicular aspirates obtained from in vitro fertilization (IVF) patients at oocyte retrieval may be a good source of immature follicles. Wu and coworkers obtained approximately 52 follicles per patient from this source (Wu, J *et al.*, 1998). We studied if this is repeatable, and we found only 14 follicles in total from 86 patients, 0.16 follicle per patient (Zhang, P *et al.*, 2002). At the same time, similar result is obtained by another group: they found 0.26 follicle per patient from follicular aspirates (Moskovtsev *et al.*, 2002).

It has been suggested that ovarian surface epithelium contains germline stem cells in postnatal mouse and these germline stem cells are able to generate new primordial follicles (Johnson *et al.*, 2004). This is, however, an unlike source of human follicles and oocytes.

Recently, primordial germ cells and male gametes have been derived from mouse embryonic stem cells (Geijsen *et al.*, 2004). Success in the generation of male gametes

suggests a possible way to produce follicles and oocytes. If follicles and oocytes could be cultured from human embryonic stem cells, it could become a research source.

2.2 CRYOPRESERVATION OF HUMAN OVARIAN TISSUE

2.2.1 Indications and significance

Incidence of cancer among females increased continuously by 0.4% per year (Jemal *et al.*, 2004). Approximately 8% of female cancer cases occur in women under 40 years of age (Oktay, KH *et al.*, 2002). As the survival rate increases (Jemal *et al.*, 2004), more and more young cancer females are facing infertility, as a consequence of anti-cancer treatment. Therefore, fertility preservation should be considered seriously before the treatment starts, although not all anti-cancer therapy is associated with infertility. (Sonmezer *et al.*, 2004). Currently, preservation of fertility can be achieved by three options: cryopreservation of embryos, cryopreservation of oocytes and cryopreservation of ovarian cortical tissue.

Cryopreservation of embryos is well established and commonly used in assisted reproduction centers worldwide. Survival rates per thawed embryo range between 35% - 90%, implantation rates between 8% - 30%, and cumulative pregnancy rates can be greater than 60% (Sonmezer *et al.*, 2004). Children (up to 18 months) born from cryopreserved embryos do not differ from those born after spontaneous pregnancies in growth and health (Wennerholm *et al.*, 1998). However, embryo cryopreservation is not applicable for female cancer patients who have no partners or do not have enough time for IVF stimulation prior to treatment.

Oocyte cryopreservation bypasses the legal and ethical debates associated with embryo cryopreservation. It is suitable for single women and also appropriate for couples that have no sperm available on the day of oocyte retrieval. Although the pregnancy rate is increasing with time, limited success is demonstrated by the small number of live births (Sonmezer *et al.*, 2004). In addition, oocyte cryopreservation also requires IVF

stimulation cycles, so patients who need immediate anti-cancer treatment cannot wait for this. Cryopreservation of immature oocytes does not require IVF stimulation, but the procedure is still in the experimental stages (Paynter, 2000; Wu, J *et al.*, 2001).

Cryopreservation of ovarian tissue eliminates many disadvantages related with cryopreservation of embryos and oocytes. It is feasible in almost all cancer cases and it is the only option for pre-pubertal patients. Most follicles in ovarian tissue are at primordial stage. Primordial follicles have several characteristics that make them less vulnerable to cryodamage, such as small size (contain less water), low metabolic rate, less differentiated, lack of zona pellucida and metaphase spindle (Shaw *et al.*, 2000; Gosden *et al.*, 2002). Another advantage of primordial follicles is that they have more time to repair sub-lethal damage to organelles and other structures during their prolonged growth phase (Picton, HM *et al.*, 2000). Owing to the robust nature and abundant number of the primordial follicles in ovarian tissue, together with other advantages over the storage of embryos and oocytes, cryopreservation of ovarian tissue appears to be the most attractive strategy to preserve ovarian function for the time being. Banking of ovarian tissue benefits not only female cancer patients but also women who have a family history of premature ovarian failure (Hovatta, 2000). In the latter cases, ovarian tissue can be banked beforehand and used when the remaining ovary stops functioning.

2.2.2 Current achievements

Cryopreserved ovarian cortical tissue can be utilized in two ways (**Fig. 2**): grafting (transplantation) or in vitro culture. Human ovarian tissue can be transplanted to human (autografting) or non-human species (xenografting). In the case of autografting, grafting ovarian tissue to original ovary site is referred as orthotopic grafting, to non-ovary site is referred as heterotopic grafting.

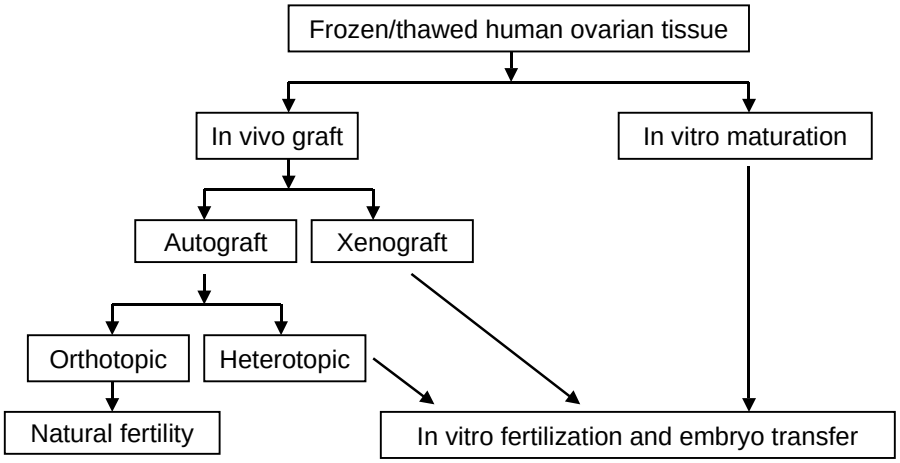

Figure 2 Current utilization of cryopreserved human ovarian tissue (Modified from Newton, 1998)

Orthotopic grafting after cryopreservation has been studied. In earlier reports, restoration of ovarian function after orthotopic grafting is indicated by follicle enlargement (up to 17-20 mm), ovulation, resumption of menstruation and secretion of estradiol and progesterone. Grafted ovarian tissue has been shown to begin functioning within 3 - 7months after grafting and has continued to function for 2 - 7months. The functioning duration depends on the quality and quantity of grafted ovarian tissue and the grafting technique (Oktay, K *et al.*, 2000; Callejo *et al.*, 2001; Radford *et al.*, 2001). Recently, the first live birth using this technique has been reported (Donnez *et al.*, 2004). In this report, cryopreserved ovarian tissue was orthotopicaly transplanted into a 32 year-old woman who suffered Hodgkin's lymphoma and had her ovarian tissue banked 7 years earlier, before the treatment started. Five months after the transplantation, the regular ovulatory cycle recovered, and 6 months later the woman got pregnant spontaneously. A healthy girl was born after full-term gestation (Donnez *et al.*, 2004).

To eliminate the complex procedures of anesthesia and surgery that are obligatory in orthotopic transplantation, **heterotopic transplantations** have been tested. Frozen/

thawed ovarian tissue was transplanted underneath lower abdominal skin. In addition to follicle development and oestrogen production, 20 oocytes were retrieved percutaneously. Of these 20 oocytes, eight were suitable for in-vitro fertilization and one fertilized normally and developed into a 4-cell embryo. No pregnancy occurred after transfer of the embryo (Oktay, K *et al.*, 2004). The same group also transplanted fresh ovarian tissue slices to forearm subcutaneously. Restoration of ovarian function is indicated by resumption of menstruation, decreased FSH and LH levels, elevated estradiol level, and development of follicles. Ovulation occured spontaneously or after hormone stimulation. The grafts were active for three years (Oktay, K *et al.*, 2001). Heterotopic transplantation is easy to perform and convenient for monitoring follicular development and retrieving oocytes. Thus, it is a good alternative to autografting. Although live birth has not been achieved by this technique, the restoring of ovulatory cycles is already attractive enough, as patients can avoid hormonal replacement therapy by utilizing their own ovarian tissue.

Xenograftings has been a method used to study the function of the cryopreserved tissue. Xenografting is usually performed on immunodeficient mice, under the kidney capsule or subcutaneously (Oktay, K *et al.*, 2000; Gook *et al.*, 2001; Van den Broecke *et al.*, 2001; Abir *et al.*, 2003; Gook *et al.*, 2003). Follicles can grow to antral stages, up to 6 mm in diameter. Ovulation and corpus luteum formation have been observed occasionally (Kim *et al.*, 2002). The most promising result has been reported by Gook et al. They observed 14 MII oocytes in total after transplanting human cryopreserved ovarian tissue to immunodeficient mice (Gook *et al.*, 2003; Gook *et al.*, 2005). Application of xenografting in clinical use is under debate, concerning possible genetic mutations and transmission of animal pathogens to humans (Kim *et al.*, 2002). Nevertheless, xenografting is valuable in assessment of the post-thaw developmental potential of stored ovarian tissue (Gook *et al.*, 2005).

A potential risk of re-transmission of malignant cells to the recipient limits the application of autografting. This risk can be avoided by maturation of follicles in vitro. Without all the disadvantages of in vivo procedures, in vitro culturing would be a safe

and simple way to utilize banked ovarian tissue. Frozen-thawed human ovarian tissue has been cultured up to 15 days, and secondary follicles have been observed (Hovatta *et al.*, 1997). Optimal culture techniques are under development (see details in section 2.3).

2.2.3 Current methods

Cells undergoing cryopreservation are liable to be damaged mainly due to the formation of intracellular ice crystals. Protection from damage during freezing and thawing can be achieved by using cryoprotective agents. Cryoprotective agents (CPAs) create osmotic gradients and assist the cell in dehydration during freezing. Moreover, permeable CPAs can lower the freezing points of the extracellular solution, allowing cells enough time to become dehydrated before the temperature reach the freezing point of the cytoplasm. Therefore, the main function of CPAs is to avoid intracellular ice formation, the main cause of cryoinjury. Cryoprotective agents are also proposed to participate in the membrane modifications during freezing (from a relatively fluid state to a rigid state) (Rall *et al.*, 1984). Human ovarian tissue frozen- thawed without CPA was composed of only traces of fibrous tissue (Newton *et al.*, 1996).

For cryopreservation of human ovarian tissue, three permeable CPAs have been tested: dimethyl sulphoxide (DMSO), 1,2 propanediol/propylene glycol (PrOH) and ethylene glycol (EG). No significant difference has been found between DMSO and PrOH regarding morphology of the ovarian cells after cryopreservation (Hovatta *et al.*, 1996; Newton *et al.*, 1996). The largest success to date with DMSO procedure is the live birth after orthotopic transplantation of cryopreserved ovarian tissue (Donnez *et al.*, 2004), while the best achievement with PrOH is the growth of 17 mm follicle and stimulated ovulation following autografting of cryopreserved ovarian tissue (Oktay, K *et al.*, 2000). Ethylene glycol is more commonly used in vitrification than in slow freezing/rapid thawing of ovarian tissue (Newton *et al.*, 1998). Limited data leave the application of EG in cryopreservation of human ovarian tissue in question.

In addition to permeable CPAs, non-permeable additives such as serum, albumin and sucrose are also used in cryopreservation of ovarian tissue. They act as osmotic buffers against cell shrinking and swelling during freezing and thawing (Picton, HM *et al.*, 2000). The concentration of sucrose is usually 0.1-0.2 M while serum and albumin are used in a 'random' manner (See Table I in article II.). We showed that cryoprotectant solutions containing either serum or human serum albumin (HSA) are equally efficient in cryopreservation of human ovarian cortical tissue (Hreinsson, J *et al.*, 2003). Human serum is usually not subject to good quality control as commercially available human serum albumin, thus the albumin is recommended over serum in clinical work.

Slow freezing /rapid thawing is the most widely used procedure for cryopreservation of human ovarian cortical tissue. Ovarian tissue is cooled slowly in 2 - 3 hours from room temperature or 4ºC to –196 ºC using a programmable freezer and thawed rapidly (1 minute) from –196 ºC to room temperature, followed by a warm bath for 2 - 5 min. Before the start of freezing, ovarian tissue is first equilibrated in cryoprotectant solution, allowing the cryoprotectant to penetrate the cells. For DMSO, the optimal equilibration temperature and time was 4ºC for 30min (Newton *et al.*, 1998). PrOH penetrates the tissue slowly at 4ºC while higher temperatures enhance the toxicity. PrOH has been used at room temperature, and the equilibration time varies between 15 - 90 min according to different experiences. Both DMSO and PrOH have been applied at the concentration of 1.5 M. **Table 1** demonstrates the standard protocol and basic principles of slow freezing/rapid thawing.

Table1. Standard protocol and basic principles of slow freezing/rapid thawing

Procedure	Principle
Stepwise equilibration: 1.5 M DMSO, 4ºC, 30 min or 1.5 M PrOH, room temperature, 15 - 90min	Water moves out of the cell due to osmotic gradient
From 0ºC or room temperature to -7ºC, -2ºC/min	Equilibration completed at the end of this step, no osmotic gradient between cytoplasm and extracellular solution, no water movement
Hold at -7ºC for 10 min, seeding manually	Induce ice crystal formation in the extracellular solution
From -7ºC to -40ºC, -0.3ºC/min	As the ice crystal gets bigger, the non-frozen part is concentrated and the osmotic pressure increases in extracellular solution. Water moves out of the cell. At the end of this step or earlier, all available water moves out of the cell and the cell is completely "dry". At the same time, cytoplasm gets frozen as the temperature reaches the freezing point of the cytoplasm.
From -40ºC to -140ºC, -30ºC/min	Necessary? Some studies skip this step.
From -140ºC to liquid nitrogen (-196ºC)	Storage
Start thawing, from liquid nitrogen (-196ºC) to room temperature, around 1min	Avoid intracellular ice formation
Warm water bath, 22ºC to 37ºC, 2-3 min	Necessary? Some studies skip this step.
Stepwise washing, progressive dilution of cryoprotectants (1.0M, 0.5M, 0M)	Gradually rehydration of the cell, reduce cell swell and avoid cell burst. At the end of this step, no cryoprotectant is left in the cell.
Post-thawing culture	May help cell recover to normal condition

Vitrification is another processes of cryopreservation using high concentration of cryoprotectant to solidify the cell in a glass state without formation of intracellular ice. Cells are cooled rapidly by direct immersion in liquid nitrogen. EG and DMSO have been

studied as cryoprotectants in vitrification of human ovarian tissue. Necrotic areas were always seen in the thawed tissue (Isachenko *et al.*, 2003; Rahimi *et al.*, 2003; Rahimi *et al.*, 2004). In addition to the high cytotoxic potential due to the high concentration of cryoprotectant, limited data on vitrification of human ovarian tissue make it impossible to draw any conclusion at the moment.

2.2.4 Perspective

Cryopreservation of human ovarian cortical tissue is a complex procedure, requiring preservation of multiple cell types that vary in volume and membrane permeability. Although a live birth has been achieved, cryopreservation still needs improvement.

An optimal freezing protocol should bear the best balance between the dehydration rate and the cooling rate. That is, as soon as the cells are completely dehydrated, the temperature decreases to the freezing point of the cytoplasm. Based on this consideration, the slow freezing /rapid thawing can be optimized at: the thickness of the tissue slice, the duration and temperature of the equilibration (for PrOH), the seeding temperature, and the cooling rate between -7ºC and -30ºC.

Ischaemic injury to the ovarian grafts occurs after transplantation. Therefore, improving the revascularization of the grafted tissue might benefit the utilization of frozen/thawed ovarian tissue. Study on sheep suggests that microvascular anastomosis of the ovarian vasculature after intact ovary cryopreservation could restore the vascular supply immediately and therefore minimize post-transplantation ischaemic follicular loss (Bedaiwy *et al.*, 2003). In fact, cryopreservation of intact human ovary has been tried recently. In that report, follicles, stromal cells and small vessels survived well with a considerable survival rate of follicles (75% after thawing *vs.* 99% before freezing) (Martinez-Madrid *et al.*, 2004). The result is promising considering the feasibility of revascularization in transplantation. This would, however, require the removal of a whole ovary and would only be indicated before high dose chemotherapy.

2.3 CULTURING OF OVARIAN CORTICAL TISSUE

2.3.1 Significance of human ovarian cortical tissue culture

Obtaining human mature oocytes from primordial follicles via complete in vitro culture can be achieved by two steps as in mice (Eppig *et al.*, 1996; O'Brien *et al.*, 2003). The first step is to mature primordial follicles to the preantral or antral stage, and the second step is to mature oocytes in oocyte-granulosa complexes that are isolated from preantral or antral follicles.

Culturing ovarian cortical tissue is the only option to mature in vitro early stage (primordial, primary and secondary) follicles in humans. Culturing of isolated early stage follicle has been attempted but all ended with failure. Isolated follicles degenerate after 24 hours of culture and the whole life span of these follicles never exceed 48 hours (Abir *et al.*, 1999). The poor survival of follicles can be due to the breakdown of the basement membrane and other intrafollicular components during enzymatic and/or mechanical isolation (Hovatta *et al.*, 1999; Gosden *et al.*, 2002). In contrast, culturing follicles in ovarian cortical tissue slices maintains normal oocyte-granulosa-theca-stroma interactions and has achieved promising results. Follicles within the tissue slices can grow to secondary and sometimes small antral stages after 14 - 28 days in culture (Hovatta *et al.*, 1997; Hovatta *et al.*, 1999; Wright *et al.*, 1999; Louhio *et al.*, 2000; Hreinsson, JG *et al.*, 2002).

Currently, culturing of human ovarian cortical tissue is not optimal enough evidenced by the rare occurrence of small antral follicles in culture. Investigating the regulation of follicle development by testing different growths factors and hormones will aid the improvement of the techniques. If ovarian tissue culture succeeds, the potential clinical benefits are profound. This will not only provide an additional option of utilizing cryopreserved ovarian tissue but will also benefit IVF patients, allowing them to avoid hormone stimulation.

2.3.2 Factors studied in tissue culture

Early follicular development consists of two phases: the first is the recruitment of primordial follicles from quiescence into the growing pool, and the second is the proliferation and differentiation of granulosa cells and thecal cells accompanied by the growth of the oocytes. The first phase mainly depends on locally produced factors (autocrine or paracrine) while the second phase starts response to extraovarian (endocrine) factors. A wealth of information is available on the roles of growths factors and hormones in early follicle development. Here we focus on several well-known factors that have been tested in ovarian tissue culture.

Growth differentiation factor-9 (GDF-9)

GDF-9 is a member of TGFβ (transforming growth factor β) superfamily. It is produced predominantly by oocytes and crucial throughout folliculogenesis (Mazerbourg *et al.*, 2003). In humans, both GDF-9 mRNA and protein are abundantly expressed in oocytes of primary follicles (Aaltonen *et al.*, 1999). Human ovarian tissue culture shows that GDF-9 recruits more primordial follicles into the growing pool, promotes the growth of activated follicle, and enhances the survival of the follicle during 14 days of culture (Hreinsson, JG *et al.*, 2002). Studies on animal also confirm the importance of GDF-9 in early follicular development. GDF-9 is expressed in rodent oocytes in follicles from early primary stage and onwards (McGrath *et al.*, 1995; Jaatinen *et al.*, 1999). An in vivo study shows that GDF-9 treatment led to a decreased number of primordial follicles and an increased number of primary and preantral follicles, indicating the enhanced initial activation of primordial follicles in rats (Vitt *et al.*, 2000). Female GDF-9 deficient mice were infertile and folliculogenesis was blocked at the primary stage (Dong *et al.*, 1996; Carabatsos *et al.*, 1998). In sheep and bovines, GDF-9 mRNA has been found in the oocytes of follicles at all stages of development, including primordial follicles (Bodensteiner *et al.*, 1999). Sheep with inactive GDF-9 only have a few follicles passed the primary stage (Juengel *et al.*, 2002).

Follicle stimulating hormone (FSH)

In addition to its well-known roles in the development and survival of preantral and antral follicle, FSH is also involved in earlier follicular development. FSH receptors are expressed in granulosa cells of the primary follicle and onward (Oktay, K *et al.*, 1997). Women with FSH receptor mutation are infertile due to impaired follicle development beyond primary stage (Aittomaki *et al.*, 1996). Human ovarian tissue culture shows that FSH increase the diameters of the follicles and improves the survival of follicles (Wright *et al.*, 1999). Human ovarian follicles growing in recipient mice after xenografting also require FSH to proceed to the early secondary stage (Oktay, K *et al.*, 1998). FSH and its receptor act through the adenylate cyclase pathway. Two secondary messengers of FSH, cGMP (cyclic guanosine 3', 5'-monophosphate), and cAMP (cyclic adenosine 3', 5'-monophosphate), are shown to be able to enhance the development and survival of the early stage follicles during 14 - 21 day cultures of human ovarian tissue (Scott *et al.*, 2004; Zhang, P *et al.*, 2004). Taken together, these data show that FSH seems not to affect initial recruitment of primordial follicles but does promote the growth and survival of activated follicles.

Kit ligand (KL) and c-kit

The interactions between c-kit and its ligand KL (Kit ligand, also termed stem cell factor-SCF, mast cell factor or steel factor) are essential for the initial recruitment of primordial follicles, follicular development until antral stage, oocyte maturation, and protection of preantral follicles from apoptosis (Driancourt *et al.*, 2000). Both in mice and sheep, KL is expressed in granulosa cells of primordial, preantral and antral follicles while c-kit is produced in all oocytes of follicles from primordial to antral stage (Driancourt *et al.*, 2000). During ovarian organ culture in rat, KL promotes the initial recruitment of primordial follicles while c-kit antibody completely blocks the activation of primordial follicles (Parrott *et al.*, 1999). Similarly, injection of c-kit antibody into mice results in disturbed onset of primordial follicle development and impaired growth of primary and preantral follicles (Yoshida *et al.*, 1997). In c-kit receptor knockout mice, follicle growth arrests at the primary stage (but initiation of primordial follicles appears not to be affected) (Kuroda *et al.*, 1988; Huang *et al.*, 1993). Mutation in KL gene causes sterility in female mice by affecting the initiation and maintenance of ovarian follicle

development (Bedell *et al.*, 1995). The effect of KL/c-kit on human early follicles has not been reported.

Anti-müllerian hormone (AMH)

AMH is also known as müllerian inhibiting substance (MIS) due to its repressive effect on the müllerian duct during male fetal development. AMH is a member of the TGFβ superfamily and it plays an important role in the regulation of ovarian follicle growth. In both the mouse and rat ovary, AMH and its type II receptor (AMHRII) are expressed in granulosa cells follicles from primary to small antral stage (Baarends *et al.*, 1995). AMHRII may be expressed also in the granulosa cells of primordial follicles, as expression of AMHRII is found during the fetal period and remains present after birth when the ovary mainly contains primordial follicles (Durlinger *et al.*, 2002). A similar expression pattern is observed in humans: granulosa cells of primordial follicles do not express AMH, whereas granulosa cells of most primary follicles showed at least a weak signal in the granulosa cells. The highest level of AMH expression is present in the granulosa cells of secondary, preantral and small antral follicles (Weenen *et al.*, 2004). AMH inhibits initial recruitment of primordial follicles in mice evidenced by the studies using AMH null mice or ovary organ culture (Durlinger *et al.*, 1999; Durlinger *et al.*, 2002). In agreement with these data, our group has found that AMH at certain concentration inhibits the activation of human primordial follicles in tissue culture (Carlsson et al, unpublished data). AMH also inhibits follicular growth by decreasing the sensitivity of ovarian follicles to FSH. In AMH-deficient mice, more follicles start to grow under the influence of exogenous FSH than in their wild-type littermates (Durlinger *et al.*, 2001).

Basic fibroblast growth factor (Basic FGF)

Basic FGF has broad function in cell proliferation, differentiation and angiogenesis. In both the rat and bovine ovary, it is produced by oocytes of primordial and primary follicles and granulosa cells of antral follicle (van Wezel *et al.*, 1995; Nilsson, E *et al.*, 2001). Basic FGF induces the recruitment of primordial follicles during tissue culture in rat (Nilsson, E *et al.*, 2001; Nilsson, EE *et al.*, 2004). In the human ovary, bFGF mRNA

are detected in follicles from primordial to secondary stage while granulosa cells of preantral and antral follicles contains bFGF protein (Yamamoto *et al.*, 1997; Quennell *et al.*, 2004). The location of bFGF in the human ovary suggests the potential role of bFGF in follicular development.

Other factors

Insulin and IGF I and II (insulin-like growth factors I and II) have been shown to enhance the development and survival of early ovarian follicular development in human ovarian tissue culture (Louhio *et al.*, 2000). During organ culture of rat ovary, both LIF (leukemia inhibitory factor) and BMP-4 (bone morphogenetic protein 4) facilitate the transition from primordial to primary follicles (Nilsson, EE *et al.*, 2002; Nilsson, EE *et al.*, 2003). In addition, BMP-4 also suppresses the cellular apoptosis in the culture. In mouse ovary cultures, BMP-7 (bone morphogenetic protein 7) promotes the primordial-primary follicle transition and increases the expression of FSH receptor as well (Lee *et al.*, 2004).

2.3.3 Survival of ovarian cells in long-term culture

Survival of ovarian cells is a problem in long-term ovarian tissue culture. Ovarian cells begin to degenerate within 24 hours of culture. By the end of 21 days of culture, the proportion of healthy tissue is only 30% and the proportion of viable follicles decreased to 40% from almost 100% at the beginning of the culture (Otala *et al.*, 2002).

Under physiological conditions, ovarian cells die through apoptosis. In adult human ovaries, apoptosis is detected in oocytes and granulosa cells of follicles from the primordial to pre-ovulatory stage (Tilly *et al.*, 1991; Mikkelsen *et al.*, 2001; Depalo *et al.*, 2003). Apoptosis also seems responsible for the cell death in ovarian tissue culture as suggested by the morphological criteria, detection of nuclear DNA fragmentation and activated caspase-3 in the ovarian cells (Otala *et al.*, 2002). Therefore, in principle, any inhibitor of apoptosis would promote the survival of the ovarian cells in vitro.

Besides the survival factors mentioned above, such as FSH, GDF, cGMP, cAMP and BMP-4, dihydrotestosterone (**DHT**) is another promising candidate. DHT suppresses

stromal cell apoptosis in human ovarian tissue culture. This suppressive effect is blocked by the antagonist of androgen receptor, suggesting that DHT acts through androgen receptors that are localized in stromal cells and granulosa cells of primordial, primary and secondary follicles (Otala *et al.*, 2004). In addition to its role as a survival enhancer, DHT also stimulates the initial recruitment and sustained follicular growth in monkey ovaries (Vendola, KA *et al.*, 1998; Vendola, K *et al.*, 1999). A similar effect is observed in culturing of isolated mouse antral follicles: follicles develop faster in the presence of DHT and grow slower in the anti-androgen treatment (Murray *et al.*, 1998).

X-linked inhibitor of apoptosis protein (**XIAP**) can be another candidate for improving the survival of ovarian cells in tissue culture. XIAP is a powerful intrinsic inhibitor of cell death and it blocks apoptosis via various pathways. It has been observed that over expression of XIAP protects cells from various apoptotic triggers, including ultraviolet irradiation, radio irradiation and chemotherapy drugs (Holcik *et al.*, 2001). XIAP has been shown to be able to block the apoptosis of rat ovarian follicles (Wang, Y *et al.*, 2003) and ovarian cell lines (Sauerwald *et al.*, 2002) in culture. XIAP also mediates the anti-apoptosis effect of FSH (Wang, Y *et al.*, 2003).

Oxidative stress serves as a trigger for apoptosis. The generation of oxidative free radical occurs in all cells as a consequence of normal cellular metabolism. Excessive production of free oxygen radicals results in oxidative stress, apoptosis or necrosis (Fridovich, 1986; Yu, 1994). The survival of ovarian cells could be impaired by oxidative stress, as oxygen concentration is much higher in culture than the physiological level in vivo. It can be hypothesized that antioxidant would have the potential to improve cell survival. This hypothesis is verified by several studies. N-acetylcysteine **(NAC)**, a free radical scavenger, reduces cell death in cultured human ovarian tissue (Otala *et al.*, 2002). **Ascorbic acid,** another free radical scavenger, decreases apoptosis in serum-free culturing of isolated preantral and antral follicles from mice (Murray *et al.*, 2001), rats (Tilly *et al.*, 1995) and bovine (Thomas *et al.*, 2001). Ascorbic acid is also found to be able to promote basement membrane integrity of isolated preantral follicles in culture both in mice (Murray *et al.*, 2001) and bovine (Thomas *et al.*, 2001). Two antioxidant

enzymes, including **superoxide dismutase** and **catalase**, also inhibit the apoptosis of isolated rat antral follicles when they are supplied in culture medium. The production of intrinsic antioxidant enzymes can be induced by FSH (Tilly *et al.*, 1995).

Culture medium significantly influences the viability of ovarian cells in tissue culture. In the culture medium that is used to achieve live births from mouse primordial follicles, fetuin is applied in all serum-free medium (O'Brien *et al.*, 2003). **Fetuin** is a glycoprotein first detected in fetal bovine serum and later shown to be a homologue of a human plasma protein: α_2HS glycoprotein (Brown *et al.*, 1992). Fetuin is a major component of bovine fetal serum and comprises approximately 45% of the total serum proteins. It is synthesized in liver and degraded through lysosomal pathway. In vitro, fetuin is prepared from bovine serum and is soluble in water. Fetuin has been shown to promote the attachment, growth, and differentiation of various types of cells in serum-free culture systems, although it is not clear if the effects reside in fetuin itself or other contaminant(s) in fetuin preparation (Nie, 1992). Although it has been reported that follicles in tissue cultured for 10 days in serum-free medium are significantly larger and less atretic than those cultured with serum alone (Wright *et al.*, 1999), more supplements to serum-free medium, besides FSH, HSA and ITS (insulin/transferrin/selenium), may benefit long-term culture. Fetuin could be one of the supplemental candidates for human ovarian tissue culture, considering the success in mice.

Deficient diffusion of nutrients and metabolites could be another cause that hinders the cell survival in tissue culture. This is supported by the observations that almost all the well-developed follicles, such as large secondary or small antral follicles, are located at the peripheral sites of the tissue slice. It has been shown that ovarian follicles cultured in tissue cubes grow and survive better than those cultured in tissue slices, possibly because the larger surface area to volume ratio of the cubes facilitates substance transfer (Scott *et al.*, 2004).

2.3.4 Perspective

Culturing of human ovarian cortical tissue is still in its infant stage. In addition to testing different factors that are necessary for early follicular development, improvement of ovarian cell survival also requires more work.

The localization of growth factors, hormones and their receptors in the human ovary can provide good candidates that might benefit ovarian tissue culture. Lessons can be taken from other species. However, species difference should always be kept in mind, especially regarding the dense fibrous nature of the human ovarian tissue, which hinders the exchange of the metabolites between cells and culture medium. In this sense, ovarian tissue from domestic animals would be a good model to investigate, as the ovarian tissue has a similar structure to the human ovary.

2.4 MOLECULAR MECHANISMS OF OOCYTE MATURATION AND MZT

"Oogenesis is the foundation of embryogenesis" (Gosden, 2002). Maternal products that accumulate during oocyte maturation direct and support the early embryonic development, especially before maternal embryonic transition (MZT) is completed. The developmental potential of an embryo is decided by the developmental potential of the oocyte that is acquired during oocyte maturation. Oocyte maturation consists of a long phase of cytoplasmic maturation and a short dramatic nuclear maturation at the end. This part of the review will outline the current understanding of the molecular mechanism of oocyte maturation and MZT, with emphasis on nuclear maturation of the oocyte.

2.4.1 Cytoplasmic maturation of the oocyte

In mammals, the oocytes in the primordial follicles can arrest at the prophase stage of first meiosis for weeks (as in mice) to decades (as in humans). Very little is known about the transcriptional activity in these non-growing oocytes. It is generally thought that these oocytes are transcriptionally quiescent. However, one study reveals that 95 genes are highly expressed in the monkey primordial oocytes (Arraztoa *et al.*, 2004), suggesting

possible transcription activity in the primordial oocytes. When follicular growth initiates, the oocytes inside the follicles begin growing as well. During oocyte growth, the oocyte diameter expands from 35 μm to 120 μm and the volume of the oocyte increases 100-fold (Gougeon, 1996; Picton, H *et al.*, 1998). It has been estimated that a human oocyte takes approximately 200 days to grow to its full size (Gougeon, 1986). Oocyte RNA and proteins accumulate in both cytoplasm and nucleus with time. The RNA and protein synthesis are high at the early and middle growth phases, and then become reduced during the late growth period of the oocyte.

Total RNA synthesis ceases when the oocyte reaches full size. A fully-grown GV oocyte contains 0.6 ng of total RNA in mice (Sternlicht *et al.*, 1981), and 2.0 ng of total RNA in the human (Neilson *et al.*, 2000). Eight percent of total RNA in oocytes is mRNA, which is rather more than that in somatic cells (Gosden, 2002). The fate of RNA transcribed during oocyte growth can differ considerably. Much of the RNA is degraded during meiotic maturation of the oocyte. Mouse MII oocytes contain 19% less total RNA than fully-grown germinal vesicle (GV) oocytes (Bachvarova, R *et al.*, 1985). A similar decline pattern is observed in human oocytes as well: MII oocytes contain approximately 40% less mRNA than fully-grown GV oocytes (Dobson *et al.*, 2004). Some mRNA is immediately translated, and others are temporally stored and recruited for translation at a defined period of oogenesis or embryogenesis. The mRNA transcripts with long poly-A tails of ~ 150A residues are for immediate use, whereas the mRNAs with shorter poly-A tails of < 90A constitute a storage form of RNA to be used only following elongation of the poly-A tail (Bachvarova, RF, 1992).

The nucleolus enlarges while the oocyte grows. Nuclear proteins, such as fibrillarin, nucleophosmin, uncleolin, RNA polymerase I, and nucleolar upstream binding factor, are synthesized during this time. In addition to the accumulation of RNAs and proteins, the oocyte also acquires polarization (Edwards, 2003), genomic imprinting (Lucifero *et al.*, 2004) and multiplying of cytoplasmic organelles during its growth.

The capacity of the oocyte to mature and constitute a high quality embryo after fertilization is finely controlled by a timed and spatially programmed gene expression. Notably, several oocyte-specific genes have been found to play crucial roles in oocyte development (well reviewed by Henneold and Rajkovic) (Rajkovic *et al.*, 2002; Hennebold, 2004). They are *Gdf9*, *Gdf 9B*, *Zp1, Zp2, Zp3* (zona pellucida glycoproteins), *Figla* (factor in the germline alpha), *Connexin 37* (Amleh *et al.*, 2002), *Nobox* (newborn ovary homeobox, also named Og2) (Rajkovic *et al.*, 2004), *Zfp 393* (zinc finger protein 393) (Yan *et al.*, 2002), and *ePab* (embryonic polyA-binding protein) (Seli *et al.*, 2005).

2.4.2 First meiosis: from GV to MII

A fully-grown GV oocyte stores all transcripts and proteins that are necessary for the first zygotic cell divisions and later zygotic genome activation (Henery *et al.*, 1995; Nothias *et al.*, 1995). Most transcription stops and translation of mRNA are reduced in the GV oocyte (Bachvarova, RF, 1992). The silence remains through oocyte meiotic resumption, fertilization and the first zygotic cell cycles. Despite the fully matured cytoplasm, the nucleus in the GV oocyte remains immature.

Resumption and completion of the first meiosis occurs by the time of ovulation, shortly after the LH surge. The LH surge triggers a decline of the intra-oocyte concentration of cAMP, leading to the activation of *MPF* (M-phase promoting factor or maturation promoting factor). The activated *MPF* precedes GVBD, therefore the GV oocyte completes first meiosis and matures to MII (metaphase II).

Cyclic AMP, produced by the granulosa cells and transported via gap junctions (composed of *connexin 37*) into the oocytes, has an important role in maintaining the meiotic arrest of the oocyte at the GV stage prior to ovulation (Tornell *et al.*, 1993; Downs, 1995). LH surge triggers the decline of the intra-oocyte cAMP via two ways: disruption of the gap junction between the oocyte and the granulosa cells (Heikinheimo *et al.*, 1998); and decreasing cAMP production in granulosa cells by I*nsl3-Lgr8* system (I*nsl3*: Leydig insulin-like 3; *Lgr8*: leucine-rich repeat-containing G protein-coupled receptor 8) (Kawamura *et al.*, 2004). Activation of *Pde3A* (phosphodiesterase 3A) in the

oocyte may also account for the cAMP decline (Conti *et al.*, 1998; Richard *et al.*, 2001), because oocytes arrest at the GV stage in *Pde3A* deficient mice (Masciarelli *et al.*, 2004). It has been shown that cAMP-dependent protein kinase A (*PKA*) results in phosphorylation of the $p34^{cdc2}$ subunit of *MPF* and therefore maintains *MPF* inactivated (Rime *et al.*, 1992). Associated with this function, cAMP-*PKA* also inhibit the activity of *c-Mos*, a *MPF* activator (Lazar *et al.*, 2002). Therefore, when the cAMP level decreases, the inhibition is removed and *MPF* becomes activated.

MPF is a cell cycle modulator comprised of two subunits, $p34^{cdc2}$ (cell division cycle 2, G1 to S and G2 to M) serine/threonine kinase and *cyclin B1*, and is responsible for inducing spindle assembly, chromatin condensation and nuclear envelope breakdown (Dunphy *et al.*, 1988; Gautier *et al.*, 1988; Pines *et al.*, 1989; Gautier *et al.*, 1990; Moreno *et al.*, 1990). *MPF* activity is low in GV oocytes. It increases during the first meiosis and reaches its first peak at metaphase I, and then the activity decreases progressively, retaining a low level. *MPF* activity re-increases at the onset of the second meiosis with the second peak at metaphase II. The high activity maintains until fertilization (Fulka *et al.*, 1992; Wu, B *et al.*, 1997). It is proposed that the decline of *MPF* activity during the transition between the two meiotic divisions is necessary for the extrusion of the first polar body (Ledan *et al.*, 2001).

MPF is regulated by several molecules and remains inactive via phosphorylation of $p34^{cdc2}$. Despite cAMP mentioned above, *Wee1* and *Mik1* kinases are two other phosphorylation inhibitors (Lundgren *et al.*, 1991; McGowan *et al.*, 1993). *MPF* becomes active when the $p34^{cdc2}$ subunit is dephosphorylated. Cdc25-phosphatase activates MPF (Gautier *et al.*, 1991; Millar *et al.*, 1992), likely though *Plk1* (polo-like kinase 1) (Anger *et al.*, 2004). In turn, activated *MPF* can enhance the activation of cdc25-phosphatase (Galaktionov *et al.*, 1991; Hoffmann *et al.*, 1993), forming a auto-amplification loop between cdc25-phosphatase and *MPF*. *C-Mos* kinase, also referred as cytostatic factor (*CSF*), is another enhancer of *MPF* activity. Besides *MAPK*, the most common pathway to activate *MPF*, *Mos* also activates *MPF* via *myt1* in xenopus oocytes (Peter *et al.*, 2002). Two TIS11 zinc finger-containing proteins, *Oma1* and *Oma2*, act upstream of

myt1 in c. elegans oocyte meiosis (Detwiler *et al.*, 2001), but the relationship between *mos* and *oma* is not yet known. In addition, Mos can inhibit degradation (proteolysis) of cyclin-B (O'Keefe *et al.*, 1991). Again, activated MPF induces c-Mos stability, forming another positive loop. *JNK, PI-3K* (Mood *et al.*, 2004) and *XGef* (Reverte *et al.*, 2003) may be involved in this loop as well. Ras activates MPF via either raf1-MAPK pathway (Lu *et al.*, 1995) or rasGAP (Ras-GTPase-activating protein) (Pomerance *et al.*, 1996). In addition, speedy (*spy1*) (Lenormand *et al.*, 1999) and *leptin* (Craig *et al.*, 2004) also active *MPF* via *MAPK*. Ribosomal S6 protein kinase (*p90rsk*) is proposed to be a downstream factor of *MAPK* (Bhatt *et al.*, 1999; Gross *et al.*, 1999). The degradation of *MPF* is mediated by *APC* (anaphase-promoting complex)-ubiquitin-proteasome pathway (Huo *et al.*, 2004).

Some targets of *MPF* are known. They are thought to be histon H1, nuclear laminins, pp60c-src kinase and RNA polymerase II (Heikinheimo *et al.*, 1998).

The *mos-MAPK-MPF* pathway is questioned by several experiments. These data suggest a reversed sequential order: *MPF-mos-MAPK*, as elevated *MPF* activity is observed prior to *MAPK* activation (Verlhac *et al.*, 1994; Zernicka-Goetz *et al.*, 1997) and *c-mos* action is inhibited in oocytes with low *MPF* activity (Josefsberg *et al.*, 2003; Lazar *et al.*, 2004). Taken together with other information, instead of concluding that "*MPF* governs *mos-MAPK*", these data are more consistent with the existence of *mos* and/or *MAPK* independent pathways and a positive feed back loop between *c-mos* and *MPF*. **Figure 3** Illustrates the activation (+) and inhibition (-) of Mos-MPF pathway.

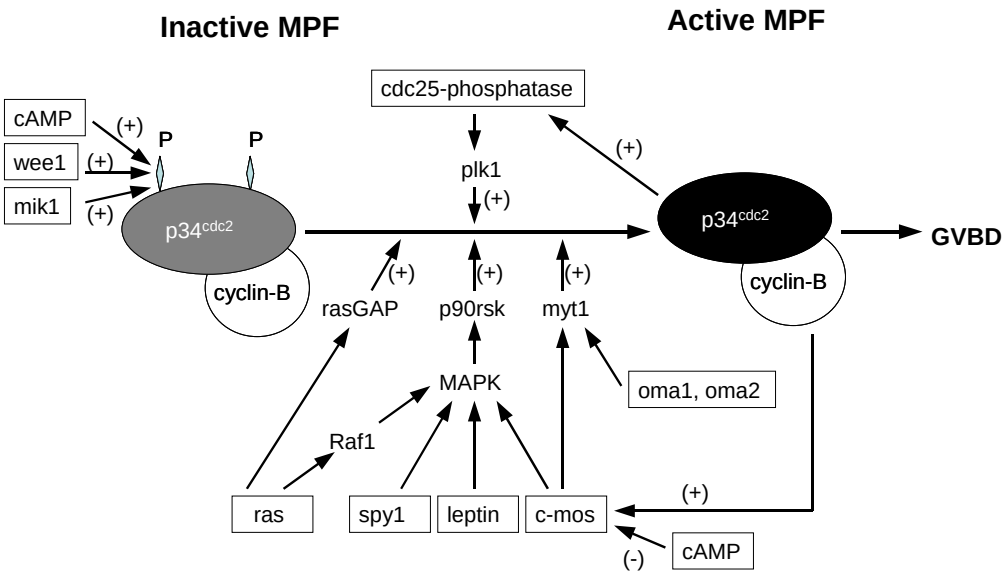

Figure 3. Activation (+) and inhibition (-) of Mos-MPF pathway

The activation of *mos-MAPK-MPF* pathway is not only crucial for the completion of the first meiotic division, but also important for maintaining oocytes at MII stage (the second meiotic metaphase arrest) before fertilization. Parthenogenetic activation of unfertilized oocytes is observed in *c-mos* deficient mice due to failed MII arrest (Colledge *et al.*, 1994; Hashimoto, N *et al.*, 1994).

More information regarding *MPF* independent factors is emerging. A recent study suggest that phosphoinositide 3-kinase (*PI3*), *jnk* (jun N-terminal kinases) and a*urora-A* are likely to be involved in the regulation of bovine oocyte maturation, independent of *MPF* (Vigneron *et al.*, 2004). *Aurora-A* regulates mouse oocyte meiosis probably via regulation spindle organization (Yao *et al.*, 2004). *Mad2* may also regulate spindle organization in rats and mice (Zhang, D *et al.*, 2004; Zhang, D *et al.*, 2005). *Brca2* is another candidate regulator in oocyte meiosis, although the underlying mechanism is not known (Sharan *et al.*, 2004).

2.4.3 Second meiosis and fertilization: from MII to zygote

Fertilization triggers completion of oocyte meiosis and formation of a one- cell embryo containing a haploid maternal pronucleus derived from the oocyte and a haploid paternal pronucleus derived from sperm.

At fertilization, sperm penetrates the cumulus cell layer, zona pellucida, and oocyte plasma membrane and the whole sperm enters the oocyte. This process is known as sperm-oocyte fusion. A protein within sperm called "sperm factor", most likely being oscillin, activates oocyte *Plc* (phosphoinositide-specific phospholipase C). The oocyte *Plc* catalyses the hydrolysis of PIP_2 to form IP_3 (inositol 1,4,5-trisphosphate) and *Dag* (sn-1, 2 diacylglycerol). Binding of IP_3 to IP_3R which is located in the smooth endoplasmic reticuli (SER, the major stores of Ca^+ in the oocyte) triggers the release of Ca^+ from the SER. Calcium, together with other modulators of IP_3R, enhance further calcium release from the SER, resulting in repetitive oscillations in Ca^+ levels in the oocyte (Wilding *et al.*, 1997). The precise frequency, duration and amplitude of calcium oscillations induce successful oocyte activation (Machaca, 2004). The Ca^{2+} oscillation has been characterized in human oocytes following in vitro fertilization or ICSI (Taylor, 1994; Tesarik *et al.*, 1994).

Elevations in intracellular calcium cause the extocytosis of cortical granules that are located in the cortical region of the oocyte below the plasma membrane (Abbott *et al.*, 2001). These granules contain enzymes that are released into the perivitelline space, resulting in modification of zone pellucida to prevent additional sperm from binding and penetrating the *ZP* (Bleil *et al.*, 1981). The Ca^{2+} oscillation is also needed for the resumption of the second meiosis. In xenopus oocytes, the Ca^{2+} oscillation activates proteolytic enzymes, which can degrade *c-Mos* and *cyclic B*, resulting in the inactivation of *MPF*, and therefore the completion of the second meiosis (Lorca *et al.*, 1993; Ito *et al.*, 2004). The oocyte extrudes the second polar body and a zygote with two pronuclei is formed.

The downstream molecules that are regulated by calcium oscillation are not yet well defined in mammals. There is evidence that *calmodulin* (a calcium-binding protein), *CaMKII* (calmodulin-dependent protein kinase II) (Lorca *et al.*, 1993), *Calpain* (a calcium-dependent cysteine protease), *PKC*, *calcineurin* (also named calcium/calmodulin-dependent serin/threonine protein phosphatase 2B) and *Src* family kinases (Talmor-Cohen *et al.*, 2004) are likely to be involved in centrosome duplication, cortical granule exocytosis, cytoskeletal rearrangements and resumption of the second meiosis. *Dag* is also shown to induce calcium oscillations, cortical granule exocytosis and *Zp* modification in mouse oocytes (Cuthbertson *et al.*, 1985; Endo *et al.*, 1987), although the underlying mechanism is not yet known.

2.4.4 Maternal to zygote transition (MZT)

MZT is characterized by the activation of zygote genome (or ZGA, zygotic gene activation) and the replacement of maternal mRNA with embryonic mRNA. During MZT, the majority of maternal mRNAs are depleted (DeRenzo *et al.*, 2004; Dobson *et al.*, 2004). Remaining maternal mRNAs, recruited by cytoplasmic polyadenylation in a stage-specific manner (Richter, 1999), together with stored maternal proteins are responsible for ZGA.

Although some zygote transcription activity has been noticed shortly after fertilization (Ao *et al.*, 1994; Daniels *et al.*, 1995; Fiddler *et al.*, 1995; Ma *et al.*, 2001), the major MZT occurs later on. The time point of MZT varies in different species. In mice, it is 2-cell stage, while it is 4-cell stage in pigs, 4-8 cell stage in humans, and 8-16 cell stage in cattle and sheep (Telford *et al.*, 1990). Embryos that fail to activate their own genome fail to develop further. Most of the embryo developmental block happens at the MZT stage (Memili *et al.*, 2000; Meirelles *et al.*, 2004).

ZGA proceeds in a stepwise manner with specific genes activated at specific developmental stages. Nuclear and cytoplasmic signals, mainly being maternal products, interact with one another to regulate ZGA. Multiple mechanisms have been identified in the regulation of ZGA (Memili *et al.*, 2000; Latham *et al.*, 2001). First of all, it has been

shown that levels and acetylation state of histones are related to ZGA. In newly formed zygote, enriched histones repress transcription by rendering DNA inaccessible to transcription factors. However, when zygotic genes are becoming active, the pool of histones is diluted, allowing the initiation of transcription (Prioleau *et al.*, 1994). Acetylated histones facilitate DNA replication and genome activation while diacetylated histones may contribute to establish and/or maintain transcriptional repression (Turner, 1991). Secondly, DNA replication is required to reprogram DNA molecules that are assembled into either a repressed or activated state (Nothias *et al.*, 1995). The conflicting effects of DNA replication on ZGA may be due to either inhibition or facilitation of the access of transcription factors to their target DNA sequences (Davis *et al.*, 1996; Aoki *et al.*, 1997; Memili *et al.*, 1999). Thirdly, chromatin structure also plays a role in ZGA, because paternal pronuclei have higher transcription level than maternal pronuclei (Aoki *et al.*, 1997). Furthermore, the status of the transcription factors obviously affects ZGA. Candidate transcription factors are *TBP* (TATA binding protein) (Majumder *et al.*, 1994), RNA polymerase II, *Tef1* (transcriptional enhancer factor 1) (Xiao *et al.*, 1991), *Sp1, Cbp, Maid, B-myb, Max,* DNA methyltransferase 1, HP-1 like chromobox protein M31 and *mTead2* (Latham *et al.*, 2001). Moreover, as cell cycle lengths are short before ZGA but become longer at the onset of ZGA, it is proposed that ZGA is related to the mechanism that controls cell cycle. Rapid cell divisions are suppressive for transcription (Memili *et al.*, 2000). Finally, post–translational modification of transcriptional machinery may also account for ZGA.

Studies on mice reveal a handful of oocyte-specific genes that play crucial roles in MZT, although the down-stream targets of these genes are, as yet, unclear. *Zar1* (Zygote arrest 1) null females are infertile, as most embryos from these mice arrest at the one-cell stage and no embryos develop to the four-cell stage. In the arrested embryos, maternal and paternal genomes do not unite (Wu, X *et al.*, 2003). *Mater* (Maternal antigen that embryos require) null females are sterile as their embryos arrest at the two-cell stage due to reduced embryonic transcription (Tong *et al.*, 2000). *Npm2* (Nucleoplasmin 2) knockout females have fertility defects owing to abnormal nuclear and nucleolar organization both in oocytes and embryos (Burns *et al.*, 2003). *Zp2* and *Zp3* knockout

females are infertile due to defects of zona pellucida. Their embryos cannot develop beyond the two-cell stage (Liu *et al.*, 1996; Rankin, T *et al.*, 1996; Rankin, TL *et al.*, 2001). Embryos from female mice lacking the gene encoding *Hsf1* (heat-shock factor-1) are unable to develop properly beyond the zygotic stage (Christians *et al.*, 2000). *Spindlin* participates MZT as it is associated with the first meiotic spindle formation (Oh *et al.*, 1997).

In conclusion, the molecular mechanisms underlying oocyte maturation and MZT are very complicated and involve multiple levels of gene regulation both temporally and spatially. Different signaling pathways interact with one another, forming a formidably dense network. Although much effort has been put into this research field, the comprehensive picture of the molecular mechanism is still far from clear. Information in the human is more limited due to the poor availability of research material. As genes expressed in the oocyte dominate the whole process from oocyte maturation to early embryo development, investigating oocyte-specific genes will doubtlessly help exploring the underlying molecular mechanisms.

3 AIMS OF THE STUDIES

1. To examine if follicular aspirates obtained during oocyte retrieval for IVF were a good source of human ovarian follicles.

2. To evaluate whether serum-free cryoprotectant solutions could be used in cryopreservation of human ovarian cortical tissue.

3. To test the effect of 8-br-cGMP and 8-br-cAMP on human ovarian follicles cultured in ovarian cortical slices.

4. To expose the gene expression profile of human GV oocytes and reveal different gene expression patterns between human GV oocytes, embryonic stem cells and fibroblasts.

4 MATERIALS AND METHODS

Human materials were used in all five studies. Ethical approval has been obtained from the Ethics Committee of the Karolinska Institutet and the Ethics Committee of Helsinki University. **Table 2** and **Table 3** summarize the materials and methods used in the five articles presented. The details are described in the individual articles.

Table 2. Information of samples used in the five studies

Sample name	Sample number	Sample source	Age of the patients (Mean ± SD)	Article location
Follicular aspirates	86	IVF patients undergoing oocyte retrieval	33 ± 4 years (Range 22 - 39)	I
Biopsies of ovarian cortical tissue	23	9 women undergoing sterilization and 14 undergoing caesarean section	34 ± 4 years (Range 28 - 39)	II
Biopsies of ovarian cortical tissue	27	9 women undergoing sterilization and 18 undergoing caesarean section	32 ± 5 years (Range 21 - 41)	III
Biopsies of ovarian cortical tissue	16	Women undergoing gynaecological laparoscopy	32 ± 4 years (Range 25 - 43)	IV
GV oocytes	76	55 women undergoing ICSI	35 ± 2 years (Range 25 - 39)	V

Table 3. Methods used in the five studies

Method	Assessment	Article location
Culture of granulosa cells	Check if follicles exist in the granulosa cell masses obtained from follicular aspirates	I
Culture of individual follicles	Test the survival capacity of individual follicles	I
Histological analysis	Identify the origin of a tissue piece found in follicular aspirates	I
	Study the viability, developmental stage, size and density of the follicles/oocytes	II, III, IV
Cryopreservation of human ovarian tissue	Compare the efficiency of human serum and human serum albumin in cryopreservation	II
Transmission electron microscopy (TEM)	Compare the ultrastructure of follicles and stroma cells before and after cryopreservation	II
Live/dead assay	Evaluate the viability of follicles/oocytes before and after cryopreservation	II
Culture of human ovarian tissue	Test the effects of cGMP and cAMP on early follicular development	III, IV
Hormone assay (radioimmunoassay)	Measure the production of oestradiol in the culture medium	IV
RNA isolation and cDNA amplification	Prepare cDNA for microarray assay from a small number of cells	V
Oligonucleotide microarray (Affymetrix)	Compare transcriptomes between human GV oocytes and embryonic stem cells	V
RT-PCR	Confirm the expression of four genes in human GV oocytes	V
Statistic analysis	Chi-square test, Fisher's exact test, Student's t-test and Mann-Whitney U-test are used where applicable	I, II, III, IV

5 RESULTS AND DISSCUSSION

5.1 ARTICLE I, COLLECTION OF FOLLICLES FROM FOLLICULAR ASPIRATES

In article I, we examined if follicular aspirates obtained during oocyte retrieval for IVF were a good source of ovarian follicles. The result is negative. Only a few follicles were found in the aspirates. Follicles were only obtained from 7 of the 86 aspirates. From these 7 samples a total of 14 follicles were found. Twelve follicles were primordial or primary, and two were secondary, 40-80 μm in diameter.

The small harvest of follicles from aspirates in our study is consistent with the results of another study (Moskovtsev *et al.*, 2002). However, Wu et al (Wu, J *et al.*, 1998) found many follicles in follicular aspirates. **Table 4** shows the results of follicle collection from follicular aspirates in these studies.

Table 4. Follicle collection from follicular aspirates in different reports

	Patient age (years)	Patient number	Volume of aspirates	Times of punctures per ovary	Total follicles collected	Follicle collected per patient
Our study, 2002	Mean 33 Range 22 - 39	86	Mean 27 ml bloodless aspirates per patient	1-2	14	Mean 0.2, range 0-5
Moskovtsev et al, 2002	Mean 34 Range: NP	54	All aspirates from each patient	1-3	48	Mean 0.9, range 0-10
Wu et al, 1998	Mean: NP Range 29-39	16	NP	NP	800	Mean 52, range 20-150

NP = no information provided in the paper

The collection methods described in the three studies appear to be similar to each other. The large variation in the number of follicles harvested from aspirates between different studies may be due to different oocyte retrieval procedures. As all primordial and primary follicles are located within 2 mm of the surface of ovarian cortex (Lass *et al.*, 1997), it is possible that small follicles are cut and mixed in follicular aspirates when the aspirate needle passes through the surface of the ovary or moves from one antral follicle to another. However, the chance of this is very low. In practice, the needle seldom punctures the ovarian surface more than once. Therefore, follicles are rarely present in follicular aspirates.

Follicular aspirates contain large numbers of granulosa cells, which can aggregate and look like a follicle. Pieces of tissue and squamous cells from the vaginal wall can also exist in aspirates and sometimes resemble follicles. In our study, a small piece of vaginal epithelium found in an aspirate resembled a cluster of follicles under the inverted microscope. After fixation and haematoxylin and eosin staining, these "follicles" were clearly vaginal epithelium as revealed by light microscopy (**Fig. 4**). Hence, distinguishing analysis is necessary when identifying follicles from follicular aspirates.

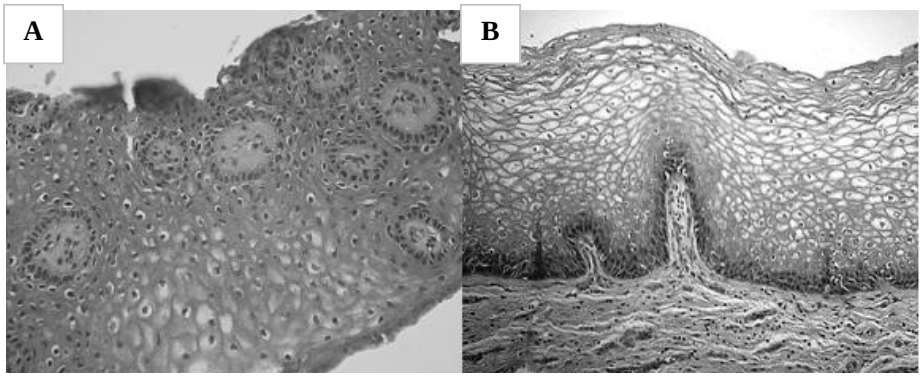

Figure 4. A Vaginal epithelium found in follicular aspirates. **B** Normal human vaginal epithelium with underlying connective tissue papillae projecting into the epithelium. Follicle-like structure in A can be formed by taking a cross section of B.

In conclusion, article I indicates that follicular aspirates are not a reliable source of human follicles and some structures found in the aspirates may be erroneously identified as follicles.

5.2 ARTICLE II, CRYOPRESERVATION OF HUMAN OVARIAN TISSUE

In article II, we evaluated whether serum-free cryoprotectant solutions could be used in cryopreservation of human ovarian cortical tissue. The answer is yes. No significant differences were observed between cryoprotectant solutions containing serum or serum-free cryoprotectant solutions containing human serum albumin (HSA), with respect to morphology and viability of follicles/oocytes.

Histological analysis on 693 follicles by light microscopy revealed that 99.3% of follicles are viable in fresh tissue; while 65% of the follicles and 75% of the oocytes were viable with serum, and 69% of follicles and 74% of the oocytes were viable with HSA. The reduction in the viability of follicles and oocytes after freezing/thawing were significant ($P<0.01$). There was no significant difference observed between solutions containing serum versus HSA regarding the viability of follicles and oocytes.

Similar results were obtained from 559 follicles analysed by live/dead assay. The viability of the follicles was 93% in fresh tissue and it was significantly decreased to 82% in tissue cryopreserved with either serum or HSA ($P<0.01$). The results are comparable with that of another group using the similar live/dead assay for evaluation of follicle viability after cryopreservation (Oktay, K *et al.*, 1997).

The higher viability rate obtained by live/dead assay compared with that obtained by light microscopy probably is due to the loss of some dead follicles during preparation, as completely dead follicles most likely disappear during enzymatic isolation. This potential cell loss could be eliminated by staining cells in tissue slides (Cortvrindt *et al.*, 2001).

Ultrastructure of 66 follicles and stromal cells were scored from TEM images. Oocytes, granulosa and stromal cells in fresh tissue had significantly higher scores than those in cryopreserved tissue regardless of the cryoprotectants used ($P<0.05$). The most remarkable reduction in score after cryopreservation was observed in the stromal cells. TEM images also showed 12.2% vacuolisation of oocytes in fresh tissue and 18.1% and 17.9% in cryopreserved tissue with serum and HSA respectively. Again, no significant differences in TEM scores of oocytes, granulosa or stromal cells were observed between serum and HSA. The real extent of vacuolisation of oocytes in our study could be lower than that we measured. Lipid drops in oocytes can be extracted and form vacuoles during our TEM preparation. These "vacuoles" are difficult to distinguish from the real vacuoles, so we considered all presented vacuoles as real vacuoles regardless of origin. To our knowledge, the TEM results of our study are the first report of ultrastructure of ovarian cells based on a large number of cells. **Figure 5** illustrates the ultrastructure of normal human ovarian cells.

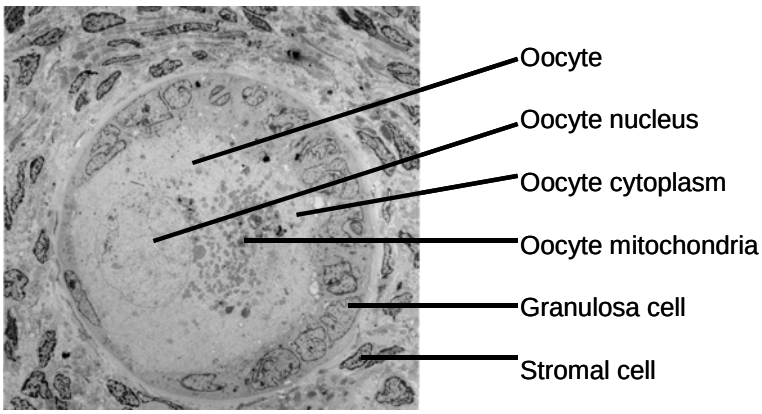

Figure 5. Ultrastructure of human oocyte, granulosa cells and stromal cells. The follicle in the picture is at primary stage. TEM 1500 X

We also studied the effect of post-thawing culture in recovering of follicles and oocytes. The results did not show any benefit of 4 hours post-thawing culture. However, we cannot draw conclusion from the results as only a small number of follicles are used in this comparison.

The use of serum in cryoprotectant solutions has been recommended previously (George *et al.*, 1992) for freezing mouse oocytes, mainly to avoide zona hardening. This does not seem to be the case for the oocyte in early follicles since the zona pellucida has not yet been formed. Moreover, serum is not subject to strict quality control in the same way as commercially available HSA, and potential risk of pathogen transmission between individuals exists when heterologous serum is used. Therefore, serum-free cryoprotectants are more suitable to cryopreserve human ovarian tissue.

Overall, article II showed that cyoprotectant solution containing HSA was equally effective as that containing serum in cryopreservation of human ovarian cortical tissue. As HSA is more safe and convenient to use, we recommend HSA in clinical work.

5.3 ARTICLE III, CULTURE OF HUMAN OVARIAN TISSUE WITH CGMP

In article III, we tested the effect of 8-br-cGMP (8-bromo- cyclic guanosine 3', 5'-monophosphate) in human ovarian cortical tissue culture. The main finding is that 8-br-cGMP improved the survival and development of early follicles during 14 days of tissue culture.

The proportion of viable follicles in uncultured tissue is near 100%. The value decreased significantly (40% - 60%) in all cultured tissue regardless of the supplements (all $P < 0.05$). However, between cultured tissues, the proportion of viable follicles was higher in the 8-br-cGMP treated ones than those without 8-br-cGMP treatment. The difference was significant on day 7. On day 14 the difference was still observed, although it was not statistically significant. The role of 8-br-cGMP as a survival factor is also observed in rat preantral ovarian follicle cultures (McGee, E *et al.*, 1997). The underlying mechanism is likely related to the suppression of apoptosis of the follicles (McGee, E *et al.*, 1997).

The percentage of follicles at different developmental stages changed dramatically after culture. In uncultured tissue, the proportion of primordial follicles was 75%. The proportion reduced significantly to the values between 14% and 30% in all cultures (all P <

0.05). Correspondingly, the proportion of primary and secondary follicles in all cultured tissues increased significantly compared with that in uncultured tissues (all $P < 0.05$). Significant differences in follicular stages were also observed between cultures with and without 8-br-cGMP. On day 7, the proportion of secondary follicles in tissues cultured with 8-br-cGMP is 45%, while it is 34% in tissues cultured without 8-br-cGMP. On day 14, the values are 51% and 36% in the presence and absence of 8-br-cGMP respectively. There is no significant difference in the proportion of primordial or primary follicles between cultures with and without 8-br-cGMP. These results suggests that 8-br-cGMP can improve the proliferation of granulosa cells and hence the development of follicles during 14 days of tissue culture.

We also measured the oestradiol concentration in the culture media. Throughout the 14-day culture period, ovarian cortical tissue cultured with 8-br-cGMP consistently produced more oestradiol than tissue cultured without 8-br-cGMP, with significance achieved at day 12 only. The finding is in agreement with the effect of 8-br-cGMP on follicle development described above. As 8-br-cGMP promoted follicular development, more follicles acquired the capability of oestradiol production.

Taken together, data in article III suggests that 8-br-cGMP may be a necessary component in culture of human ovarian tissue, as it enhances the survival and development of early follicles. This finding would help the optimisation of culture system for human ovarian tissue.

5.4 ARTICLE IV, CULTURE OF HUMAN OVARIAN TISSUE WITH CAMP

In article IV, we tested the effect of 8-br-cAMP (8-bromo- cyclic adenosine 3', 5'-monophosphate) in human ovarian cortical tissue culture. The results showed that 8-br-cAMP improved the survival and development of early follicles during 21days of tissue culture.

The proportion of viable follicles in uncultured tissue is 100%. After 7 days of culture, the value decreased significantly to 83% in culture with8-br-cAMP, and 75% in cultures without 8-br-cAMP treatment (both $P < 0.001$). Differences in the proportion of viable follicles between cultured tissues were not significant during the first 14 days of culture. However, on day 21, a significantly higher proportion of viable follicles was observed in 8-br-cAMP treated tissue compare with that in control culture without 8-br-cAMP (80% vs. 55%, $P<0.05$).

The majority of follicles initiated their growth once the culturing started. In uncultured tissue, the proportion of primordial follicles was 70%. After 7 days of culture, the proportion significantly reduced to 21% in cultures with or without 8-br-cAMP ($P < 0.001$). At the same time, the proportion of secondary follicles in all cultures significantly increased from 7.6% to 44% - 47% ($P < 0.001$). Significant differences in follicle stages between cultures with and without 8-br-cAMP were observed on day 14: the proportions of secondary follicles are 50% and 20% in tissues cultured with and without 8-br-cAMP respectively, while the proportion of primordial follicles are 9.7% and 26.7% correspondingly (all $P<0.05$). The differences in proportion of follicles at different stage between cultures with and without 8-br-cGMP were not significant on day 7 and day 21.

Moreover, we measured the diameters of follicles and oocytes at different culture points. All the follicles after 7 days culture were significantly larger that those before culture independent of 8-br-cAMP (48-52 μm vs. 41 μm, $P<0.001$). The diameters of the oocytes did not significantly change after culture. No significant difference in the size of follicles and oocytes were found between cultures at different time points. There is no difference in size of follicles or oocytes between cultures with and without 8-br-cAMP throughout the whole 21 days culture period. These observations in our study are consistent with natural follicular development. In humans, the oocyte enters the growth phase when it becomes completely enclosed by approximately 15 cuboidal granulosa cells (Gougeon *et al.*, 1987). The oocytes begin to grow when the follicles are at late primary or early secondary stage. In our study, most secondary follicles in culture were at an early stage, which explains why the oocytes did not grow significantly. Similar results are obtained

by de Bruin and coworkers (de Bruin *et al.*, 2002). They found that the sizes of oocytes do not change with the developmental stage before the primary stage.

The effects of 8-br-cAMP on follicle development in the study were in accordance with those obtained by using FSH supplementation (Wright *et al.*, 1999). This result would have potential clinical application. Some women suffer infertility due to an inactivating mutation in their FSH receptor (Aittomaki *et al.*, 1995; Aittomaki *et al.*, 1996). These patients cannot respond to FSH and only have primordial and primary follicles (Aittomaki *et al.*, 1996). Cyclic AMP is the second messenger of FSH (Skalhegg *et al.*, 2000). FSH exerts its effect on granulosa cells partly through the receptor-activated cAMP-protein kinase A pathway. Theoretically, cAMP may therefore replace some FSH action. Culturing ovarian cortical tissue from these patients with cAMP may help in obtaining mature oocytes for IVF.

To conclude, the results in article IV indicate that 8-br-cAMP may affect the early development of human ovarian follicles during a prolonged culture period, leading to enhanced follicular development and better survival. During 14-21 days of ovarian tissue culture, 8-br-cAMPcould be added in culture medium.

5.5 ARTICLE V, GENE EXPRESSION OF HUMAN GV OOCYTES

In article V, we analyzed the gene expression profile of human germinal vesicle oocytes (hGVO) and compared it with the profiles of human embryonic stem cells and human foreskin fibroblasts. The results exposed a global gene expression profile of hGVO. Further, it highlighted an abundant body of new information and revealed distinct sets of genes that appear to be involved in oocyte maturation and early embryonic development.

In total, 11,191 unigenes (16,965 probe sets) were expressed. Forty-nine percent of these genes are as yet unclassified by biological function. The data were highly reproducible as confirmed by a high correlation coefficiency (r) of the gene expression profiles between

Kommentar [h1]: I think these abbreviations are OK

duplicates: 0.95, 0.93, and 0.94 for hGVO, hESC and hFIB respectively. The quality of the data was also confirmed by comparing our data with previous studies. Over 80% genes reported in hGVO by other studies (Neilson *et al.*, 2000; Dobson *et al.*, 2004) were found to be present in our data.

In particular, eight oocyte-specific genes, which were not expressed in hESC or hFIB, were highly expressed in hGVO (4-8 times higher level as compared to *beta-ACTIN* level) They were *MATER, ZAR1, NPM2, FIGLA, GDF9, BMP15, MOS* and *ZP2*. Among these eight genes, *MATER, ZAR1, NPM2* and *FIGLA* have not been reported earlier in hGVO. The expression of these four genes in hGVO was also confirmed by RT-PCR (**Fig. 6**). *FIGLA* expression is also detected in human fetal ovary and is considered associated with primordial follicle formation (Bayne *et al.*, 2004). *Mater* and *Zar1* transcripts are detected also in bovine oocyte (Pennetier *et al.*, 2004). Studies on mice reveal the crucial roles of *Zar1* (Wu, X *et al.*, 2003), *Mater* (Tong *et al.*, 2000) and *Npm2* (Burns *et al.*, 2003) in early embryonic development. Furthermore, in females lacking *Figla* (Factor in the germline alpha), primordial follicles are not formed at birth, and massive depletion of oocytes results in shrunken ovaries and female sterility. In addition, the *Figla* null females do not express *Zp1, Zp2 or Zp3 (Soyal et al., 2000)*. The functions of these genes in humans need further exploration.

Furthermore, several imprinted genes, such as *UBE3A, MEST (PEG1), PEG3, IGF2R, GNAS, GRB10* and *SGCE* were detected in hGVO for the first time. The expression of the imprinted gene *SNRPN* in hGVO is consistent with a previous report (Geuns *et al.*, 2003).

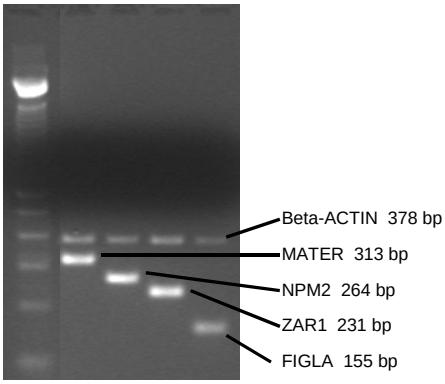

Figure 6. Detection of *MATER, NPM2, ZAR1,* and *FIGLA* in human GV oocytes by RT-PCR. The order of the lanes (from left to right) is ladder, *MATER* plus *Beta-ACTIN, NPM2* plus *Beta-ACTIN, ZAR1* plus *Beta-ACTIN,* and *FIGLA* plus *Beta-ACTIN. Beta-ACTIN* was checked at the same time as a positive control. All the PCR negative controls did not produce any visible product on the gel (data not shown).

We also found the existence of known components of three signaling pathways in hGVO. They are the *MOS-MPF* pathway, *TGF-beta* superfamily signaling pathway, and *WNT* signaling pathway. In *MOS-MPF* pathway, $p34^{cdc2}$ *(CDC2), cyclin-B (CCNB1, CCNB2), MOS, MAPKs, CDC25, MYT1,* and *SPY1* were detected, and the findings supported the previous reports in that some members such as *cyclin-B1* and *c-MOS,* are identified in hGVO (Heikinheimo *et al.*, 1995). Transforming growth factor-beta (*TGF-beta*) superfamily signaling pathway plays an important role in the development of follicles, oocytes and embryos (Chang *et al.*, 2002). Most members of this pathway have previously been identified in granulosa cells (Mazerbourg *et al.*, 2003). Here, we observed the expression of most members in hGVO as well (see **Table I** in article V). These data suggest a possible *TGF-beta* signaling loop between oocytes and granulosa cells. Little is known about the role of the *WNT* signaling pathway in human oocytes. We found that all the main components of the *WNT* pathway were expressed in the hGVO; they are *FRIZZLED (FZD4, FZD3, FZD6), LRP6, DSH (DVL1, DVL2), APC, AXIN2, GSK3, beta-CATENIN (CTNNB1)* and *JNK (MAPK8).* These findings indicate that the *WNT* pathway may participate in very early development, in line with a report that Wnt4

mutations disrupt the normal development of the female reproductive system in mice (Vainio *et al.*, 1999).

Moreover, we compared gene expression profiles of hGVO, hESC, and hFIB and found twelve gene expression patterns among these three cell types. In total, 1,752 genes were up regulated and 1785 genes were down regulated in hGVO when compared with hESC and hFIB. Among the up-regulated genes, there were 751 genes expressed in hGVO but absent in hESC and hFIB, 377 genes expressed in hGVO at 2-fold higher level against hESC and hFIB**.** Genes that were highly expressed in hGVO but were absent or had low expression level in hESC and hFIB may have unique roles in the maturation of oocytes and/or fertilization. This hypothesis is supported by the observation that both oocyte-specific genes (such as *GDF9, ZP2*) and genes involved in meiotic maturation (such as *MOS*) fall in these sets. Genes that were highly expressed in both hGVO and hESC but were absent or had low expression in hFIB might be important for both maturation of the oocytes and early embryonic development. This inference is backed up by the reports that the two genes *DNMT1* (Mhanni *et al.*, 2002; Hashimoto, H *et al.*, 2003) and *BRCA2* (Suzuki *et al.*, 1997; Sharan *et al.*, 2004) in this group are related to both oocyte maturation and embryonic development.

Finally, we compared our data to the data on mice by Wang et al.(Wang, QT *et al.*, 2004) who used very similar methods. We found mouse and human GV oocytes shared 2,951 genes. 860 genes are specific in mouse GV oocytes while 1,349 genes are specific in hGVO. We suppose that the genes expressed in both hGVO and mouse GV oocytes may have conserved functions in both species, and the genes expressed in hGVO but not in mouse GV oocytes might have a specific function only in human regarding oocyte maturation and/or early embryonic development.

In summary, article V reported, for the first time, the microarray data of normal human GV oocytes based on a large number of oocytes. An overview of the transcriptome of hGVO was generated. Functional studies of candidate genes revealed by the present study would enable the discovery of more genes involved in oocyte maturation,

fertilization and early embryo development. Overall, our study provides a rich source of information for further investigation of molecular mechanism of early human development.

6 CONCLUSIONS

1. Follicular aspirates obtained at oocyte retrieval are not a reliable source of human ovarian follicles.

2. Serum-free cryoprotectant solution is equally effective as that containing serum in cryopreservation of human ovarian cortical tissue.

3. Both 8-br-cGMP and 8-br-cAMP enhance the survival and development of human early follicles cultured in ovarian cortical tissue.

4. In total, 11,191 unigenes were expressed normal human GV oocytes. Forty-nine percent of these genes are as yet unclassified by biological function. Twelve gene expression patterns identified between human GV oocytes, embryonic stem cells and fibroblasts suggest potential candidate genes involved in oocyte maturation and embryonic development.

7 ACKNOWLEDGEMENTS

I would like to express my sincere gratitude and thanks to:

Prof. **Outi Hovatta,** my supervisor, for taking me as her student, for her endless support, understanding, trust, and encouragement. Outi, I feel so lucky to be your student. Your wonderful personality and vast knowledge will continually teach me in both life and science.

Prof. **Britt-Marie Landgren**, for giving me the opportunity to study in her department, for her encouragement and understanding. I really enjoy your humour, Britt-Marie.

Prof. **Juha Kere**, for guidance, encouragement and excellent ideas, for patient explanation of molecular techniques. Juha, what impressed me most is not your genuine knowledge, but your inspiration. You are never down. That is really great!

Dr. **Evelyn E. Telfer**, for always being a great friend, for correction of my manuscripts and wonderful advice. Evelyn, you are a lovely lady!

Julius Hreinsson, for introducing me to the IVF research field, for friendship and unconditioned help, for wonderful collaboration, pleasant travel company and delicious Icelandic food.

Thanks are not enough to express my gratitude to my colleagues **Jennifer Scott** and **Inger Britt Carlsson**. They offer me all the help they can. Thank you, girls, for the best friendship, for enormous enthusiasm and encouragement, for the careful English correction and valuable advice, for the nice atmosphere in our office. You enhanced my happiness and diluted my sadness. I own you a lot!

Erja Kerkelä and **Heli Skottman,** my "supervisors" in molecular biology. Thank you for your guidance, trust, and kindness. Working with you is so relaxing, happy and fruitful!

Karolina Kublickiene, for her warm help when I first came to Novum, for interesting chats on the science and the life, for her Swedish translations and the nice dinner! Karolina, you are so capable in everything, I am jealous of you!

Lusine Aghajanova, Lev Levkov and **Mia Lindeberg,** for warm friendship, kind support and good advise. **Lev**, you never say "no" when I need your help. Thank you for everything!

My other co-authors, **Kjell Hultenby, Marja Liisa Swahn, Henna Louhio, Timo Tuuri, Jari Sjöberg, Katja Kivinen** and **Riitta Lahesmaa,** for their contributions to the studies. Without their efforts, my thesis is not possible. I really appreciate their contributions!
All patients, for their kind donation of samples. All doctors and nurses working in the Department of Obstetrics and Gynecology, Huddinge Hospital, for their help in collecting samples.

All people working in the Fertility lab, K 59: **Linda Eklund, Sofia Johansson, Maryam Sheikhi, Lena Möller, Mirja Tolvanen, Ingalill Persson, Ami Strömberg, Eija Matilainen,** for their help in sample collection, funny jokesand big smiles. **Monica Klauert, Sari Hillblom, Kerstin Bjuresten, Mia Karlsson, Margaretha Ström and Lena Ydenius** for their kind help whenever I need it.

Mikeal Zmarzlak, Arif Kisana, Mona Fartoo, Hanna Karlsson, for their kind offer and help in mouse sample collection.

All the members of and around the lab: **Victoria Keros**, **Maria-Natalia Cruz**, **Leonid Luksha**, **Gayane Avetisyan, José Inzunza, Marina Yassenko, Monira Akhtar, Eimantas Svedas**, **Yongmei Guan**, **Karin Gertow**, **Catharina Maijgren- Steffensson**, **Marta Imreh**, **Krisztina Szöke**, **Jessica Cederwall**, **Anneli Stavreus-evers**, **Fredwell Hambiliki** for the friendship and the nice times we spent together.

Katarina Oxelbeck, Lisbeth Löfstrand, Anita Gasperoni, Agneta Wittlock and **Marie Klinta-Svensson** for their professional help and kindness.

Miina Miller, Kjell Carlström, Jan-Åke Ågren, Elsebrit Ljungström Köhl, Eva Blomén, Silwa Mengarelli, and staff in Affymetrix core facility at Novum: **Karin Dahlman-Wright, Johan Lidén, Marika Rönnholm** and **David Brodin,** for their excellent technical support.

Members in Juha's group in CBT: **Myriam Peyrard-Janvid**, **Hong Jiao**, **Hanna Peterson**, **Heidi Anthoni**, **Marco Zucchelli**, **Tiina Skoog, Cecilia Lindgren** and **Ville-Veikko Makela**, for their help in experiments, valuable advices and "emergency" help when I get "lost" in the lab. Other members in the group: **Ingegerd Fransson, Ulf Hannelius, Linda Berglind, Isabel Tapia, Sara Bruce, Anna Hellquist, Harriet Gullstén, Hannele Laivori, Päivi Onkamo, Päivi Kiviluoma, Astrid Lindsted, Erik Melen** and **Rasko Leinone**, for their kindness and the nice atmosphere created in the lab.

All my other friends: **Zhiping Zhang, Changjuan Pan, Danhuan Pan, Fanqing Xia, Quan Du, Jue Wang, Fengqing Xiang, Weihua Dong, Jinjing Pei, Huixin Wang, Shuhua Ma, Yang Yang, Shaozheng Wei, Jie Hu,** for helping us settle down and get used to life in Stockholm. **Yi Zhang, Hanjin Xie, Hui Gao, Wen Cai, Hao Mo, Hao Wu, Yongtao Xue, Mats Franzén, Qianren Jin, Xinjun Li, Yaofeng Zhao, Peipei Zhao, Sicheng Wen, Jing Ding, Wenpeng Niu, Qinyang Wu, Xiaojing Hao, Zheng**

Zhou, Yu Shi, Zhong He, Xupeng Ge, Guojun Chen, Hong Fang for understanding, help and the happy holidays we spend together.

Prof. **Suxuan Fan** and Prof. **Huifang Zheng**, my supervisors for my Master degree in China, for opening the research door to me, and for instructing me in clinical work.

My parents-in-law: **Lihua Lu** and **Jianguo Zhou**, for their understanding and selfless supporting.

My father **Zemin Zhang** and my mother **Yuelan Fang,** for their true love to me.
□ □ ,□ □ □ □ □ □ □ □ ! My sisters **Ping Zhang** and **Fan Zhang**, for taking care of me wherever I am.

Last but not least, lots of thanks to my husband **Xiaolei Zhou** and my lovely son **Robin Ziyue Zhou**, for making my life always sunny. I am so proud of you!

GOD BLESSES YOU ALL!

This work was supported in part by a grant from the Swedish Medical Research Council, the Academy of Finland, Sigrid Jusélius Foundation, and the Jalmari and Rauha Ahokas Foundation.

8 REFERENCES

Aaltonen J, Laitinen MP, Vuojolainen K, Jaatinen R, Horelli-Kuitunen N, Seppa L, Louhio H, Tuuri T, Sjoberg J, Butzow R, Hovata O, Dale L and Ritvos O (1999) Human growth differentiation factor 9 (GDF-9) and its novel homolog GDF- 9B are expressed in oocytes during early folliculogenesis. *J Clin Endocrinol Metab* **84**, 2744-2750.

Abbott AL and Ducibella T (2001) Calcium and the control of mammalian cortical granule exocytosis. *Front Biosci* **6**, D792-806.

Abir R, Orvieto R, Raanani H, Feldberg D, Nitke S and Fisch B (2003) Parameters affecting successful transplantation of frozen-thawed human fetal ovaries into immunodeficient mice. *Fertil Steril* **80**, 421-428.

Abir R, Roizman P, Fisch B, Nitke S, Okon E, Orvieto R and Ben Rafael Z (1999) Pilot study of isolated early human follicles cultured in collagen gels for 24 hours. *Hum Reprod* **14**, 1299-1301.

Aittomaki K, Herva R, Stenman UH, Juntunen K, Ylostalo P, Hovatta O and de la Chapelle A (1996) Clinical features of primary ovarian failure caused by a point mutation in the follicle-stimulating hormone receptor gene. *J Clin Endocrinol Metab* **81**, 3722-3726.

Aittomaki K, Lucena JL, Pakarinen P, Sistonen P, Tapanainen J, Gromoll J, Kaskikari R, Sankila EM, Lehvaslaiho H, Engel AR and et al. (1995) Mutation in the follicle-stimulating hormone receptor gene causes hereditary hypergonadotropic ovarian failure. *Cell* **82**, 959-968.

Amleh A and Dean J (2002) Mouse genetics provides insight into folliculogenesis, fertilization and early embryonic development. *Hum Reprod Update* **8**, 395-403.

Anger M, Klima J, Kubelka M, Prochazka R, Motlik J and Schultz RM (2004) Timing of Plk1 and MPF activation during porcine oocyte maturation. *Mol Reprod Dev* **69**, 11-16.

Ao A, Erickson RP, Winston RM and Handyside AH (1994) Transcription of paternal Y-linked genes in the human zygote as early as the pronucleate stage. *Zygote* **2**, 281-287.

Aoki F, Worrad DM and Schultz RM (1997) Regulation of transcriptional activity during the first and second cell cycles in the preimplantation mouse embryo. *Dev Biol* **181**, 296-307.

Arraztoa JA, Zhou J, Marcu D, Cheng C, Bonner R, Chen M, Xiang C, Brownstein M, Maisey K, Imarai M and Bondy C (2004) Identification of genes expressed in primate primordial oocytes. *Hum Reprod*.

Baarends WM, Uilenbroek JT, Kramer P, Hoogerbrugge JW, van Leeuwen EC, Themmen AP and Grootegoed JA (1995) Anti-mullerian hormone and anti-mullerian hormone type II receptor messenger ribonucleic acid expression in rat ovaries during postnatal development, the estrous cycle, and gonadotropin-induced follicle growth. *Endocrinology* **136**, 4951-4962.

Bachvarova R, De Leon V, Johnson A, Kaplan G and Paynton BV (1985) Changes in total RNA, polyadenylated RNA, and actin mRNA during meiotic maturation of mouse oocytes. *Dev Biol* **108**, 325-331.

Bachvarova RF (1992) A maternal tail of poly(A): the long and the short of it. *Cell* **69**, 895-897.

Bayne RA, Martins da Silva SJ and Anderson RA (2004) Increased expression of the FIGLA transcription factor is associated with primordial follicle formation in the human fetal ovary. *Mol Hum Reprod* **10**, 373-381.

Bedaiwy MA, Jeremias E, Gurunluoglu R, Hussein MR, Siemianow M, Biscotti C and Falcone T (2003) Restoration of ovarian function after autotransplantation of intact frozen-thawed sheep ovaries with microvascular anastomosis. *Fertil Steril* **79**, 594-602.

Bedell MA, Brannan CI, Evans EP, Copeland NG, Jenkins NA and Donovan PJ (1995) DNA rearrangements located over 100 kb 5' of the Steel (Sl)-coding region in Steel-panda and Steel-contrasted mice deregulate Sl expression and cause female sterility by disrupting ovarian follicle development. *Genes Dev* **9**, 455-470.

Bhatt RR and Ferrell JE, Jr. (1999) The protein kinase p90 rsk as an essential mediator of cytostatic factor activity. *Science* **286**, 1362-1365.

Bleil JD, Beall CF and Wassarman PM (1981) Mammalian sperm-egg interaction: fertilization of mouse eggs triggers modification of the major zona pellucida glycoprotein, ZP2. *Dev Biol* **86**, 189-197.

Bodensteiner KJ, Clay CM, Moeller CL and Sawyer HR (1999) Molecular cloning of the ovine Growth/Differentiation factor-9 gene and expression of growth/differentiation factor-9 in ovine and bovine ovaries. *Biol Reprod* **60**, 381-386.

Brown WM, Saunders NR, Mollgard K and Dziegielewska KM (1992) Fetuin--an old friend revisited. *Bioessays* **14**, 749-755.

Burns KH, Viveiros MM, Ren Y, Wang P, DeMayo FJ, Frail DE, Eppig JJ and Matzuk MM (2003) Roles of NPM2 in chromatin and nucleolar organization in oocytes and embryos. *Science* **300**, 633-636.

Callejo J, Salvador C, Miralles A, Vilaseca S, Lailla JM and Balasch J (2001) Long-term ovarian function evaluation after autografting by implantation with fresh and frozen-thawed human ovarian tissue. *J Clin Endocrinol Metab* **86**, 4489-4494.

Carabatsos MJ, Elvin J, Matzuk MM and Albertini DF (1998) Characterization of oocyte and follicle development in growth differentiation factor-9-deficient mice. *Dev Biol* **204**, 373-384.

Chang H, Brown CW and Matzuk MM (2002) Genetic analysis of the mammalian transforming growth factor-beta superfamily. *Endocr Rev* **23**, 787-823.

Christians E, Davis AA, Thomas SD and Benjamin IJ (2000) Maternal effect of Hsf1 on reproductive success. *Nature* **407**, 693-694.

Colledge WH, Carlton MB, Udy GB and Evans MJ (1994) Disruption of c-mos causes parthenogenetic development of unfertilized mouse eggs. *Nature* **370**, 65-68.

Conti M, Andersen CB, Richard FJ, Shitsukawa K and Tsafriri A (1998) Role of cyclic nucleotide phosphodiesterases in resumption of meiosis. *Mol Cell Endocrinol* **145**, 9-14.

Cortvrindt RG and Smitz JE (2001) Fluorescent probes allow rapid and precise recording of follicle density and staging in human ovarian cortical biopsy samples. *Fertil Steril* **75**, 588-593.

Craig J, Zhu H, Dyce PW, Petrik J and Li J (2004) Leptin enhances oocyte nuclear and cytoplasmic maturation via the mitogen-activated protein kinase pathway. *Endocrinology* **145**, 5355-5363.

Cuthbertson KS and Cobbold PH (1985) Phorbol ester and sperm activate mouse oocytes by inducing sustained oscillations in cell Ca2+. *Nature* **316**, 541-542.

Daniels R, Kinis T, Serhal P and Monk M (1995) Expression of the myotonin protein kinase gene in preimplantation human embryos. *Hum Mol Genet* **4**, 389-393.

Davis W, Jr., De Sousa PA and Schultz RM (1996) Transient expression of translation initiation factor eIF-4C during the 2-cell stage of the preimplantation mouse embryo: identification by mRNA differential display and the role of DNA replication in zygotic gene activation. *Dev Biol* **174**, 190-201.

de Bruin JP, Dorland M, Spek ER, Posthuma G, van Haaften M, Looman CW and te Velde ER (2002) Ultrastructure of the resting ovarian follicle pool in healthy young women. *Biol Reprod* **66**, 1151-1160.

Depalo R, Nappi L, Loverro G, Bettocchi S, Caruso ML, Valentini AM and Selvaggi L (2003) Evidence of apoptosis in human primordial and primary follicles. *Hum Reprod* **18**, 2678-2682.

DeRenzo C and Seydoux G (2004) A clean start: degradation of maternal proteins at the oocyte-to-embryo transition. *Trends Cell Biol* **14**, 420-426.

Detwiler MR, Reuben M, Li X, Rogers E and Lin R (2001) Two zinc finger proteins, OMA-1 and OMA-2, are redundantly required for oocyte maturation in C. elegans. *Dev Cell* **1**, 187-199.

Dobson AT, Raja R, Abeyta MJ, Taylor T, Shen S, Haqq C and Pera RA (2004) The unique transcriptome through day 3 of human preimplantation development. *Hum Mol Genet* **13**, 1461-1470.

Dong J, Albertini DF, Nishimori K, Kumar TR, Lu N and Matzuk MM (1996) Growth differentiation factor-9 is required during early ovarian folliculogenesis. *Nature* **383**, 531-535.

Donnez J, Dolmans MM, Demylle D, Jadoul P, Pirard C, Squifflet J, Martinez-Madrid B and van Langendonckt A (2004) Livebirth after orthotopic transplantation of cryopreserved ovarian tissue. *Lancet* **364**, 1405-1410.

Downs SM (1995) The influence of glucose, cumulus cells, and metabolic coupling on ATP levels and meiotic control in the isolated mouse oocyte. *Dev Biol* **167**, 502-512.

Driancourt MA, Reynaud K, Cortvrindt R and Smitz J (2000) Roles of KIT and KIT LIGAND in ovarian function. *Rev Reprod* **5**, 143-152.

Dunphy WG, Brizuela L, Beach D and Newport J (1988) The Xenopus cdc2 protein is a component of MPF, a cytoplasmic regulator of mitosis. *Cell* **54**, 423-431.

Durlinger AL, Gruijters MJ, Kramer P, Karels B, Ingraham HA, Nachtigal MW, Uilenbroek JT, Grootegoed JA and Themmen AP (2002) Anti-Mullerian hormone inhibits initiation of primordial follicle growth in the mouse ovary. *Endocrinology* **143**, 1076-1084.

Durlinger AL, Gruijters MJ, Kramer P, Karels B, Kumar TR, Matzuk MM, Rose UM, de Jong FH, Uilenbroek JT, Grootegoed JA and Themmen AP (2001) Anti-Mullerian hormone attenuates the effects of FSH on follicle development in the mouse ovary. *Endocrinology* **142**, 4891-4899.

Durlinger AL, Kramer P, Karels B, de Jong FH, Uilenbroek JT, Grootegoed JA and Themmen AP (1999) Control of primordial follicle recruitment by anti-Mullerian hormone in the mouse ovary. *Endocrinology* **140**, 5789-5796.

Edwards RG (2003) Aspects of the molecular regulation of early mammalian development. *Reprod Biomed Online* **6**, 97-113.

Endo Y, Schultz RM and Kopf GS (1987) Effects of phorbol esters and a diacylglycerol on mouse eggs: inhibition of fertilization and modification of the zona pellucida. *Dev Biol* **119**, 199-209.

Eppig JJ and O'Brien MJ (1996) Development in vitro of mouse oocytes from primordial follicles. *Biol Reprod* **54**, 197-207.

Faddy MJ (2000) Follicle dynamics during ovarian ageing. *Mol Cell Endocrinol* **163**, 43-48.

Fiddler M, Abdel-Rahman B, Rappolee DA and Pergament E (1995) Expression of SRY transcripts in preimplantation human embryos. *Am J Med Genet* **55**, 80-84.

Fridovich I (1986) Biological effects of the superoxide radical. *Arch Biochem Biophys* **247**, 1-11.

Fulka J, Jr., Jung T and Moor RM (1992) The fall of biological maturation promoting factor (MPF) and histone H1 kinase activity during anaphase and telophase in mouse oocytes. *Mol Reprod Dev* **32**, 378-382.

Galaktionov K and Beach D (1991) Specific activation of cdc25 tyrosine phosphatases by B-type cyclins: evidence for multiple roles of mitotic cyclins. *Cell* **67**, 1181-1194.

Gautier J, Minshull J, Lohka M, Glotzer M, Hunt T and Maller JL (1990) Cyclin is a component of maturation-promoting factor from Xenopus. *Cell* **60**, 487-494.

Gautier J, Norbury C, Lohka M, Nurse P and Maller J (1988) Purified maturation-promoting factor contains the product of a Xenopus homolog of the fission yeast cell cycle control gene cdc2+. *Cell* **54**, 433-439.

Gautier J, Solomon MJ, Booher RN, Bazan JF and Kirschner MW (1991) cdc25 is a specific tyrosine phosphatase that directly activates p34cdc2. *Cell* **67**, 197-211.

Geijsen N, Horoschak M, Kim K, Gribnau J, Eggan K and Daley GQ (2004) Derivation of embryonic germ cells and male gametes from embryonic stem cells. *Nature* **427**, 148-154.

George MA, Johnson MH and Vincent C (1992) Use of fetal bovine serum to protect against zona hardening during preparation of mouse oocytes for cryopreservation. *Hum Reprod* **7**, 408-412.

Geuns E, De Rycke M, Van Steirteghem A and Liebaers I (2003) Methylation imprints of the imprint control region of the SNRPN-gene in human gametes and preimplantation embryos. *Hum Mol Genet* **12**, 2873-2879.

Gook DA, Edgar DH, Borg J, Archer J, Lutjen PJ and McBain JC (2003) Oocyte maturation, follicle rupture and luteinization in human cryopreserved ovarian tissue following xenografting. *Hum Reprod* **18**, 1772-1781.

Gook DA, Edgar DH, Borg J, Archer J and McBain JC (2005) Diagnostic assessment of the developmental potential of human cryopreserved ovarian tissue from multiple patients using xenografting. *Hum Reprod* **20**, 72-78.

Gook DA, McCully BA, Edgar DH and McBain JC (2001) Development of antral follicles in human cryopreserved ovarian tissue following xenografting. *Hum Reprod* **16**, 417-422.

Gosden RG (2002) Oogenesis as a foundation for embryogenesis. *Mol Cell Endocrinol* **186**, 149-153.

Gosden RG, Mullan J, Picton HM, Yin H and Tan SL (2002) Current perspective on primordial follicle cryopreservation and culture for reproductive medicine. *Hum Reprod Update* **8**, 105-110.

Gougeon A (1986) Dynamics of follicular growth in the human: a model from preliminary results. *Hum Reprod* **1**, 81-87.

Gougeon A (1996) Regulation of ovarian follicular development in primates: facts and hypotheses. *Endocr Rev* **17**, 121-155.

Gougeon A and Chainy GB (1987) Morphometric studies of small follicles in ovaries of women at different ages. *J Reprod Fertil* **81**, 433-442.

Gross SD, Schwab MS, Lewellyn AL and Maller JL (1999) Induction of metaphase arrest in cleaving Xenopus embryos by the protein kinase p90Rsk. *Science* **286**, 1365-1367.

Hashimoto H, Suetake I and Tajima S (2003) Monoclonal antibody against dnmt1 arrests the cell division of xenopus early-stage embryos. *Exp Cell Res* **286**, 252-262.

Hashimoto N, Watanabe N, Furuta Y, Tamemoto H, Sagata N, Yokoyama M, Okazaki K, Nagayoshi M, Takeda N, Ikawa Y and et al. (1994) Parthenogenetic activation of oocytes in c-mos-deficient mice. *Nature* **370**, 68-71.

Heikinheimo O and Gibbons WE (1998) The molecular mechanisms of oocyte maturation and early embryonic development are unveiling new insights into reproductive medicine. *Mol Hum Reprod* **4**, 745-756.

Heikinheimo O, Lanzendorf SE, Baka SG and Gibbons WE (1995) Cell cycle genes c-mos and cyclin-B1 are expressed in a specific pattern in human oocytes and preimplantation embryos. *Hum Reprod* **10**, 699-707.

Henery CC, Miranda M, Wiekowski M, Wilmut I and DePamphilis ML (1995) Repression of gene expression at the beginning of mouse development. *Dev Biol* **169**, 448-460.

Hennebold JD (2004) Characterization of the ovarian transcriptome through the use of differential analysis of gene expression methodologies. *Hum Reprod Update* **10**, 227-239.

Hoffmann I, Clarke PR, Marcote MJ, Karsenti E and Draetta G (1993) Phosphorylation and activation of human cdc25-C by cdc2--cyclin B and its involvement in the self-amplification of MPF at mitosis. *Embo J* **12**, 53-63.

Holcik M and Korneluk RG (2001) XIAP, the guardian angel. *Nat Rev Mol Cell Biol* **2**, 550-556.

Hovatta O (2000) Cryopreservation and culture of human primordial and primary ovarian follicles. *Mol Cell Endocrinol* **169**, 95-97.

Hovatta O, Silye R, Abir R, Krausz T and Winston RM (1997) Extracellular matrix improves survival of both stored and fresh human primordial and primary ovarian follicles in long-term culture. *Hum Reprod* **12**, 1032-1036.

Hovatta O, Silye R, Krausz T, Abir R, Margara R, Trew G, Lass A and Winston RM (1996) Cryopreservation of human ovarian tissue using dimethylsulphoxide and propanediol-sucrose as cryoprotectants. *Hum Reprod* **11**, 1268-1272.

Hovatta O, Wright C, Krausz T, Hardy K and Winston RM (1999) Human primordial, primary and secondary ovarian follicles in long-term culture: effect of partial isolation. *Hum Reprod* **14**, 2519-2524.

Hreinsson J, Zhang P, Swahn ML, Hultenby K and Hovatta O (2003) Cryopreservation of follicles in human ovarian cortical tissue. Comparison of serum and human serum albumin in the cryoprotectant solutions. *Hum Reprod* **18**, 2420-2428.

Hreinsson JG, Scott JE, Rasmussen C, Swahn ML, Hsueh AJ and Hovatta O (2002) Growth differentiation factor-9 promotes the growth, development, and survival of human ovarian follicles in organ culture. *J Clin Endocrinol Metab* **87**, 316-321.

Huang EJ, Manova K, Packer AI, Sanchez S, Bachvarova RF and Besmer P (1993) The murine steel panda mutation affects kit ligand expression and growth of early ovarian follicles. *Dev Biol* **157**, 100-109.

Huo LJ, Fan HY, Zhong ZS, Chen DY, Schatten H and Sun QY (2004) Ubiquitin-proteasome pathway modulates mouse oocyte meiotic maturation and fertilization via regulation of MAPK cascade and cyclin B1 degradation. *Mech Dev* **121**, 1275-1287.

Isachenko E, Isachenko V, Rahimi G and Nawroth F (2003) Cryopreservation of human ovarian tissue by direct plunging into liquid nitrogen. *Eur J Obstet Gynecol Reprod Biol* **108**, 186-193.

Ito J, Kawano N, Hirabayashi M and Shimada M (2004) The role of calcium/calmodulin-dependent protein kinase II on the inactivation of MAP kinase and p34cdc2 kinase during fertilization and activation in pig oocytes. *Reproduction* **128**, 409-415.

Jaatinen R, Laitinen MP, Vuojolainen K, Aaltonen J, Louhio H, Heikinheimo K, Lehtonen E and Ritvos O (1999) Localization of growth differentiation factor-9 (GDF-9) mRNA and protein in rat ovaries and cDNA cloning of rat GDF-9 and its novel homolog GDF-9B. *Mol Cell Endocrinol* **156**, 189-193.

Jemal A, Tiwari RC, Murray T, Ghafoor A, Samuels A, Ward E, Feuer EJ and Thun MJ (2004) Cancer statistics, 2004. *CA Cancer J Clin* **54**, 8-29.

Johnson J, Canning J, Kaneko T, Pru JK and Tilly JL (2004) Germline stem cells and follicular renewal in the postnatal mammalian ovary. *Nature* **428**, 145-150.

Josefsberg LB, Galiani D, Lazar S, Kaufman O, Seger R and Dekel N (2003) Maturation-promoting factor governs mitogen-activated protein kinase activation and interphase suppression during meiosis of rat oocytes. *Biol Reprod* **68**, 1282-1290.

Juengel JL, Hudson NL, Heath DA, Smith P, Reader KL, Lawrence SB, O'Connell AR, Laitinen MP, Cranfield M, Groome NP, Ritvos O and McNatty KP (2002) Growth differentiation factor 9 and bone morphogenetic protein 15 are essential for ovarian follicular development in sheep. *Biol Reprod* **67**, 1777-1789.

Kawamura K, Kumagai J, Sudo S, Chun SY, Pisarska M, Morita H, Toppari J, Fu P, Wade JD, Bathgate RA and Hsueh AJ (2004) Paracrine regulation of mammalian oocyte maturation and male germ cell survival. *Proc Natl Acad Sci U S A* **101**, 7323-7328.

Kim SS, Soules MR and Battaglia DE (2002) Follicular development, ovulation, and corpus luteum formation in cryopreserved human ovarian tissue after xenotransplantation. *Fertil Steril* **78**, 77-82.

Kuroda H, Terada N, Nakayama H, Matsumoto K and Kitamura Y (1988) Infertility due to growth arrest of ovarian follicles in Sl/Slt mice. *Dev Biol* **126**, 71-79.

Lass A, Silye R, Abrams DC, Krausz T, Hovatta O, Margara R and Winston RM (1997) Follicular density in ovarian biopsy of infertile women: a novel method to assess ovarian reserve. *Hum Reprod* **12**, 1028-1031.

Latham KE and Schultz RM (2001) Embryonic genome activation. *Front Biosci* **6**, D748-759.

Lazar S, Galiani D and Dekel N (2002) cAMP-Dependent PKA negatively regulates polyadenylation of c-mos mRNA in rat oocytes. *Mol Endocrinol* **16**, 331-341.

Lazar S, Gershon E and Dekel N (2004) Selective degradation of cyclin B1 mRNA in rat oocytes by RNA interference (RNAi). *J Mol Endocrinol* **33**, 73-85.

Ledan E, Polanski Z, Terret ME and Maro B (2001) Meiotic maturation of the mouse oocyte requires an equilibrium between cyclin B synthesis and degradation. *Dev Biol* **232**, 400-413.

Lee WS, Yoon SJ, Yoon TK, Cha KY, Lee SH, Shimasaki S, Lee S and Lee KA (2004) Effects of bone morphogenetic protein-7 (BMP-7) on primordial follicular growth in the mouse ovary. *Mol Reprod Dev* **69**, 159-163.

Lenormand JL, Dellinger RW, Knudsen KE, Subramani S and Donoghue DJ (1999) Speedy: a novel cell cycle regulator of the G2/M transition. *Embo J* **18**, 1869-1877.

Liu C, Litscher ES, Mortillo S, Sakai Y, Kinloch RA, Stewart CL and Wassarman PM (1996) Targeted disruption of the mZP3 gene results in production of eggs lacking a zona pellucida and infertility in female mice. *Proc Natl Acad Sci U S A* **93**, 5431-5436.

Lorca T, Cruzalegui FH, Fesquet D, Cavadore JC, Mery J, Means A and Doree M (1993) Calmodulin-dependent protein kinase II mediates inactivation of MPF and CSF upon fertilization of Xenopus eggs. *Nature* **366**, 270-273.

Louhio H, Hovatta O, Sjoberg J and Tuuri T (2000) The effects of insulin, and insulin-like growth factors I and II on human ovarian follicles in long-term culture. *Mol Hum Reprod* **6**, 694-698.

Lu KP and Hunter T (1995) Evidence for a NIMA-like mitotic pathway in vertebrate cells. *Cell* **81**, 413-424.

Lucifero D, Mann MR, Bartolomei MS and Trasler JM (2004) Gene-specific timing and epigenetic memory in oocyte imprinting. *Hum Mol Genet* **13**, 839-849.

Lundgren K, Walworth N, Booher R, Dembski M, Kirschner M and Beach D (1991) mik1 and wee1 cooperate in the inhibitory tyrosine phosphorylation of cdc2. *Cell* **64**, 1111-1122.

Ma J, Svoboda P, Schultz RM and Stein P (2001) Regulation of zygotic gene activation in the preimplantation mouse embryo: global activation and repression of gene expression. *Biol Reprod* **64**, 1713-1721.

Machaca K (2004) Increased sensitivity and clustering of elementary Ca2+ release events during oocyte maturation. *Dev Biol* **275**, 170-182.

Majumder S and DePamphilis ML (1994) TATA-dependent enhancer stimulation of promoter activity in mice is developmentally acquired. *Mol Cell Biol* **14**, 4258-4268.

Martinez-Madrid B, Dolmans MM, Van Langendonckt A, Defrere S and Donnez J (2004) Freeze-thawing intact human ovary with its vascular pedicle with a passive cooling device. *Fertil Steril* **82**, 1390-1394.

Masciarelli S, Horner K, Liu C, Park SH, Hinckley M, Hockman S, Nedachi T, Jin C, Conti M and Manganiello V (2004) Cyclic nucleotide phosphodiesterase 3A-deficient mice as a model of female infertility. *J Clin Invest* **114**, 196-205.

Mazerbourg S and Hsueh AJ (2003) Growth differentiation factor-9 signaling in the ovary. *Mol Cell Endocrinol* **202**, 31-36.

McGee E, Spears N, Minami S, Hsu SY, Chun SY, Billig H and Hsueh AJ (1997) Preantral ovarian follicles in serum-free culture: suppression of apoptosis after activation of the cyclic guanosine 3',5'-monophosphate pathway and stimulation of growth and differentiation by follicle-stimulating hormone. *Endocrinology* **138**, 2417-2424.

McGee EA and Hsueh AJ (2000) Initial and cyclic recruitment of ovarian follicles. *Endocr Rev* **21**, 200-214.

McGowan CH and Russell P (1993) Human Wee1 kinase inhibits cell division by phosphorylating p34cdc2 exclusively on Tyr15. *Embo J* **12**, 75-85.

McGrath SA, Esquela AF and Lee SJ (1995) Oocyte-specific expression of growth/differentiation factor-9. *Mol Endocrinol* **9**, 131-136.

Meirelles FV, Caetano AR, Watanabe YF, Ripamonte P, Carambula SF, Merighe GK and Garcia SM (2004) Genome activation and developmental block in bovine embryos. *Anim Reprod Sci* **82-83**, 13-20.

Memili E and First NL (1999) Control of gene expression at the onset of bovine embryonic development. *Biol Reprod* **61**, 1198-1207.

Memili E and First NL (2000) Zygotic and embryonic gene expression in cow: a review of timing and mechanisms of early gene expression as compared with other species. *Zygote* **8**, 87-96.

Mhanni AA and McGowan RA (2002) Variations in DNA (cytosine-5)-methyltransferase-1 expression during oogenesis and early development of the zebrafish. *Dev Genes Evol* **212**, 530-533.

Mikkelsen AL, Host E and Lindenberg S (2001) Incidence of apoptosis in granulosa cells from immature human follicles. *Reproduction* **122**, 481-486.

Millar JB and Russell P (1992) The cdc25 M-phase inducer: an unconventional protein phosphatase. *Cell* **68**, 407-410.

Mood K, Bong YS, Lee HS, Ishimura A and Daar IO (2004) Contribution of JNK, Mek, Mos and PI-3K signaling to GVBD in Xenopus oocytes. *Cell Signal* **16**, 631-642.

Moreno S and Nurse P (1990) Substrates for p34cdc2: in vivo veritas? *Cell* **61**, 549-551.

Moskovtsev SI, Griffin JT, Peterson CM and Carrell DT (2002) Primordial and pre-antral follicles are not commonly observed in IVF aspirates. *Hum Reprod* **17**, 1783-1787.

Murray AA, Gosden RG, Allison V and Spears N (1998) Effect of androgens on the development of mouse follicles growing in vitro. *J Reprod Fertil* **113**, 27-33.

Murray AA, Molinek MD, Baker SJ, Kojima FN, Smith MF, Hillier SG and Spears N (2001) Role of ascorbic acid in promoting follicle integrity and survival in intact mouse ovarian follicles in vitro. *Reproduction* **121**, 89-96.

Neilson L, Andalibi A, Kang D, Coutifaris C, Strauss JF, 3rd, Stanton JA and Green DP (2000) Molecular phenotype of the human oocyte by PCR-SAGE. *Genomics* **63**, 13-24.

Newton H, Aubard Y, Rutherford A, Sharma V and Gosden R (1996) Low temperature storage and grafting of human ovarian tissue. *Hum Reprod* **11**, 1487-1491.

Newton H, Fisher J, Arnold JR, Pegg DE, Faddy MJ and Gosden RG (1998) Permeation of human ovarian tissue with cryoprotective agents in preparation for cryopreservation. *Hum Reprod* **13**, 376-380.

Nie Z (1992) Fetuin: its enigmatic property of growth promotion. *Am J Physiol* **263**, C551-562.

Nilsson E, Parrott JA and Skinner MK (2001) Basic fibroblast growth factor induces primordial follicle development and initiates folliculogenesis. *Mol Cell Endocrinol* **175**, 123-130.

Nilsson EE, Kezele P and Skinner MK (2002) Leukemia inhibitory factor (LIF) promotes the primordial to primary follicle transition in rat ovaries. *Mol Cell Endocrinol* **188**, 65-73.

Nilsson EE and Skinner MK (2003) Bone morphogenetic protein-4 acts as an ovarian follicle survival factor and promotes primordial follicle development. *Biol Reprod* **69**, 1265-1272.

Nilsson EE and Skinner MK (2004) Kit ligand and basic fibroblast growth factor interactions in the induction of ovarian primordial to primary follicle transition. *Mol Cell Endocrinol* **214**, 19-25.

Nothias JY, Majumder S, Kaneko KJ and DePamphilis ML (1995) Regulation of gene expression at the beginning of mammalian development. *J Biol Chem* **270**, 22077-22080.

O'Brien MJ, Pendola JK and Eppig JJ (2003) A revised protocol for in vitro development of mouse oocytes from primordial follicles dramatically improves their developmental competence. *Biol Reprod* **68**, 1682-1686.

Oh B, Hwang SY, Solter D and Knowles BB (1997) Spindlin, a major maternal transcript expressed in the mouse during the transition from oocyte to embryo. *Development* **124**, 493-503.

O'Keefe SJ, Kiessling AA and Cooper GM (1991) The c-mos gene product is required for cyclin B accumulation during meiosis of mouse eggs. *Proc Natl Acad Sci U S A* **88**, 7869-7872.

Oktay K, Briggs D and Gosden RG (1997) Ontogeny of follicle-stimulating hormone receptor gene expression in isolated human ovarian follicles. *J Clin Endocrinol Metab* **82**, 3748-3751.

Oktay K, Buyuk E, Veeck L, Zaninovic N, Xu K, Takeuchi T, Opsahl M and Rosenwaks Z (2004) Embryo development after heterotopic transplantation of cryopreserved ovarian tissue. *Lancet* **363**, 837-840.

Oktay K, Economos K, Kan M, Rucinski J, Veeck L and Rosenwaks Z (2001) Endocrine function and oocyte retrieval after autologous transplantation of ovarian cortical strips to the forearm. *Jama* **286**, 1490-1493.

Oktay K and Karlikaya G (2000) Ovarian function after transplantation of frozen, banked autologous ovarian tissue. *N Engl J Med* **342**, 1919.

Oktay K, Newton H and Gosden RG (2000) Transplantation of cryopreserved human ovarian tissue results in follicle growth initiation in SCID mice. *Fertil Steril* **73**, 599-603.

Oktay K, Newton H, Mullan J and Gosden RG (1998) Development of human primordial follicles to antral stages in SCID/hpg mice stimulated with follicle stimulating hormone. *Hum Reprod* **13**, 1133-1138.

Oktay K, Nugent D, Newton H, Salha O, Chatterjee P and Gosden RG (1997) Isolation and characterization of primordial follicles from fresh and cryopreserved human ovarian tissue. *Fertil Steril* **67**, 481-486.

Oktay KH and Yih M (2002) Preliminary experience with orthotopic and heterotopic transplantation of ovarian cortical strips. *Semin Reprod Med* **20**, 63-74.

Otala M, Erkkila K, Tuuri T, Sjoberg J, Suomalainen L, Suikkari AM, Pentikainen V and Dunkel L (2002) Cell death and its suppression in human ovarian tissue culture. *Mol Hum Reprod* **8**, 228-236.

Otala M, Makinen S, Tuuri T, Sjoberg J, Pentikainen V, Matikainen T and Dunkel L (2004) Effects of testosterone, dihydrotestosterone, and 17beta-estradiol on human ovarian tissue survival in culture. *Fertil Steril* **82 Suppl 3**, 1077-1085.

Parrott JA and Skinner MK (1999) Kit-ligand/stem cell factor induces primordial follicle development and initiates folliculogenesis. *Endocrinology* **140**, 4262-4271.

Paynter SJ (2000) Current status of the cryopreservation of human unfertilized oocytes. *Hum Reprod Update* **6**, 449-456.

Pennetier S, Uzbekova S, Perreau C, Papillier P, Mermillod P and Dalbies-Tran R (2004) Spatio-temporal expression of the germ cell marker genes MATER, ZAR1, GDF9, BMP15,andVASA in adult bovine tissues, oocytes, and preimplantation embryos. *Biol Reprod* **71**, 1359-1366.

Peter M, Labbe JC, Doree M and Mandart E (2002) A new role for Mos in Xenopus oocyte maturation: targeting Myt1 independently of MAPK. *Development* **129**, 2129-2139.

Picton H, Briggs D and Gosden R (1998) The molecular basis of oocyte growth and development. *Mol Cell Endocrinol* **145**, 27-37.

Picton HM, Kim SS and Gosden RG (2000) Cryopreservation of gonadal tissue and cells. *Br Med Bull* **56**, 603-615.

Pines J and Hunter T (1989) Isolation of a human cyclin cDNA: evidence for cyclin mRNA and protein regulation in the cell cycle and for interaction with p34cdc2. *Cell* **58**, 833-846.

Pomerance M, Thang MN, Tocque B and Pierre M (1996) The Ras-GTPase-activating protein SH3 domain is required for Cdc2 activation and mos induction by oncogenic Ras in Xenopus oocytes independently of mitogen-activated protein kinase activation. *Mol Cell Biol* **16**, 3179-3186.

Prioleau MN, Huet J, Sentenac A and Mechali M (1994) Competition between chromatin and transcription complex assembly regulates gene expression during early development. *Cell* **77**, 439-449.

Quennell JH, Stanton JA and Hurst PR (2004) Basic fibroblast growth factor expression in isolated small human ovarian follicles. *Mol Hum Reprod* **10**, 623-628.

Radford JA, Lieberman BA, Brison DR, Smith AR, Critchlow JD, Russell SA, Watson AJ, Clayton JA, Harris M, Gosden RG and Shalet SM (2001) Orthotopic reimplantation of cryopreserved ovarian cortical strips after high-dose chemotherapy for Hodgkin's lymphoma. *Lancet* **357**, 1172-1175.

Rahimi G, Isachenko E, Isachenko V, Sauer H, Wartenberg M, Tawadros S, Hescheler J, Mallmann P and Nawroth F (2004) Comparison of necrosis in human ovarian tissue after conventional slow freezing or vitrification and transplantation in ovariectomized SCID mice. *Reprod Biomed Online* **9**, 187-193.

Rahimi G, Isachenko E, Sauer H, Isachenko V, Wartenberg M, Hescheler J, Mallmann P and Nawroth F (2003) Effect of different vitrification protocols for human ovarian tissue on reactive oxygen species and apoptosis. *Reprod Fertil Dev* **15**, 343-349.

Rajkovic A and Matzuk MM (2002) Functional analysis of oocyte-expressed genes using transgenic models. *Mol Cell Endocrinol* **187**, 5-9.

Rajkovic A, Pangas SA, Ballow D, Suzumori N and Matzuk MM (2004) NOBOX deficiency disrupts early folliculogenesis and oocyte-specific gene expression. *Science* **305**, 1157-1159.

Rall WF, Reid DS and Polge C (1984) Analysis of slow-warming injury of mouse embryos by cryomicroscopical and physiochemical methods. *Cryobiology* **21**, 106-121.

Rankin T, Familari M, Lee E, Ginsberg A, Dwyer N, Blanchette-Mackie J, Drago J, Westphal H and Dean J (1996) Mice homozygous for an insertional mutation in the Zp3 gene lack a zona pellucida and are infertile. *Development* **122**, 2903-2910.

Rankin TL, O'Brien M, Lee E, Wigglesworth K, Eppig J and Dean J (2001) Defective zonae pellucidae in Zp2-null mice disrupt folliculogenesis, fertility and development. *Development* **128**, 1119-1126.

Reverte CG, Yuan L, Keady BT, Lacza C, Attfield KR, Mahon GM, Freeman B, Whitehead IP and Hake LE (2003) XGef is a CPEB-interacting protein involved in Xenopus oocyte maturation. *Dev Biol* **255**, 383-398.

Richard FJ, Tsafriri A and Conti M (2001) Role of phosphodiesterase type 3A in rat oocyte maturation. *Biol Reprod* **65**, 1444-1451.

Richter JD (1999) Cytoplasmic polyadenylation in development and beyond. *Microbiol Mol Biol Rev* **63**, 446-456.

Rime H, Haccard O and Ozon R (1992) Activation of p34cdc2 kinase by cyclin is negatively regulated by cyclic amp-dependent protein kinase in Xenopus oocytes. *Dev Biol* **151**, 105-110.

Sauerwald TM, Betenbaugh MJ and Oyler GA (2002) Inhibiting apoptosis in mammalian cell culture using the caspase inhibitor XIAP and deletion mutants. *Biotechnol Bioeng* **77**, 704-716.

Scott JE, Carlsson IB, Bavister BD and Hovatta O (2004) Human ovarian tissue cultures: extracellular matrix composition, coating density and tissue dimensions. *Reprod Biomed Online* **9**, 287-293.

Scott JE, Zhang P and Hovatta O (2004) Benefits of 8-bromo-guanosine 3',5'-cyclic monophosphate (8-br-cGMP) in human ovarian cortical tissue culture. *Reprod Biomed Online* **8**, 319-324.

Seli E, Lalioti MD, Flaherty SM, Sakkas D, Terzi N and Steitz JA (2005) An embryonic poly(A)-binding protein (ePAB) is expressed in mouse oocytes and early preimplantation embryos. *Proc Natl Acad Sci U S A* **102**, 367-372.

Sharan SK, Pyle A, Coppola V, Babus J, Swaminathan S, Benedict J, Swing D, Martin BK, Tessarollo L, Evans JP, Flaws JA and Handel MA (2004) BRCA2 deficiency in mice leads to meiotic impairment and infertility. *Development* **131**, 131-142.

Shaw JM, Oranratnachai A and Trounson AO (2000) Fundamental cryobiology of mammalian oocytes and ovarian tissue. *Theriogenology* **53**, 59-72.

Skalhegg BS and Tasken K (2000) Specificity in the cAMP/PKA signaling pathway. Differential expression,regulation, and subcellular localization of subunits of PKA. *Front Biosci* **5**, D678-693.

Sonmezer M and Oktay K (2004) Fertility preservation in female patients. *Hum Reprod Update* **10**, 251-266.

Soyal SM, Amleh A and Dean J (2000) FIGalpha, a germ cell-specific transcription factor required for ovarian follicle formation. *Development* **127**, 4645-4654.

Sternlicht AL and Schultz RM (1981) Biochemical studies of mammalian oogenesis: kinetics of accumulation of total and poly(A)-containing RNA during growth of the mouse oocyte. *J Exp Zool* **215**, 191-200.

Suzuki A, de la Pompa JL, Hakem R, Elia A, Yoshida R, Mo R, Nishina H, Chuang T, Wakeham A, Itie A, Koo W, Billia P, Ho A, Fukumoto M, Hui CC and Mak TW (1997) Brca2 is required for embryonic cellular proliferation in the mouse. *Genes Dev* **11**, 1242-1252.

Talmor-Cohen A, Tomashov-Matar R, Eliyahu E, Shapiro R and Shalgi R (2004) Are Src family kinases involved in cell cycle resumption in rat eggs? *Reproduction* **127**, 455-463.

Taylor CT (1994) Calcium signals and human oocyte activation: implications for assisted conception. *Hum Reprod* **9**, 980-984.

Telford NA, Watson AJ and Schultz GA (1990) Transition from maternal to embryonic control in early mammalian development: a comparison of several species. *Mol Reprod Dev* **26**, 90-100.

Tesarik J, Sousa M and Testart J (1994) Human oocyte activation after intracytoplasmic sperm injection. *Hum Reprod* **9**, 511-518.

Thomas FH, Leask R, Srsen V, Riley SC, Spears N and Telfer EE (2001) Effect of ascorbic acid on health and morphology of bovine preantral follicles during long-term culture. *Reproduction* **122**, 487-495.

Tilly JL, Kowalski KI, Johnson AL and Hsueh AJ (1991) Involvement of apoptosis in ovarian follicular atresia and postovulatory regression. *Endocrinology* **129**, 2799-2801.

Tilly JL and Tilly KI (1995) Inhibitors of oxidative stress mimic the ability of follicle-stimulating hormone to suppress apoptosis in cultured rat ovarian follicles. *Endocrinology* **136**, 242-252.

Tong ZB, Gold L, Pfeifer KE, Dorward H, Lee E, Bondy CA, Dean J and Nelson LM (2000) Mater, a maternal effect gene required for early embryonic development in mice. *Nat Genet* **26**, 267-268.

Tornell J and Hillensjo T (1993) Effect of cyclic AMP on the isolated human oocyte--cumulus complex. *Hum Reprod* **8**, 737-739.

Turner BM (1991) Histone acetylation and control of gene expression. *J Cell Sci* **99 (Pt 1)**, 13-20.

Vainio S, Heikkila M, Kispert A, Chin N and McMahon AP (1999) Female development in mammals is regulated by Wnt-4 signalling. *Nature* **397**, 405-409.

Van den Broecke R, Liu J, Handyside A, Van der Elst JC, Krausz T, Dhont M, Winston RM and Hovatta O (2001) Follicular growth in fresh and cryopreserved human ovarian cortical grafts transplanted to immunodeficient mice. *Eur J Obstet Gynecol Reprod Biol* **97**, 193-201.

Van Den Broecke R, Van Der Elst J, Liu J, Hovatta O and Dhont M (2001) The female-to-male transsexual patient: a source of human ovarian cortical tissue for experimental use. *Hum Reprod* **16**, 145-147.

van Wezel IL, Umapathysivam K, Tilley WD and Rodgers RJ (1995) Immunohistochemical localization of basic fibroblast growth factor in bovine ovarian follicles. *Mol Cell Endocrinol* **115**, 133-140.

Vendola K, Zhou J, Wang J, Famuyiwa OA, Bievre M and Bondy CA (1999) Androgens promote oocyte insulin-like growth factor I expression and initiation of follicle development in the primate ovary. *Biol Reprod* **61**, 353-357.

Vendola KA, Zhou J, Adesanya OO, Weil SJ and Bondy CA (1998) Androgens stimulate early stages of follicular growth in the primate ovary. *J Clin Invest* **101**, 2622-2629.

Verlhac MH, Kubiak JZ, Clarke HJ and Maro B (1994) Microtubule and chromatin behavior follow MAP kinase activity but not MPF activity during meiosis in mouse oocytes. *Development* **120**, 1017-1025.

Vigneron C, Perreau C, Dupont J, Uzbekova S, Prigent C and Mermillod P (2004) Several signaling pathways are involved in the control of cattle oocyte maturation. *Mol Reprod Dev* **69**, 466-474.

Vitt UA, McGee EA, Hayashi M and Hsueh AJ (2000) In vivo treatment with GDF-9 stimulates primordial and primary follicle progression and theca cell marker CYP17 in ovaries of immature rats. *Endocrinology* **141**, 3814-3820.

Wang QT, Piotrowska K, Ciemerych MA, Milenkovic L, Scott MP, Davis RW and Zernicka-Goetz M (2004) A genome-wide study of gene activity reveals developmental signaling pathways in the preimplantation mouse embryo. *Dev Cell* **6**, 133-144.

Wang Y, Rippstein PU and Tsang BK (2003) Role and gonadotrophic regulation of X-linked inhibitor of apoptosis protein expression during rat ovarian follicular development in vitro. *Biol Reprod* **68**, 610-619.

Weenen C, Laven JS, Von Bergh AR, Cranfield M, Groome NP, Visser JA, Kramer P, Fauser BC and Themmen AP (2004) Anti-Mullerian hormone expression pattern in the human ovary: potential implications for initial and cyclic follicle recruitment. *Mol Hum Reprod* **10**, 77-83.

Wennerholm UB, Albertsson-Wikland K, Bergh C, Hamberger L, Niklasson A, Nilsson L, Thiringer K, Wennergren M, Wikland M and Borres MP (1998) Postnatal growth and health in children born after cryopreservation as embryos. *Lancet* **351**, 1085-1090.

Wilding M and Dale B (1997) Sperm factor: what is it and what does it do? *Mol Hum Reprod* **3**, 269-273.

Wright CS, Hovatta O, Margara R, Trew G, Winston RM, Franks S and Hardy K (1999) Effects of follicle-stimulating hormone and serum substitution on the in-vitro growth of human ovarian follicles. *Hum Reprod* **14**, 1555-1562.

Wu B, Ignotz G, Currie WB and Yang X (1997) Dynamics of maturation-promoting factor and its constituent proteins during in vitro maturation of bovine oocytes. *Biol Reprod* **56**, 253-259.

Wu J, Zhang L and Liu P (1998) A new source of human oocytes: preliminary report on the identification and maturation of human preantral follicles from follicular aspirates. *Hum Reprod* **13**, 2561-2563.

Wu J, Zhang L and Wang X (2001) In vitro maturation, fertilization and embryo development after ultrarapid freezing of immature human oocytes. *Reproduction* **121**, 389-393.

Wu X, Viveiros MM, Eppig JJ, Bai Y, Fitzpatrick SL and Matzuk MM (2003) Zygote arrest 1 (Zar1) is a novel maternal-effect gene critical for the oocyte-to-embryo transition. *Nat Genet* **33**, 187-191.

Xiao JH, Davidson I, Matthes H, Garnier JM and Chambon P (1991) Cloning, expression, and transcriptional properties of the human enhancer factor TEF-1. *Cell* **65**, 551-568.

Yamamoto S, Konishi I, Nanbu K, Komatsu T, Mandai M, Kuroda H, Matsushita K and Mori T (1997) Immunohistochemical localization of basic fibroblast growth factor (bFGF) during folliculogenesis in the human ovary. *Gynecol Endocrinol* **11**, 223-230.

Yan W, Burns KH, Ma L and Matzuk MM (2002) Identification of Zfp393, a germ cell-specific gene encoding a novel zinc finger protein. *Mech Dev* **118**, 233-239.

Yao LJ, Zhong ZS, Zhang LS, Chen DY, Schatten H and Sun QY (2004) Aurora-A is a critical regulator of microtubule assembly and nuclear activity in mouse oocytes, fertilized eggs, and early embryos. *Biol Reprod* **70**, 1392-1399.

Yoshida H, Takakura N, Kataoka H, Kunisada T, Okamura H and Nishikawa SI (1997) Stepwise requirement of c-kit tyrosine kinase in mouse ovarian follicle development. *Dev Biol* **184**, 122-137.

Yu BP (1994) Cellular defenses against damage from reactive oxygen species. *Physiol Rev* **74**, 139-162.

Zernicka-Goetz M, Verlhac MH, Geraud G and Kubiak JZ (1997) Protein phosphatases control MAP kinase activation and microtubule organization during rat oocyte maturation. *Eur J Cell Biol* **72**, 30-38.

Zhang D, Li M, Ma W, Hou Y, Li YH, Li SW, Sun QY and Wang WH (2005) Localization of Mitotic Arrest Deficient 1 (MAD1) in Mouse Oocytes During the First Meiosis and Its Functions as a Spindle Checkpoint Protein. *Biol Reprod* **72**, 58-68.

Zhang D, Ma W, Li YH, Hou Y, Li SW, Meng XQ, Sun XF, Sun QY and Wang WH (2004) Intra-oocyte localization of MAD2 and its relationship with kinetochores, microtubules, and chromosomes in rat oocytes during meiosis. *Biol Reprod* **71**, 740-748.

Zhang J, Liu J, Xu KP, Liu B and DiMattina M (1995) Extracorporeal development and ultrarapid freezing of human fetal ova. *J Assist Reprod Genet* **12**, 361-368.

Zhang P, Hreinsson JG, Telfer E and Hovatta O (2002) Few instead of many: human follicle collection from follicular aspirates at oocyte retrieval. *Hum Reprod* **17**, 3190-3192.

Zhang P, Louhio H, Tuuri T, Sjoberg J, Hreinsson J, Telfer EE and Hovatta O (2004) In vitro effect of cyclic adenosine 3', 5'-monophosphate (cAMP) on early human ovarian follicles. *J Assist Reprod Genet* **21**, 301-306.