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**PRESERVATION OF  
FERTILITY THROUGH  
CRYOPRESERVATION AND  
IN VITRO MATURATION OF  
HUMAN OVARIAN  
FOLLICLES AND OOCYTES**

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"The boys just call me Camouflage"  
Stan Ridgeway



## **ABSTRACT**

One of the most rapidly expanding fields in assisted reproduction is the preservation of fertility for young women at risk of premature ovarian failure. This may be caused by cytotoxic therapy or other reasons. Cryopreservation of follicles in ovarian tissue has been successful in animal models with live young being born. Furthermore, the survival of human ovarian follicles after cryopreservation and thawing has been shown. In vitro maturation of oocytes (IVM) is important for patients having ovarian tissue cryopreserved and stored for further use. This method has also applications in clinical assisted reproduction in order to avoid ovarian hyperstimulation syndrome, which is a serious side effect of ovarian hyperstimulation during IVF treatment.

The aim of this thesis was to develop methods for cryopreservation of follicles in human ovarian tissue, to identify new groups of patients who might benefit from such methods and to develop techniques for in-vitro maturation of ovarian follicles and oocytes.

The effect of growth differentiation factor-9 (GDF-9) on early human ovarian follicles was studied in an organ culture system. A significantly higher proportion of cultured primordial follicles showed initiation of growth, reaching the secondary stage of development in the presence of GDF-9 compared to controls. Follicle viability was also improved with GDF-9 resulting in a smaller reduction in follicle numbers due to atresia. GDF-9 thus promoted growth, development, and survival of human ovarian follicles in organ culture.

In a study comparing the use of serum and human serum albumin (HSA) for cryoprotectant solutions in cryopreservation of human ovarian cortical tissue, good viability of the follicles after thawing was shown using light microscopy, transmission electron microscopy and live/dead assay for analysis. A cryoprotectant solution containing HSA was equally effective as a cryoprotectant containing serum.

Recombinant LH and recombinant hCG were equally efficient in promoting the maturation of oocytes in clinical in-vitro maturation (IVM). An IVM programme was established in the process, giving a powerful method to study the final stages of oocyte maturation in addition to clinical applications.

Follicular density was analysed in ovarian cortical tissue from nine out of ten adolescent girls with Turner's syndrome, coming for preservation of ovarian tissue for possible fertility treatment later in life. Follicles were found in the biopsy tissue from eight subjects, the highest numbers being seen in the youngest girls and in those with mosaicism where not all of the cells in the body have the XO karyotype. Follicular density was negatively correlated with serum levels of FSH. The finding that adolescent girls with Turner's syndrome still have follicles in their ovaries runs contrary to previous assumptions that these women in most cases have no hope of having their own genetic offspring and raises the possibility of future fertility through cryopreservation of ovarian tissue.

A culture system for human ovarian follicles and oocytes has been established through these projects and the process of cryopreservation of follicles in ovarian tissue has been studied. These methods are of great importance for women at risk for premature ovarian failure and cryopreservation of ovarian tissue is now offered for these women at Huddinge University Hospital. The need to further develop these methods is evident and more studies along these lines are being conducted.



## LIST OF PUBLICATIONS

- I. **Hreinsson J**, Scott JE, Rasmussen C, Swahn ML, Hsueh AJ, 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.
  
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- III. **Hreinsson J**, Zhang P, Swahn ML, Hultenby K, Hovatta O. 2003. Cryopreservation of follicles in human ovarian cortical tissue. Comparison of serum and human serum albumin in the cryoprotectant solutions. *Human Reproduction*, in press.
  
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## 1 ABBREVIATIONS

AMH	Anti-Mullerian Hormone
ART	Assisted Reproductive Techniques
BMP	Bone Morphogenetic Protein
DMSO	Di Methyl Sulfoxide
FSH	Follicle stimulating hormone
GDF-9	Growth Differentiation Factor 9
GnRH	Gonadotrophin Releasing Hormone
GnRHa	Gonadotrophin Releasing Hormone Agonist
GV	Germinal Vesicle
Gy	Gray, absorbed dose of irradiation
hCG	Human Chorionic Gonadotrophin
HSA	Human Serum Albumin
ICSI	Intracytoplasmic Sperm Injection
IVF	In vitro fertilization
kDa	KiloDalton
LH	Luteinizing Hormone
MII	Metaphase II of meiosis
PCO	Polycystic Ovaries
PrOH	Propane Diol
PGD	Preimplantation Genetic Diagnosis
TGF $\beta$	Transforming Growth Factor $\beta$



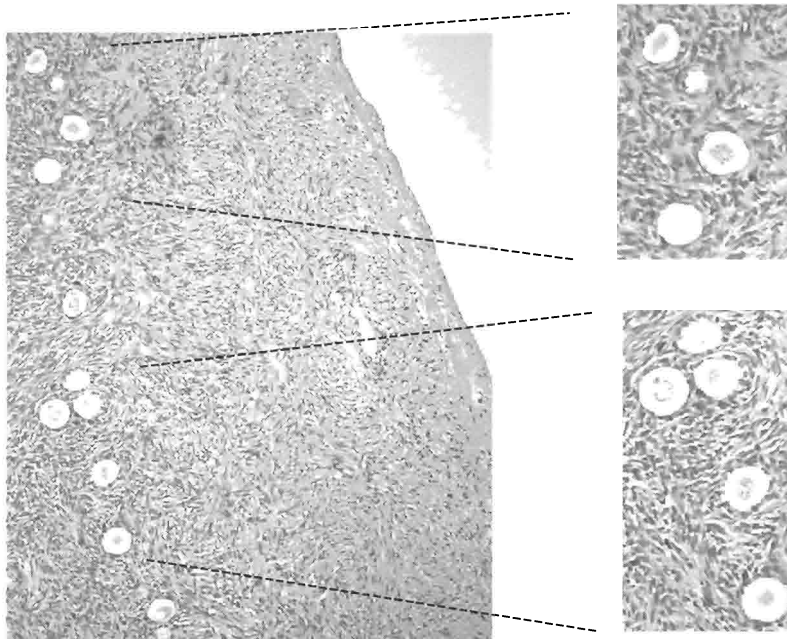
## 2 INTRODUCTION AND LITERATURE REVIEW

### 2.1 FOLLICULOGENESIS AND FOLLICULAR GROWTH

#### 2.1.1 Primordial germ cells

The ovaries are almond-shaped structures approximately 3 cm in length, 1.5 cm wide and 1 cm thick. Before puberty the surface of the ovary is smooth, but it becomes scarred and irregular during reproductive life due to repeated ovulations. After menopause, the ovaries are approximately one fourth the size observed during the reproductive period (Ross *et al.*, 2003). The ovary consists of two regions. The central portion of the ovary is called the medulla and contains loose connective tissue, blood vessels, lymphatic vessels and nerves. The peripheral portion of the ovary surrounding the medulla is called the ovarian cortex. The cortex contains the ovarian follicles at a depth of approximately 1-2 mm, embedded in a compact richly cellular connective tissue (Figure 1).

**Figure 1**



The ovarian follicles lie at a depth of 1-2 mm in the ovarian cortex. Two enlarged images are shown on the right. The follicles are approximately 40 microns in diameter.

Oocytes in the ovarian follicles originate from primordial germ cells (PGC). These cells are thought to have an identical early history in both sexes, originating soon after gastrulation (Buehr, 1997). The PGC's migrate from the primary ectoderm during the second week of development into an extraembryonic structure called the yolk sac. Between week 4 and 6 of fetal life, these cells continue to migrate to the wall of the gut tube and via the mesentery to the dorsal body wall. They come to rest on either side of the midline and colonize the developing gonads by 7 weeks gestation (Witschi, 1948; Gondos *et al.*, 1971; Fujimoto *et al.*, 1977; Motta and Makabe, 1986; Larsen, 2001). The PGC's continue to multiply by mitosis and stimulate cells of the adjacent epithelium to form the primitive sex chords, which contribute to the formation of the follicular cells. At approximately 10 weeks gestation, female PGC's start to enter meiosis, while male PGC's continue to divide until they are arrested in mitosis at 16–18 weeks gestation (Gondos and Hobel, 1971; Gondos *et al.*, 1986).

After colonization of the gonadal primordium the sex specific differences appear, the germ cells differentiate into oogonia or spermatogonia, depending both on their chromosomal constitution and on the environment in which they develop. The initial "decision" to embark on oogenesis or spermatogenesis depends on their environment, and not on their own chromosomes (McLaren 1995). In the female fetus, the somatic sex cord cells do not contain a Y chromosome, no expression of Y-specific proteins is present and therefore differentiation into Sertoli cells is not seen. In the absence of Sertoli cells and the Y-specific proteins Leydig cells are not formed and testosterone is not produced. The absence of testosterone leads to the degeneration of the Wolffian duct. Male development of the sexual structures is not stimulated, and female development ensues through further development of the Müllerian duct, which is the precursor of the female sexual structures (Larsen 2001).

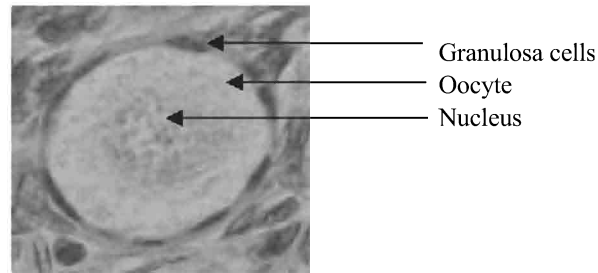
This traditional view of female development as a default state resulting primarily from the absence of the testis-determining factor is undoubtedly oversimplified. A recently identified member of the nuclear hormone-receptor superfamily, Dax-1, has been implicated in the control of female development and may be responsible for a sex-reversal syndrome in humans, referred to as dosage-sensitive sex reversal (Swain *et al.*, 1998). In this syndrome, XY individuals carrying duplications of Xp21, a part of the small arm of the X chromosome, develop as females.

A locally acting cell signal, Wnt-4 has also been shown to be essential for development of the female reproductive system. Wnt-4 appears to be required for formation of the Müllerian duct, which is the progenitor of the oviduct, uterus and upper part of the vagina. Wnt-4 also appears to suppress inappropriate development of Leydig cell precursors in the developing ovary and to aid in maintaining oocyte development (Cadigan and Nusse, 1997; Vainio *et al.*, 1999).

The oogonia proliferate by mitotic divisions until they enter meiosis and arrest in the prophase of the first meiotic division. These primary oocytes form clusters, each surrounded by a flat layer of follicle cells, thus forming functional units of primordial follicles (Figure 2). The nucleus of these dormant primary oocytes becomes very large and is called a germinal vesicle (GV). The total number of germ cells reaches a maximum of 6 to 7 million at five months of gestational age in the female fetus. At this time, two thirds of the total germ cells are primary oocytes; the remaining third can still be viewed as oogonial. The follicles are depleted through atresia to approximately one

to two million primordial follicles at birth and 3-400.000 follicles at menarche (Baker, 1963; Himelstein-Braw *et al.*, 1976; Faddy, 2000).

**Figure 2**



A primordial follicle with thin granulosa cells surrounding the oocyte.

### 2.1.2 Growth factors and pre-antral follicle growth

The oocytes in the primordial follicles play a key role in follicular development. They are essential for the initial assembly of the follicles, and bidirectional communication between the oocyte and the surrounding follicular cells is important through the entire growth phase of the follicle (Eppig, 2001). Growth factors secreted by the oocytes in growing follicles affect the growth and differentiation of the surrounding granulosa cells and follicular development may be orchestrated and coordinated according to a developmental program intrinsic to the oocyte. The oocyte, previously thought to be a passive passenger in the follicle, now has been suggested to be the captain of the ship (Eppig *et al.*, 2002).

The early stages of follicle growth depend on a multitude of factors, including follicle stimulating hormone (FSH) (Yen *et al.*, 1999). The nature of the signal that causes initiation of growth of the follicles that have been resting for up to 50 years has yet to be determined. Whether this is a positive signal or a release from an inhibitory factor has been discussed (Gougeon and Busso, 2000). Controlling factors regarding the mechanisms of apoptosis of the follicles are also being studied.

To enable the evaluation of follicular development, a classification system has been described (Gougeon, 1986; Gougeon, 1996) which we have used in our evaluations of follicular development. Briefly, the resting primordial follicles are characterized by a single layer of flattened granulosa cells surrounding the oocyte, primary follicles have at least some cuboidal granulosa cells and secondary follicles have attained more than one layer of granulosa cells. The early growth phase is characterised by granulosa cell proliferation and increase in size of the oocyte. As the follicle becomes larger, the surrounding stroma stratifies and differentiates, developing the theca interna and theca externa. The theca cells produce androgens under the control of LH, and the androgens are aromatized to estrogen in the granulosa cells of the growing follicles. During follicle growth, the oocyte becomes surrounded by a thick extracellular coat, the zona

pellucida. Zona pellucida glycoproteins are synthesized exclusively by oocytes during their growth phase and are then assembled in a highly specific manner. These unique glycoproteins play important roles during mammalian oogenesis, fertilization, and preimplantation development and construction of the zona pellucida is a major activity of growing oocytes as they prepare for ovulation and fertilization (Wassarman, 1991). The granulosa cells closest to the oocyte retain direct contact with the plasma membrane of the oocyte through cellular processes traversing the zona pellucida. During the final stages of oocyte maturation before ovulation these processes are disrupted and the direct contact between the oocyte and granulosa/cumulus cells is lost (Gilula *et al.*, 1978; Yen *et al.*, 1999).

The smallest early growing follicles lack an independent blood supply, but secondary follicles of 80-100  $\mu\text{m}$  in diameter are served by one or two arterioles (Gougeon, 1996). Vascular endothelial growth factor (VEGF) has been shown to be important in this respect as inhibition of VEGF effect resulted in decreases in theca cell proliferation, decreased endothelial cell area, decreased development of antral follicles, less granulosa cell proliferation, decreased follicular diameter and lack of development of ovulatory follicles (Wulff *et al.*, 2002). Furthermore, the rate of atresia was significantly increased. VEGF has also been seen to stimulate preantral follicular development in the rat ovary (Danforth *et al.*, 2003). However, the follicle itself is essentially avascular and thus concentration gradients of growth factors may be established during its growth and development.

A description follows of the growth factors involved in early follicular growth, especially those currently under most intensive study.

#### 2.1.2.1 TGF- $\beta$ superfamily

Several growth factors have been identified as being of importance in early follicular growth. Among these are the members of the Transforming Growth Factor  $\beta$  (TGF- $\beta$ ) superfamily of peptide growth factors, shown to be important at these early stages (Erickson and Shimasaki, 2000). These include AMH (Anti Müllerian Hormone, also known as Mullerian Inhibiting Substance or MIS), activins, inhibins, Growth Differentiation Factor-9 (GDF-9), GDF-9B and the bone morphogenetic proteins, or BMP's.

The TGF- $\beta$  superfamily consists of some 40 genes; the first ones identified over 20 years ago (Roberts *et al.*, 1981). They are a family of 25 kDa structurally related polypeptides and exist as homo- or heterodimers. They have been proposed to have regulatory effects on follicular growth (Gougeon and Busso, 2000).

Two types of receptors have been identified for these polypeptides, Type I and Type II, each including several subtypes. These are structurally related transmembrane serine/threonine kinases responsible for the binding almost all of TGF- $\beta$  superfamily members (Massagué, 1998). To mediate signalling by the TGF- $\beta$  superfamily members, a tetrameric complex of two type I and two type II receptors is required (Wrana *et al.*, 1992). Binding proteins that exert antagonistic effects to these growth factors have also been identified, for example blocking of BMP signalling has been shown by binding of such proteins to the ligand and thus preventing receptor binding (Miyazono, 2000). Follistatin, first purified from follicular fluid (Robertson *et al.*, 1987; Ueno *et al.*, 1987), is an important inhibitor of activin function and exerts its effect by binding to their common  $\beta$  subunits (Shimonaka *et al.*, 1991). Follistatin also



blocks the biological effects of other TGF- $\beta$  superfamily members, such as several BMP's including GDF-9B (Otsuka *et al.*, 2001a). The importance of follistatin in regulating growth factor activity is supported by the observation of multiple defects and perinatal death in mice with a targeted disruption of the follistatin gene (Matzuk *et al.*, 1995).

Once the TGF- $\beta$  ligand has initiated signalling by binding to and bringing together type I and type II receptor serine/threonine kinases on the cell surface, receptor II phosphorylates the receptor I kinase domain. The signal is further propagated through phosphorylation of intracellular proteins, the so-called Smad proteins. There are eight distinct Smad proteins known (Shi and Massague, 2003) and they are divided into three functional classes. Receptor-regulated Smad (R-Smad), Co-mediator Smad (Co-Smad), and the inhibitory Smad (I-Smad). R-Smads are directly phosphorylated and activated by the type I receptor kinases, they undergo homotrimerization and formation of heteromeric complexes with Co-Smad. The activated Smad complexes are translocated into the nucleus and, in conjunction with other nuclear cofactors, regulate the transcription of target genes. The I-Smads negatively regulate TGF- $\beta$  signaling by competing with R-Smads for receptor or Co-Smad interaction and by targeting the receptors for degradation (Shi and Massague, 2003).

TGF- $\beta$  signaling generally has a negative effect on cell growth and inactivation of this pathway contributes to malignancies. Tumor-derived mutations have been observed in both TGF- $\beta$  family receptors and the Smad proteins and many other somatic and hereditary disorders arise as a result of mutations or malfunctions in the TGF- $\beta$  pathway (Massagué *et al.*, 2000).

#### 2.1.2.2 GDF-9 and GDF-9 B

GDF-9 is expressed and secreted by the oocytes during early follicular development (McGrath *et al.*, 1995) and a morphogen gradient of GDF-9 has been proposed to be partly responsible for granulosa cell differentiation in-vivo, using rodents (Erickson and Shimasaki, 2000). GDF-9 is obligatory for folliculogenesis and fertility and its expression is correlated with recruitment of follicles for growth (Dong *et al.*, 1996). GDF-9 was the first oocyte derived growth factor shown to be necessary for somatic cell function in vivo. In GDF-9 deficient mice, several aberrations are observed. Among the most serious is that although the oocytes grow in their follicles the growth of the follicular cells is retarded which results in an arrest in follicle growth and infertility (Carabatsos *et al.*, 1998).

In the human ovary, the signaling pathway for GDF-9 has been studied and some of its molecular components have been identified in isolated human granulosa-luteal cells. GDF-9 was seen to activate Smad2 phosphorylation, acting in a similar way as TGF- $\beta$  and activin, although parallel pathways may exist. GDF-9 was also seen to stimulate inhibin-B production in these cells, indicating that local paracrine control of inhibin production may occur via oocyte derived factors (Kaivo-Oja *et al.*, 2003).

Several studies have addressed the biological effects exerted by GDF-9. Growth of preantral follicles isolated from immature rats was enhanced by treatment with either GDF-9 or FSH with the combined treatment showing an additive effect (Hayashi *et al.*, 1999). In oocyte free cumulus cell complexes, GDF-9 was able to induce cumulus expansion in vitro. The key events in the production of the hyaluronic acid-rich extracellular matrix produced during cumulus expansion are the induction of

hyaluronan synthase 2 (HAS2) and suppression of urokinase plasminogen activator (uPA) mRNA synthesis. GDF-9 was able to induce these and other important effects in cultured mouse granulosa cells demonstrating that GDF-9 binds to receptors on granulosa cells and regulates expression of a number of gene products (Elvin *et al.*, 1999). GDF-9 has been shown to be a proliferation factor for cultured granulosa cells from early antral and preovulatory rat follicles but it suppressed FSH-induced differentiation of the same cells (Vitt *et al.*, 2000a). Intraperitoneal injections of GDF-9 twice a day for 7 or 10 days in 5-day-old female rats increased ovarian weight and increased the number of primary and small preantral follicles concomitant with a decrease in the number of primordial follicles. The number of large preantral follicles was only minimally affected (Vitt *et al.*, 2000b).

GDF-9 is expressed in human oocytes during early follicular development (Dube *et al.*, 1998). Another homologue GDF-9B, also known as BMP-15, was identified and cloned from human ovaries by Aaltonen and co-workers (Aaltonen *et al.*, 1999). Both of these oocyte-derived homologues have been shown to be essential for normal follicular development in sheep, including both the early and later stages of growth (Juengel *et al.*, 2002). The major biological functions of GDF-9B in the ovary are considered to be to stimulate granulosa cell mitosis, to inhibit the expression of FSH receptor mRNA in granulosa cells and to stimulate the expression of kit ligand mRNA in granulosa cells (Otsuka *et al.*, 2000; Otsuka *et al.*, 2001b; Otsuka and Shimasaki, 2002). Mutations in GDF-9B are associated with the infertility and the increased ovulation rate seen in Inverdale and Hanna sheep in such a way that sheep which are heterozygous for the mutation show an increase in ovulation rate whereas homozygotes are infertile (Galloway *et al.*, 2000; Galloway *et al.*, 2002). The sheep are an oligoovulatory species and are therefore more likely to have aspects of follicle regulation in common with humans than rodents. This observation confirms the profound effect of these growth factors on the regulation of follicular growth.

### 2.1.2.3 AMH

A strong negative correlation exists between the total number of follicles in the ovaries and the proportion of growing follicles. As the follicles are depleted with age through atresia or growth initiation, the proportion of growing follicles at any given time increases (Gougeon *et al.*, 1994). This observation has resulted in the hypothesis that an inhibitory signal produced by the primordial and primary follicles themselves is responsible for maintaining the growth arrest in the primordial follicles and as the follicle pool is depleted more follicles are released from these inhibitory effects (Gougeon, 1996). This is supported by the observations that an activation of follicle growth is seen during culture in-vitro, possibly because of a release from inhibitory effects in the ovary. This has been observed both in bovine (Wandji *et al.*, 1996; Wandji *et al.*, 1997) and in human ovarian follicles (Hovatta *et al.*, 1997).

Another TGF- $\beta$  superfamily member, anti mullerian hormone or AMH, has been proposed as this putative inhibitory factor. AMH induces regression of the Mullerian ducts during male sexual differentiation, however the expression patterns of AMH in the ovary indicate that it may play a role in folliculogenesis (Durlinger *et al.*, 2002a). The role of AMH in this respect is indirectly supported by observations that AMH concentrations in serum decrease with age (de Vet *et al.*, 2002) and that a strong correlation exists between AMH concentration in serum and ovarian follicle status

reflected by early antral follicle counts on day 3 of the menstrual cycle. This correlation was shown to be stronger with AMH than with other hormonal parameters, such as serum levels of inhibin B, estradiol, FSH and LH (Fanchin *et al.*, 2003). AMH has been proposed as a marker for the ovarian reserve. AMH was positively correlated with the ovarian response, as expressed by the number of oocytes retrieved after oocyte collection. Poor response in IVF, indicative of a diminished ovarian reserve, was associated with reduced baseline serum AMH concentrations (van Rooij *et al.*, 2002). Studies on mice have shown that AMH is an important regulator of follicle growth. Mice with an inactivating mutation in the AMH gene show an increased number of growing follicles compared with wild-type mice (Durlinger *et al.*, 1999). In 13-month-old AMH null females, hardly any primordial follicles were observed and a reduced number of antral follicles was seen, indicating an early depletion of primordial follicles in these mice. A reduction in the number of growing follicles was seen in mouse ovaries cultured in the presence of AMH (Durlinger *et al.*, 2002b) showing the inhibitory effect on the growth recruitment of these follicles. Thus, AMH is the first growth factor to show inhibition of follicle growth, making it a candidate for the putative inhibitor of initiation of follicle growth in the ovary. However it is unlikely to be the only one.

#### 2.1.2.4 Inhibins and Activins

Inhibins and activins are important subclasses of the TGF- $\beta$  superfamily. They are disulphide-linked dimers where inhibins share a common  $\alpha$  subunit and differ on the basis of the A or B  $\beta$  subunits: inhibin A and inhibin B. Activins are dimers of the  $\beta$  subunits of inhibin, either homodimers of the  $\beta$  subunit (Activin A:  $\beta A\beta A$ . Activin B:  $\beta B\beta B$ ) or a heterodimer (Activin AB:  $\beta A\beta B$ ) (de Kretser *et al.*, 2002).

Inhibins and activins are produced by the granulosa cells (Findlay *et al.*, 2001; Findlay *et al.*, 2002), additionally the Activin  $\beta A$  and  $\beta B$  subunits are produced by the gonadotrophs in the pituitary and other organs (Corrigan *et al.* 1991).

Inhibins are so named because they are implicated in a feedback regulatory mechanism to inhibit FSH secretion. Inhibin B is secreted mainly during the early follicular phase, then decreases and is undetectable after the LH surge (Groome *et al.*, 1996) whereas inhibin A levels are low during the first half of the follicular phase and then increase to peak in the luteal phase. This indicates that inhibin A is produced by the dominant follicle (Hayes *et al.*, 1998) whereas inhibin B has been suggested as a marker for the ovarian follicular reserve (Welt *et al.*, 1999). The hypothesis is that the rising FSH levels that accompany female reproductive aging occur as a result of lower inhibin B levels since fewer follicles are left in the ovaries to contribute to inhibin B production. However, a clear causal relationship has not been unequivocally confirmed and individual variation is great. The strongest correlation between inhibin B levels and antral follicle development has been shown during FSH stimulation for assisted reproduction treatment (Yong *et al.*, 2003).

Activin has been shown to promote granulosa cell proliferation and to enhance FSH actions by increasing the expression of FSH receptor in granulosa cells. Activin also modulates steroidogenesis in granulosa and theca cells (Findlay *et al.*, 2002). Activin may play a role in controlling early follicle growth; results in mice suggest that secondary follicles cause primary follicles to become dormant at the resting stage by secreting activin (Mizunuma *et al.*, 1999). Activin may also have age-specific effects

since activin-A was shown to stimulate the growth of follicles from immature animals, but to block the FSH-induced growth of preantral follicles from adult animals (Liu *et al.*, 1999). The effects of activin are partly regulated by follistatin, a product of the granulosa cells, which is an activin-binding protein that neutralizes the actions of activin by forming an inactive complex (Shintani *et al.*, 1997).

Activin A, inhibin A and inhibin B are produced by the placenta, decidua, and fetal membranes and a role for activins and inhibins has been suggested in pregnancy. Higher levels of activin were associated with multiple gestations while rapidly falling levels were seen to be associated with early miscarriage. Low levels and a rapid decline in inhibin A was seen in pregnancies with embryonic failure (Lockwood *et al.*, 1997). Involvement of inhibin A and activin A in the pathogenesis of gestational diseases and a clinical usefulness of measuring inhibin-related proteins in the diagnosis, prevention, prognosis and follow-up of different gestational pathologies has been suggested. This includes the viability of early pregnancy, Down's syndrome, fetal demise, pre-eclampsia, pregnancy-induced hypertension, preterm delivery and intrauterine growth restriction (Florio *et al.*, 2001). Measurement of activin levels during ovarian stimulation for assisted reproduction on the other hand has not been useful for clinical purposes, presumably because of the extragonadal source of activin (Lockwood *et al.*, 1996; Casper *et al.*, 2001).

#### 2.1.2.5 *KIT Ligand*

The Kit gene encodes a receptor (KIT), present on the oocyte and theca cells, which belongs to a family of type III transmembrane tyrosine kinase receptors. KIT and its ligand, KIT ligand or stem cell factor which is encoded by the Mgf gene, are important regulators of oogenesis and folliculogenesis (Driancourt *et al.*, 2000). KIT is produced by the oocytes and theca-interstitial cells whereas KIT ligand is produced by the granulosa cells. Follicle growth appears to involve KIT-KIT ligand interactions; blocking these interactions in the mouse disturbed the onset of primordial follicle development, primary follicle growth, follicular fluid formation in preantral follicles and final follicle maturation (Yoshida *et al.*, 1997). Using the rat as a model system, KIT ligand was seen to promote primordial follicle development (Parrott and Skinner, 1999). In addition to this, an anti-apoptotic effect of KIT ligand on primordial germ cells, oogonia and oocytes has been demonstrated, and KIT ligand may influence theca cell differentiation as well as cytoplasmic maturation of the oocytes in antral follicles (Driancourt *et al.*, 2000).

In human, the effects of KIT ligand are different from those seen in rodents. Blocking KIT by an antibody caused atresia of the primordial follicles within one week in culture of human ovarian tissue (Laitinen *et al.*, unpublished observations).

#### 2.1.2.6 *FSH*

Binding of FSH to granulosa cells from rat follicles of all sizes has been observed (Nimrod *et al.*, 1976) whereas the LH receptor is found only in granulosa cells from large pre-ovulatory follicles (Kammerman and Ross, 1975). In human, primordial follicles do not express the FSH receptor, however 33% of primary and two-layer follicles and all multilayered follicles express the FSH receptor (Oktay *et al.*, 1997). FSH-deficient female mice have secondary follicles in the ovaries but are infertile due to a block prior to antral follicle formation (Kumar *et al.*, 1997). Women with an

inactivating mutation in the FSH receptor have only occasional early secondary follicles (Aittomäki *et al.*, 1996).

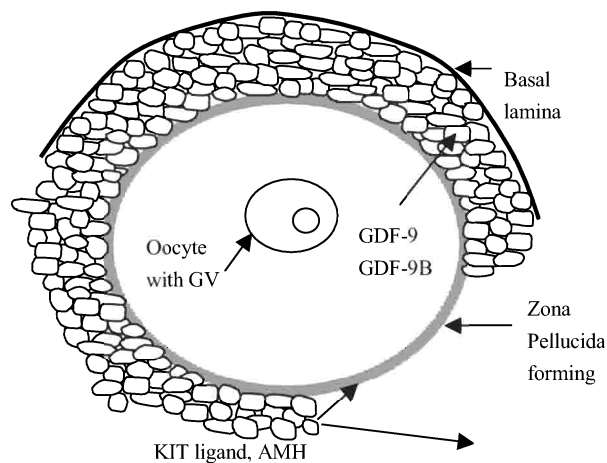
The crucial role of FSH for follicle growth and dominant follicle selection has long since been realized (Erickson and Shimasaki, 2001) and FSH has also been implicated in early follicle growth and survival. After enzymatic isolation of human preantral follicles and their long-term culture *in vitro*, FSH was seen to induce growth and differentiation of preantral follicles (Roy and Treacy, 1993). In culture of human ovarian cortical biopsies, FSH reduced levels of atresia and increased the mean diameter of healthy follicles (Wright *et al.*, 1999; Figure 3).

#### 2.1.2.7 Other growth factors

Other systems have been identified that influence early follicle growth. One of these is the insulin-like growth factor (IGF) system, which has been proposed to amplify the effects of FSH (Adashi, 1995). The mRNAs encoding the type 1 IGF receptor, and IGF binding proteins 2 and 3 have been demonstrated in preantral follicles (Armstrong *et al.*, 2002) and the IGF system is considered important for the regulation of follicle growth and corpus luteum function (Armstrong and Webb, 1997). In culture of human ovarian tissue, IGF I, IGF II and insulin promoted follicle development and served as survival factors (Louhio *et al.*, 2000).

Keratinocyte growth factor has been seen to promote the survival, growth, and differentiation of cultured preantral rat follicles (McGee *et al.*, 1999). Basic fibroblast growth factor induces proliferation of granulosa, theca and ovarian stromal cells in an organ culture system of rat ovaries (Nilsson *et al.*, 2001) and leukaemia inhibitory factor (LIF) has been seen to increase the primordial to primary follicle transition, also in cultured rat ovaries, and may interact with KIT ligand to promote primordial follicle development (Nilsson *et al.*, 2002).

**Figure 3**



Secondary ovarian follicle with multiple layers of granulosa cells surrounding the oocyte. GDF-9 and GDF-9B are secreted by the oocyte and act on the growth and differentiation of the granulosa cells. KIT ligand and AMH are produced by the granulosa cells. Endocrine and paracrine factors affecting follicle growth include FSH, Activins and Inhibins.

### 2.1.3 Gonadotrophins and antral follicles

During growth, the follicles migrate into the medullar region of the ovary (Yen *et al.*, 1999). The development of antral follicles can be divided into seven classes, in addition to the first pre-antral stages, based on the number of granulosa cells in these follicles (Gougeon, 1986). As the follicles grow, the number of granulosa cells increases from approximately 2-5 million during the early follicular phase to 50-100 million at the time of ovulation (Gougeon, 1996). The time required for complete follicle growth *in vivo* has been estimated to be approximately 85 days from the preantral stage to ovulation (Gougeon, 1986) with the growth process being affected by the gonadotrophin fluctuations in the menstrual cycles during that time.

Gonadotrophins, especially FSH, are of primary importance for follicular growth and they sustain follicular steroidogenesis. According to the "two-cell, two gonadotrophins" theory, theca interstitial cells are stimulated by LH to produce aromatizable androgens that are transported to the granulosa cells where they are converted to estrogens by aromatising enzymes which are induced by FSH (Hillier *et al.*, 1994).

The occurrence of continuous basal follicle growth from infancy to menopause is supported by morphological evidence. Throughout infancy, when circulating levels of gonadotrophins are low, 2-6 mm or even larger follicles are observed in the ovaries (Peters *et al.*, 1978). This is also observed in patients with Kallman's syndrome where levels of gonadotrophins are very low, FSH and LH/hCG treatment is required to attain follicle maturation and ovulation in these patients (Sungurtekin *et al.*, 1995).

When the follicles have reached a size of 2 mm, they become more dependent on FSH for growth and the steroid production increases. When the middle part of the menstrual cycle approaches, there is a dramatic increase in estrogen, followed by an LH and to a lesser extent an FSH surge. This triggers the dominant follicle to ovulate (Yen *et al.*, 1999). After ovulation, the follicle reorganizes to become the corpus luteum, responsible for progesterone production and early maintenance of pregnancy.

#### 2.1.3.1 Final follicle and oocyte maturation

The signals initiated by gonadotrophins control complex patterns of gene expression required for follicle maturation and ovulation. The action of gonadotrophins on the oocyte is indirect since oocytes lack gonadotrophin receptors (Amsterdam *et al.*, 1975; Dekel *et al.*, 1988). Cyclic AMP (cAMP) acts as a second messenger for the gonadotrophins and cAMP signalling is involved in most aspects of these processes in the granulosa cells (Adashi *et al.*, 1990; Conti, 2002). The occupied gonadotrophin receptor causes an activation of the heterotrimeric G proteins, which stimulate the adenylyl cyclase in the granulosa cells. The increase in cAMP causes the activation of protein kinase A (PKA) that phosphorylates cytoplasmic substrates or transcription factors in the nucleus to control gene expression (Conti, 2002). This linear signalling cascade is not sufficient however, to fully explain the divergent outcomes of cAMP activation in granulosa cells. Both the FSH and the LH receptor, expressed in granulosa cells from mature follicles use cAMP as the primary signalling pathway, and whereas FSH stimulates growth of the granulosa cells and promotes androgen aromatisation, LH promotes exit from the cell cycle and suppression of aromatase expression and aromatisation (Richards, 2001). A number of hypotheses have been put forward to explain these divergent signalling mechanisms. The gonadotrophin receptors may be

coupled to different G proteins and therefore to more than one signalling pathway or to other signalling pathways not including G proteins and that this branching occurs at the cell membrane. The cAMP signal originating from the two receptors may also activate signalling cascades that are specific for the state of differentiation of granulosa cells or according to compartmentalisation of cAMP in the granulosa cells (Conti, 2002).

The oocytes in the preovulatory follicles are maintained in meiotic arrest throughout follicular growth by a signal arising from the granulosa/cumulus cells, which is mediated through the gap junctions present at the processes protruding through the zona pellucida. The nature of this signal is debated, but the intracellular concentration of cAMP in the oocyte is of crucial importance in this respect (Conti *et al.*, 2002). High levels of cAMP in the oocyte are shown to maintain meiotic arrest and the resumption of meiosis is associated with a decrease in cAMP concentration (Schultz *et al.*, 1983). The cAMP has traditionally been considered to be produced by the granulosa/cumulus cells surrounding the oocyte and that it diffuses through the gap junctions to maintain high intracellular cAMP levels in the oocyte. Although this is undoubtedly an important source of cAMP for the oocyte, recent evidence suggests that the oocytes themselves are able to produce and control intracellular cAMP levels (Horner *et al.*, 2003). Spontaneous maturation in-vitro after the release of the oocyte from the inhibitory follicular environment was, however observed decades ago (Pincus and Enzmann, 1935; Edwards, 1965).

Recent evidence suggests that release from the inhibitory cAMP levels in the oocyte is not the only signal important for oocyte maturation. An intermediate of the cholesterol biosynthetic pathway, follicular fluid-meiosis-activating sterol (FF-MAS), was recently identified (Byskov *et al.*, 1995; Byskov *et al.*, 1997) and has been shown to be important in reinitiating meiosis in oocytes. FF-MAS is present in follicular fluid and ovarian tissue, is produced by the granulosa/cumulus cells and it is capable of initiating meiosis in oocytes from several species in the presence of meiotic inhibitors (Grøndahl *et al.*, 1998; Hegele-Hartung *et al.*, 1999; Byskov *et al.*, 2002).

In addition to the positive effects of FF-MAS on the progression of meiosis, recent evidence indicates that FF-MAS may protect mammalian oocytes from precocious chromatid segregation (Cukurcam *et al.*, 2003). FF-MAS has also been suggested as an important factor for human assisted reproduction (Byskov *et al.*, 2002) since it has been seen to improve the survival and quality of human oocytes in in-vitro maturation studies (Cavilla *et al.*, 2001).

### 2.1.3.2 Ovulation and fertilization

Mechanically, ovulation entails rapid follicular enlargement and simultaneous protrusion of the follicle from the ovarian cortex. LH (or hCG) stimulates a cascade of proteolytic enzymes, including plasminogen activator, plasmin and matrix metalloproteinase 1 (MMP-1). These enzymes bring about the degradation of the perifollicular matrix and the decomposition of the collagen fibers, which provide strength to the follicular wall (Tsafriri, 1995). Cellular death involving both apoptosis and inflammatory necrosis occurs at the formative site of ovulation (Murdoch, 1995a, 1995b). This sequence of events may begin five to six days before the onset of the LH surge, however the LH surge marks the end of the follicular phase of the menstrual cycle and rupture of the follicle follows up to 36 hours later (Schmidt-Gollwitzer *et al.*, 1977; Yen *et al.*, 1999). Rupture of the follicle results in the expulsion of the oocyte-

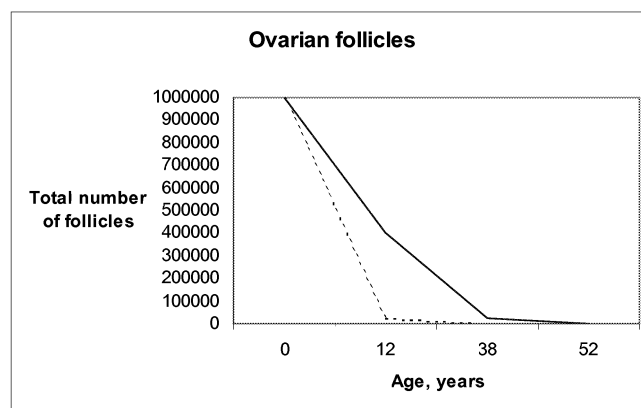
cumulus complex along with the follicular fluid. The oocyte-cumulus complex is transported into the fallopian tube and it is there that fertilization and early embryo development take place in-vivo.

The oocyte, released from the meiotic arrest by the LH surge, proceeds to the metaphase of the second meiotic division (MII) visible by the extrusion of the first polar body. Again the oocyte is arrested, now at the MII stage and only if the oocyte is fertilized and activated by a spermatozoon, can meiosis be completed. The second polar body is then extruded and interphase follows with pronucleus formation and DNA synthesis (Sirard, 2001). The transition from maternal to zygotic gene transcription, where the developmental programme of the oocyte is replaced by the programme of the early embryo, occurs at the 4-8 cell stage in the human (Braude *et al.*, 1988; Telford *et al.*, 1990). The three major functions of this transition are to destroy oocyte-specific transcripts, replace them with zygotic transcripts and to promote reprogramming in gene expression. This reprogramming is important for the transformation of the differentiated oocyte into the totipotent blastomeres of the cleavage stage embryo (Schultz, 2002).

#### 2.1.3.3 Dynamics of the follicular reserve through the fertile period to menopause

The number of follicles in the ovaries of a young woman at menarche is estimated to be 400,000. Assuming 400 ovulations during the fertile period, approximately 1000 follicles are recruited towards ovulation or degenerate in each menstrual cycle. Given the fact that only one or two follicles reach full maturity and produce a fertilizable oocyte in each cycle, most potential oocytes go to waste. Some of these oocytes may however be rescued by controlled hyperstimulation for assisted reproduction treatments.

**Figure 4**



Schematic diagram representing the decline in follicle numbers with age (according to Faddy, 2000). After cytotoxic therapy, the curve is shifted to the left (broken line).



The dynamics of this process are characterised by dramatic changes as the ovary ages (Faddy 2000). High rates of growth of small primordial follicles occur in the young ovary. As the ovary ages, the rate of atresia is increased and growth rates are reduced, with a dramatic increase in the rate of atresia apparent at the age of 38 when approximately 25 000 follicles remain in the ovaries (Faddy 2000).

Follicle loss occurs at an exponential rate in the ovaries with advancing age and when the number of follicles falls below a critical threshold level, menopause is imminent. The number of follicles left in the ovaries at the onset of menopause has been estimated to be 1000, corresponding to the median age for menopause at 51 years in the population (Faddy and Gosden 1996), Figure 4. After that the ovary is unable to provide enough antral follicles to sustain menstrual cyclicality.

## **2.2 FERTILITY PRESERVATION**

### **2.2.1 Effects of cytotoxic therapy**

The survival rate of children after treatment against malignant disease has increased to over 70% in the last three decades, primarily due to improvements in treatment therapies (Landis *et al.*, 1998). One of the side effects of such treatments is the frequent loss of fertility, which can seriously affect their adult lives. Options for fertility preservation for men through cryopreservation and storage of sperm have been routinely available since the 1970's. Through recent advances in ART treatments, namely intracytoplasmic sperm injection - ICSI, good pregnancy rates can be expected using cryopreserved and thawed sperm and this procedure is considered an essential part of any comprehensive cancer care programme (Kelleher *et al.*, 2001). Even men who are persistently azoospermic after chemotherapy, traditionally considered sterile unless sperm was frozen before therapy, may be able to father their own genetic offspring using testicular biopsy to obtain sperm for ICSI (Damani *et al.*, 2002; Meseguer *et al.*, 2003). This is a very important result for survivors of treatment against cancer and is a prime example of how new groups of patients may benefit from advances in assisted reproduction treatments.

The gonadotoxic effect of chemotherapy is dose and age-dependent and varies according to drug regimens used. In women, a reduction in follicle numbers is seen after chemotherapy (Nicosia *et al.*, 1985) and the course of ovarian dysfunction is consistent with the destruction of a fixed number of follicles (Howell and Shalet, 1998). Temporary or permanent amenorrhoea may follow therapy and older patients who have a smaller ovarian reserve are more likely to undergo premature menopause following therapy than younger women. A much higher rate of premature menopause was found by studying the menstrual histories of more than 1000 women receiving treatment against malignancy in childhood, compared with a control population (Byrne *et al.*, 1992). Among the treated women, 42% had reached menopause by the age of 31 years, compared with 5% of the controls.

Thus, maintenance of a normal menstrual cycle immediately after chemotherapy does not guarantee a comparable menopausal age as in the overall population and also amenorrhoea following chemotherapy does not necessarily imply permanent ovarian failure. This is especially true in younger patients (Howell and Shalet, 1998). Survivors of cancer in childhood have been shown to have a diminished ovarian reserve in spite of regular menstrual cycles and may have a shortened reproductive life span and early

menopause (Larsen *et al.*, 2003). This must be taken into account when counselling these patients, since delaying childbirth may not be advisable even for the individuals retaining ovarian function after treatment against cancer.

Radiotherapy also causes destruction of the oocytes and reduction of the follicular reserve. In a study of eight patients who received total body irradiation in childhood or early adolescence and 19 patients who received abdominal irradiation, only three retained normal ovarian function (Wallace *et al.*, 1989; Wallace *et al.*, 2003), two in the first group and one in the second group. Follow-up until menopause for these three patients was not reported. Ovarian failure was diagnosed in the remaining patients between the ages of 10 and 15 years. The authors calculated the amount of radiation required to destroy 50% of the oocytes in the ovaries and estimated that less than 2 Gy is required. The patients receiving total body irradiation were in first or second remission of leukaemia and had been treated with 14.4 Gy (Wallace *et al.*, 2003), only 0.4 - 1.7 % of the oocytes were estimated to remain after treatment.

Other consequences of follicle depletion following chemotherapy or radiotherapy are estrogen deficiency, possible cardiovascular mortality and reduction in bone mineral density. Hormone replacement therapy (HRT) is indicated for these patients (Howell and Shalet, 1998).

Patients who do conceive after treatment against cancer do not seem to have a higher risk of genetic disease or birth defects in their offspring (Dodds *et al.*, 1993; Boice *et al.*, 2003).

### **2.2.2 Premature ovarian failure**

Premature ovarian failure (POF) is defined as menopause before the age of 40 years and it is estimated to occur in 1% of women. Underlying causes of POF are of genetic, autoimmune, iatrogenic and environmental origin (Gersak *et al.*, 2003). Gene defects, such as FSH receptor mutations, which preclude follicle maturation (Aittomäki *et al.*, 1996) in spite of the presence of follicles in the ovaries, are a clear indication for cryopreservation of ovarian tissue since the only way to obtain mature oocytes in these cases would be in-vitro culture. These mutations are recessive and the risk of transmitting them to the offspring is minimal. This potential risk could be avoided by preimplantation genetic diagnosis (PGD) if the partner happens to be a carrier of the same mutation. Chemotherapy for autoimmune diseases may cause POF (Packham and Hall, 2003) and is also an indication for ovarian tissue cryopreservation.

### **2.2.3 Turner's syndrome**

A normal female karyotype consists of 46 chromosomes including two sex chromosomes, 46 XX. Turner's syndrome is caused by the total or partial absence of one X chromosome, 45 X. Approximately half of the patients with Turner's syndrome have the 45 X genotype, others have a mixture of 45 X and 46 XX in their cells and are referred to as mosaics (Lippe, 1991; Saenger, 1996). Furthermore 5-10% of these patients may have Y chromosomal material in some cells. In these cases gonadectomy is generally recommended because of the risk for gonadoblastoma; however, this consensus has been questioned (Gravholt *et al.*, 2000). Turner's syndrome is a relatively common chromosomal disorder, affecting approximately 1 in 2000 female births, although only about 1% of 45X fetuses survive to term and as many as 10% of

spontaneous miscarriages have a 45X karyotype (Hall and Gilchrist, 1990; Robinson, 1990).

Turner's syndrome is often diagnosed at an early age because of the short stature of these girls, or by prenatal diagnosis. Other findings which may be indicative of Turner's syndrome are webbing of the neck, low set ears, impaired hearing, low posterior hair-line, high arched palate, small lower jaw, defective dental development, lymphedema of hands and feet, increased number of pigmented naevi, nail dysplasia, broad chest, widely spaced nipples, inverted nipples, cubitus valgus (bent elbows), coarctation of the aorta, bicuspid aortic valve and renal malformations. The presence of these features varies and some girls are easily recognised by the clinician whereas other girls are indistinguishable from girls with a normal karyotype. Even though spatial perception and mathematical abilities may be hampered, the patients usually have average intelligence or above (Saenger *et al.*, 2001).

The ovarian function is affected in girls with Turner's syndrome; accelerated atresia reduces the ovarian reserve at an early age. The time when the follicles start to disappear is not clear but the number of follicles has been estimated to be normal at least up to the 18th week of foetal life (Singh and Carr 1966; Weiss 1971). The ovarian dysgenesis results in streak ovaries of white fibrous stromal tissue, which are devoid of follicles and oocytes. Nevertheless, spontaneous pregnancies occur in 2–5% of these women, and up to 30% achieve at least some pubertal development (Pasquino *et al.*, 1997). Pregnancies are more common in subjects with chromosomal mosaicism, but they also occur in women with non-mosaic Turner's syndrome (Hovatta, 1999; Schwack and Schindler, 2000).

Many of the conditions associated with Turner's syndrome can be treated; growth hormone therapy should be initiated early, at the age of two to five years, to increase the final height of the patient (Nilsson *et al.*, 1996). Hormone replacement therapy (HRT) is used to achieve secondary sexual characteristics and to prevent osteoporosis. Other problems such as cardiac problems can be monitored closely and severe morbidity or mortality can often be avoided. Audiogram should be performed regularly as a means of detecting hearing problems. A Swedish study on middle-aged women with Turner's syndrome reported that ovarian failure and infertility were major problems for these women and that infertility had caused them deep grief, (Sylvén *et al.*, 1993).

Although early diagnosis has facilitated interventions leading to better quality of life in many aspects for these patients, the issue of infertility remains a great problem for most women with Turner's syndrome.

Miscarriages are more common in the spontaneous pregnancies of women with Turner's syndrome than in the general population of women (Bryman *et al.*, 2000). Concerns have been raised that this might be caused by frequent chromosomal abnormalities, as Turner's syndrome is more common in children born to these women. However, miscarriage rates after oocyte donation are also high among these women so this is not the only explanation. The elevated frequency of miscarriages may also be the result of a poorly developed uterus and low uterine blood flow, and can be overcome by adequate HRT (Hovatta, 1999).

In vitro fertilization and oocyte donation has brought new options for women with Turner's syndrome and this is now a well-established treatment for women with this condition. Good results have been achieved; clinical pregnancy rates of up to 46% per embryo transfer and implantation rates of 30% have been obtained (Foudila *et al.*, 1999; Hovatta, 1999).

Estradiol and progesterone treatment are necessary to prepare the endometrium for implantation and to sustain early pregnancy. Women with Turner's syndrome may need a more extensive hormone therapy than other oocyte donation patients. The pregnancies are considered high-risk pregnancies because of the cardiovascular risk. Cardiovascular anomalies occur in 19-44% of women with Turner's syndrome and at least two fatal aortic dissections have been described in pregnancies for these women (Nagel and Tesch, 1997). The risk for hypertension does not seem to be increased compared to other women undergoing oocyte donation but disturbances in carbohydrate metabolism may occur. A careful health check-up before treatment is important, with a cardiological assessment and an echocardiogram being crucial. A glucose tolerance test can be recommended and frequent monitoring during pregnancy is also of great import (Saenger *et al.*, 2001). The short stature of women with Turner's syndrome makes them predisposed to fetopelvic disproportion. This has resulted in an increased frequency of caesarean sections among them. For the present generation receiving growth hormone therapy from childhood, this may not be a problem.

The increased risk of complications in pregnancy for these women makes it unacceptable to further increase the risk by inducing twin (or even higher order) pregnancies. Single embryo transfer must be applied in their fertility treatment. Good pregnancy rates have been achieved with single embryo transfer for these and other patients (Martikainen *et al.*, 2001; Söderström-Anttila *et al.*, 2003).

#### **2.2.4 Options for fertility preservation**

Preservation of fertility for patients at risk for premature ovarian failure relies in most cases on cryopreservation of gametes, fertilized oocytes or embryos. Attempts have been made to prevent the damage afflicted by chemotherapy on the ovarian follicles through administering a GnRH agonist parallel to treatment (Blumenfeld *et al.*, 1996) and preliminary results were promising. However, since the early growth of the follicles seems to be gonadotrophin independent, the mechanism behind this possible protection remains unclear and the preliminary results have not been verified in larger clinical trials (Janson, 2000).

Shielding of the ovaries through surgical transposition before pelvic irradiation has also been used with some success (Bisharah and Tulandi, 2003) however scatter irradiation cannot be avoided and this method offers no protection against chemotherapy. Cryopreservation of gametes and fertilized oocytes has in recent years received more attention and the following discussion will focus on these aspects.

Important advances in reproductive biology were made already in the 1940s with the advent of successful cryopreservation of spermatozoa using glycerol as the protective agent (Polge *et al.*, 1949). Attempts with ovarian follicles were also successful a decade later, when live births were obtained in mice after autografting of frozen-thawed ovarian cortical tissue (Parrott, 1960). Cryopreservation of spermatozoa is the only proven method to preserve fertility in men with cancer (Ragni *et al.*, 2003). This

method is now well established and is widely offered to men before treatment against cancer and as a supplement to fertility treatment (Hovatta, 2001).

#### 2.2.4.1 Cryopreservation of embryos

The advent of in vitro fertilization (Steptoe and Edwards, 1978) enabled studies on human preimplantation embryos. The potential of cryopreservation of surplus embryos after gonadotrophin stimulation was soon realized. Using dimethyl sulfoxide (DMSO) the first human pregnancy after transfer of cryopreserved and thawed embryos was announced only five years later (Trounson and Mohr, 1983). Propanediol (PrOH) was also used for cryopreservation of embryos in these early years of human ART (Lassalle *et al.*, 1985) and is now the cryoprotectant most commonly used in the ART laboratory.

Cryopreservation of embryos before imminent chemotherapy or radiotherapy is suitable for women who have a partner and who are willing to undergo ART. However the three to five weeks needed for a stimulated cycle to reach oocyte retrieval may represent an unacceptable delay in the anti-cancer treatment (Amorim *et al.*, 2003) even though the use of GnRH antagonists can shorten this time down to two weeks (Anderson *et al.*, 1999). Tamoxifen, a non-steroidal anti-estrogen, originally used as a contraceptive has also been used for follicle stimulation, IVF and cryopreservation of embryos in breast cancer patients (Oktay *et al.*, 2003). Cryopreservation of embryos is not considered an option for young girls facing anti-cancer therapy and the need for a willing partner for sexually mature women contributes to make cryopreservation of embryos a relatively seldom used option for fertility preservation in these cases.

#### 2.2.4.2 Cryopreservation of oocytes

Cryopreservation of mature oocytes after conventional stimulation, antagonist cycles or from natural cycles does not require a partner. However lengthy stimulation is often required and this method is not an option for young girls.

Recent improvements in the methods for the cryopreservation of oocytes make this an attractive alternative in many cases. The first reports of infants born after cryopreservation of oocytes and thawing followed by IVF came soon after the first successes with cryopreservation of embryos (Chen, 1988; Van Uem *et al.*, 1988). For many years following these reports, success rates remained low. Using 1.5 M PrOH, significant improvements were seen by increasing the sucrose concentration from 0.1 M, as is commonly used, to 0.2 M or 0.3 M (Fabbri *et al.*, 2001). Slightly increased time of exposure to cryoprotectants, from 10 to 15 minutes, was also beneficial. Raising the sucrose concentration to 0.2 M has also been reported to increase the survival of biopsied embryos cryopreserved after PGD cycles (Jericho *et al.*, 2003).

The established success of cryopreservation of oocytes with live births and success rates approaching that of embryo cryopreservation (Coticchio, 2003) makes this an attractive option when there is time for gonadotrophin stimulation. Immature oocytes (germinal vesicle, or GV stage oocytes) can be successfully cryopreserved after stimulation for ART (Tucker *et al.*, 1998) and during tissue processing of ovarian biopsies for ovarian tissue cryopreservation, oocytes from antral follicles can be aspirated and cryopreserved (Revel *et al.*, 2003). Cryopreservation of mature or immature oocytes can thus be recommended to patients at risk of premature ovarian failure whenever practically possible.

#### 2.2.4.3 Cryopreservation of ovarian cortical tissue

A revival of interest in cryopreservation of follicles in ovarian cortical tissue was seen in the 1990s when survival of human ovarian follicles after cryopreservation and thawing was shown using DMSO, PrOH and ethylene glycol (EG) (Hovatta *et al.*, 1996; Newton *et al.*, 1996).

The main functions of the cryoprotective agents are to dehydrate the cells and to avoid ice-crystal formation during cryopreservation and thawing. This is accomplished by the permeating cryoprotectants such as DMSO, PrOH and EG, and the non-permeating cryoprotectants such as sucrose, which dehydrates the cells by osmotic pressure. The primordial follicle with its oocyte has several characteristics that make it more likely to survive cryopreservation than at later stages of development. These are the small size of the oocyte and its support cells, facilitating equilibration with cryoprotectants; low metabolic rate; absence of a zona pellucida and the meiotic arrest in the prophase of the first meiotic division (Shaw *et al.*, 2000). The inherent quiescence of primordial follicles may also make them more likely to survive the initial ischaemia following replantation before the tissue has been revascularized.

Cryopreservation of follicles in ovarian cortical tissue necessitates ovarian biopsy by laparoscopy. In some cases a unilateral or bilateral oophorectomy is performed, but more commonly an ovarian biopsy is taken. It is noteworthy that the density of follicles varies significantly in pieces from different parts of the ovary (Lass *et al.*, 1997; Schmidt *et al.*, 2003) so care should be taken to take a sufficiently large biopsy to obtain a representative part of the ovarian cortex. Unilateral oophorectomy is recommended by some groups in order to get sufficient material for cryopreservation, but this must be weighed against the possibility of survival of the ovarian follicles through the various modes of cancer treatment.

The follicles are cryopreserved while still embedded in the ovarian tissue using slow, controlled-rate cooling followed by storage in liquid nitrogen at -196 °C. The tissue is divided into small fragments with a scalpel to facilitate equilibration with cryoprotectant. After storage the tissue is thawed rapidly in a water bath at room temperature or at 30 °C.

The cryoprotectants used in most laboratories today are DMSO, PrOH or EG at 1.5 M with sucrose at 0.1 M (Hovatta *et al.*, 1996; Newton *et al.*, 1996; Gook *et al.*, 1999). No significant differences have been observed between DMSO and PrOH in terms of follicle survival or other aspects and EG has also been used with good results (Newton *et al.*, 1996). Serum supplementation has been used for the cryopreservation at 10 - 20%, but human serum albumin (HSA) has also been used at 10 mg/ml (Gook *et al.*, 1999). Isolation of the follicles from the ovarian tissue has been used for research purposes to show their viability (Oktay *et al.*, 1997) but is not applied in a clinical setting.

Viability of the follicles after thawing has been studied by various methods. Histological evaluation where eosinophilia of the cytoplasm, clumping of the chromatin material, wrinkling of the nuclear membrane and pyknosis of the granulosa cells are markers of atresia (Gougeon, 1986) is a reliable method for evaluating follicle viability (Hovatta *et al.*, 1996; Gook *et al.*, 1999). Transmission electron microscopy (TEM) permits a more detailed examination of the ultrastructure of the cells (Oktay *et al.*, 1997; Gook *et al.*, 1999; Nisolle *et al.*, 2000). Fluorescent viability markers have been

used, both in isolated follicles (Oktay *et al.*, 1997) and in tissue slices (Cortvrind and Smitz, 2001). In these methods, enzymatic esterase activity in the cytoplasm in living cells is shown by green fluorescence and membrane integrity is tested by blocking the influx of red fluorescent colour in cells with intact cell membranes. Initiation of growth of frozen-thawed follicles in organ culture has also been demonstrated (Hovatta *et al.*, 1997). Varying results have been obtained in these studies, but at least 70% of the follicles can be expected to survive the process of cryopreservation and thawing.

Hormonal assays have been used after transplantation of cryopreserved/thawed ovarian tissue to follow follicular development (Salle *et al.*, 1999; Kim *et al.*, 2002) and in-vitro culture can also be used to evaluate follicle viability (Sugimoto *et al.*, 1996; Hovatta *et al.*, 1997; Paynter *et al.*, 1999; Newton and Illingworth, 2001).

Viability of the follicles has been shown by xenografting to mice with severe combined immunodeficiency (SCID-mice), (Newton *et al.*, 1996; Van den Broecke *et al.*, 2001; Gook *et al.*, 2001; Kim *et al.*, 2002). These studies show good recovery of follicles and follicular growth to late secondary and antral stages. Oocyte maturation within mucified oocyte-cumulus complexes, ovulation and subsequent formation of corpus luteum has also been shown after xenotransplantation of cryopreserved and thawed ovarian tissue (Gook *et al.*, 2003).

Concern has been raised regarding autotransplantation of the tissue after thawing to patients with malignant disease, such as lymphoma (Shaw *et al.*, 1996), because of the danger of transmitting the original disease back to the patient. In Hodgkin's disease there appears to be smaller risk of ovarian involvement (Meirow *et al.*, 1998) and in another study on transplantation of ovarian tissue from patients with Hodgkin's lymphoma or non-Hodgkin's lymphoma to SCID-mice the authors did not detect any transmission (Kim *et al.*, 2001). The concern of possible transmission of malignant disease after autotransplantation of cryopreserved and thawed tissue pieces is still valid and the potential risk must be evaluated in each case depending on the nature of the original disease.

Live born individuals after cryopreservation and thawing of ovarian tissue followed by autotransplantation have so far only been seen in animals. In mice this procedure has been successful (Parrott, 1960; Gunasena *et al.*, 1997; Sztejn *et al.*, 1998; Liu *et al.*, 2001; Snow *et al.*, 2002), as well as in sheep (Gosden *et al.*, 1994; Salle *et al.*, 2002). In the rat, live born young were obtained after transplantation of a whole frozen-thawed ovary, fallopian tube and segment of uterus (Wang *et al.*, 2002). In various animals, such as the rat, rabbit, marmoset, elephant, cow, wombat and tammar wallaby, follicular survival and growth after cryopreservation, thawing and transplantation or culture has been seen (Daniel *et al.*, 1983; Candy *et al.*, 1995; Aubard *et al.*, 1998; Gunasena *et al.*, 1998; Paynter *et al.*, 1999; Wolvekamp *et al.*, 2001; Mattiske *et al.*, 2002).

In human, autotransplantation of thawed ovarian tissue has also been described and evidence of follicular function after the procedure has been obtained. Follicular development induced by FSH stimulation was accompanied by estradiol production (Oktay and Karlikaya, 2000) and ovulation was confirmed. Follicular and endometrial development have been observed (Radford *et al.*, 2001) and another study showed a temporary decrease in FSH levels and estradiol production (Callejo *et al.*, 2001). No pregnancies have as yet been reported in human using this procedure.

Cryopreservation of follicles in ovarian cortical tissue is considered a promising method for preservation of fertility among young women undergoing cytotoxic therapy against cancer and for women at risk of premature ovarian failure (Oktay, 2001). The results so far show that a large proportion of the ovarian follicles survives the cryopreservation process and will start growing during in-vitro culture or after transplantation (Amorim *et al.*, 2003). These recent advances have inspired cautious optimism regarding the possibility of offering this method for preservation of fertility for women at risk of premature ovarian failure. Currently, cryopreservation of ovarian tissue is offered by many fertility clinics around the world and intensive research is aimed at improving the methods for cryopreservation and utilization of the stored ovarian reserve of patients.

### **2.2.5 Culture of ovarian follicles**

When cryopreservation of ovarian tissue is performed because of blood borne malignancies, such as leukaemia, replantation of the tissue to the patient cannot be performed because of the risk of reintroducing the original disease. In these cases the follicles have to be cultured from the primordial to antral stages of development and the oocytes matured in vitro. This is a complex procedure, where the whole process of in-vitro growth and maturation from the primordial stage has until now, only been successful with live born individuals in the mouse (Eppig and O'Brien, 1996; O'Brien *et al.*, 2003). Using growing follicles, also from mice, developmentally competent mature oocytes and live born young have been obtained (Eppig and Schroeder, 1989; Spears *et al.*, 1994; Cortvrindt *et al.*, 1996). In the human, the time for follicle growth until ovulation is longer than in rodents.

The high density of the stroma in the human ovarian cortical tissue has contributed to delaying a successful system for isolating follicles. Enzymatic isolation of preantral follicles has been successful using human ovarian cortical tissue (Roy and Treacy, 1993) where culture was performed for up to five days. In these follicles FSH was seen to induce follicular DNA synthesis, antrum formation in larger follicles and estradiol production (Roy and Treacy, 1993). Culture of isolated human primordial follicles for over 24 hrs has not been successful however (Abir *et al.*, 1999). In a comparative study of isolated and non-isolated follicles, significantly better survival was seen for follicles cultured within tissue slices than among partially isolated follicles (Hovatta *et al.*, 1999).

Research regarding culture techniques for human ovarian follicles relies on donated cortical biopsies from women undergoing gynaecological surgery or caesarean section. Attempts have been made to find alternative sources of follicles for research. Follicular aspirates from patients undergoing oocyte recovery in ART treatments have been examined under the hypothesis that small pieces of cortical tissue or early follicles may be found from the passage of the needle through the cortex at the initial puncture. A few follicles have been found in these aspirates, but this was shown not to be a useful source of early follicles (Moskovtsev *et al.*, 2002; Zhang *et al.*, 2002).

In organ culture, extracellular matrix has been shown to be beneficial for the survival of the follicles (Hovatta *et al.*, 1997). An activation of growth of the primordial follicles has been observed with a majority of the follicles leaving the latent primordial stage after one week of culture (Hovatta *et al.*, 1999). Using this culture system, follicle



growth up to the secondary stage regularly occurs, and antral follicles are occasionally observed (Hovatta *et al.*, 1997, 1999).

Several factors have been identified that are important for growth of the follicles in-vitro. FSH was seen to reduce atresia and to increase the mean diameter of healthy follicles in organ culture. HSA supplemented with insulin/transferrin/selenium mix was also beneficial compared with serum (Wright *et al.*, 1999). Insulin, insulin-like growth factors I (IGF-I) and IGF-II have been seen to improve survival of follicles and to increase the proportion of primary follicles during two weeks of culture (Louhio *et al.*, 2000). Elucidation of the growth factors involved is of great importance and requirements of the follicles at different stages during culture must be defined. Further work is required to optimise culture conditions for in-vitro growth of ovarian follicles.

### **2.3 IN VITRO MATURATION OF OOCYTES (IVM)**

In classical in-vitro fertilization treatment, simultaneous growth and development of multiple follicles through hyperstimulation of the ovaries is achieved by FSH injections following downregulation by GnRH agonist or antagonist (Hughes *et al.*, 1992; Liu *et al.*, 1992; Minaretzis *et al.*, 1995). Final oocyte maturation is induced by hCG administration followed by transvaginal oocyte retrieval before spontaneous ovulation occurs. Ovarian hyperstimulation syndrome (OHSS) is a serious side effect of the hyperstimulation treatment. OHSS is characterized by massive enlargement of the ovaries, ascites, pleural effusion, oligurea, hemoconcentration and thromboembolic phenomena and is a life threatening complication of ART treatment. Women with PCO syndrome may be especially at risk for developing OHSS (Aboulghar and Mansour, 2003). Different strategies have been proposed to reduce the incidence of OHSS, such as cancellation of treatment while continuing GnRH agonist administration, withholding FSH and delaying oocyte retrieval until reduction in estradiol levels has been observed (coasting) but the optimal solution to this problem has not yet been realised (Al-Shawaf and Grudzinkas, 2003). Complete prevention of OHSS remains one of the major challenges of ART treatment today.

Maturation of oocytes in vitro was observed decades before clinical IVF was started (Pincus and Enzmann, 1935; Edwards, 1965) and the first children (triplets) born after in-vitro maturation of oocytes (IVM) originated from oocytes extracted from ovarian tissue (Cha *et al.*, 1991). Since then this technique has been further developed and healthy children have been born from IVM oocytes (Barnes *et al.*, 1995; Cha and Chian, 1998; Chian *et al.*, 1999, 2001; Mikkelsen *et al.*, 1999; Jaroudi *et al.*, 1999; Suikkari *et al.*, 2000; Child *et al.*, 2001; Tan *et al.*, 2002). Cryopreservation of embryos using oocytes matured in vitro has also resulted in normal pregnancies (Suikkari *et al.*, 2000; Chian *et al.*, 2001).

IVM treatments utilize low doses of FSH for initiation of follicular development, or indeed no FSH stimulation at all, although FSH may be helpful in timing oocyte retrieval (Mikkelsen and Lindenberg, 2001). In women with PCO, hCG given before oocyte retrieval in IVM treatments appears useful, with relatively high pregnancy rates achieved (Chian *et al.*, 1999; Son *et al.* 2002). IVM may thus be the optimal treatment for women at risk of developing OHSS since minimal or no FSH stimulation is involved. IVM has been especially recommended for women with PCO and is also

considered a safe and cost-effective alternative to conventional stimulated IVF/ICSI treatments (Tan and Child, 2002).

IVM is a rapid treatment in cancer patients who require cryopreservation of oocytes or embryos, and is particularly useful for women who have disseminated lupus erythematosus before chemotherapy for their autoimmune disorder, because such women poorly tolerate hormonal stimulation. It is also the final stage of maturation of oocytes from ovarian primordial follicles cryopreserved within cortical tissue slices (Hovatta *et al.*, 1996; Gook *et al.* 1999).

IVM involves aspiration of oocytes from pre-ovulatory follicles. We perform oocyte aspiration when a leading follicle of 10-14mm is observed and the endometrium has reached at least 5mm in thickness. The in-vitro maturation time is 32-36 hrs, which is practically applicable in a clinical setting and has previously been shown to be adequate in IVM (Smith *et al.*, 2000). Maturation is defined as nuclear maturation of the oocytes from the GV stage to MII observed by the extrusion of the second polar body. This is the standard definition of maturation used in ICSI treatments to select oocytes for microinjection. Immature oocytes are aspirated two to four days before ovulation would have occurred, hence there may arise an asynchrony between nuclear and cytoplasmic maturation of these oocytes which could partially account for the lower success rates achieved through IVM compared with regular IVF/ICSI. The importance of cytoplasmic maturation for embryo development has been shown, including microtubule dynamics and acquisition of cytoplasmic proteins as well as chromatin phosphorylation and other imprinting events (Trounson *et al.*, 2001; Combelles *et al.*, 2002; Kerjean *et al.*, 2003). The timing of oocyte aspiration is critical, allowing as many follicles as possible to reach sufficient size for cytoplasmic competence of the oocytes while avoiding a negative effect of the dominant follicle (Trounson *et al.*, 2001). The dialogue between the oocyte and granulosa/cumulus cells during the maturation process is also critical for correct expression of genes important in the maturation process (Eichenlaub-Ritter and Peschke, 2002).

Recently, concerns have been raised regarding diseases associated with imprinting defects in children born after ART treatments. Reports have shown a higher frequency of Beckwith-Wiedemann syndrome and Angelman syndrome in children born after ART (Gosden *et al.*, 2003). Many other studies do not, however, show an increased frequency of congenital abnormalities associated with IVF and the increased risk of genetic abnormalities associated with microinjection techniques (ICSI) is thought to be related to parental factors. In essence, men with low sperm counts who need ICSI have an increased risk of carrying these underlying defects (Ludwig and Katalinic, 2002). The risk for imprinting defects may be considered to be higher when prolonged culture is applied, such as in IVM treatments (Gosden *et al.*, 2003). In studies presented so far however, children born after IVM are seen to be healthy and an increased risk associated with this technique has not been observed (Mikkelsen *et al.*, 2003).

Optimising the culture systems in IVM is of great importance and the definition of the various factors necessary for in-vitro maturation is of paramount importance for the success of these treatments. One of these factors, follicular fluid-meiosis activating sterol (FF-MAS), an intermediate of cholesterol biosynthesis, has been shown to induce oocyte maturation in-vitro (Byskov *et al.*, 1995; Hegele-Hartung C *et al.*, 1999; Grondahl *et al.*, 2003). Manipulation of this and other factors, such as metabolites and growth factors (Sutton *et al.*, 2003), in IVM culture requires further investigation and will be crucial for the development of IVM treatments.

### **3 AIMS OF THE PROJECTS**

The aim of the thesis projects was to develop methods for cryopreservation of follicles in human ovarian tissue, to identify new groups of patients who might benefit from such methods and to develop techniques for in vitro maturation of ovarian follicles and oocytes.

Specific aims

- To study the effect of Growth differentiation factor-9 (GDF-9) on human ovarian follicles in organ culture.
- To analyse the numbers and densities of follicles in ovarian cortical tissue from adolescent girls with Turner's syndrome who came to our clinics with an aim to preserve ovarian tissue for possible infertility treatment later in life.
- To compare the use of serum and human serum albumin (HSA) for cryoprotectant solutions containing propanediol and sucrose to evaluate whether serum-free medium could be used for cryopreservation of follicles in human ovarian tissue.
- To compare recombinant LH and recombinant hCG for the in-vitro maturation of human oocytes in a clinical study. A secondary objective was to optimise the final stages of oocyte maturation in the IVF laboratory and to establish a functioning clinical IVM program.

## **4 MATERIALS AND METHODS**

### **4.1 SUBJECTS, COLLECTION AND PROCESSING OF OVARIAN TISSUE BIOPSIES**

Ethical approval was obtained for all the studies presented in this thesis from the Ethics Committee of the Karolinska Institutet at Huddinge University Hospital.

Small biopsies of ovarian cortical tissue, up to five millimetres in diameter, were obtained as donations from 57 women who had given informed consent for participation in the studies (articles I and III). Of these women, 45 underwent Caesarean section and 12 underwent gynaecologic operations by laparoscopy. The mean age of the women was 33.4 years  $\pm$  4.5 (SD), range 19–41 years. There was no overlap of patient biopsies between different studies.

The tissue was collected into warm, pre-equilibrated HEPES-buffered medium containing HSA (Gamete<sup>®</sup>, Vitrolife, Göteborg) and immediately taken to the laboratory. Working under a stereomicroscope, most of the medullar tissue was removed. The cortical tissue was then cut into pieces of approximately 1–1.5 mm<sup>3</sup> with a scalpel, keeping the tissue immersed in collection medium during initial processing.

In article II, ovarian tissue from nine girls aged 12–19 years, was cryopreserved to store ovarian follicles that might still be left in their ovaries. One of these girls, aged 15 years, had a marker Y-chromosome and she underwent oophorectomy because of the risk of cancer. A part of the ovary (25%) to one whole ovary was laparoscopically removed for the procedure. In addition to these nine girls, one 17-year-old girl underwent laparoscopy, but no ovarian tissue was found.

For the IVM study (article IV), patients were recruited from our IVF programme. Inclusion criteria were the same as for regular IVF/ICSI treatments, however couples with male factor infertility requiring sperm extraction from a testicular biopsy were not considered for the study. The mean age of the women was 31.5  $\pm$  3.7 (SD), range 23–38 years. Indications for treatment were in most cases unexplained infertility, anovulation or male factor infertility. A total of 84 couples were assessed for eligibility for the randomised study and 73 completed their treatments, 36 for oocyte maturation in culture medium containing recombinant hCG, and 37 using recombinant LH. Eleven cycles were cancelled before oocyte retrieval (OR), in most cases due to suboptimal endometrial or follicular development. Parallel to these treatments, 38 individually tailored IVM cycles were carried out among 32 couples, two thirds of which were repeated cycles among couples previously included in the study.

### **4.2 CRYOPRESERVATION AND THAWING OF OVARIAN TISSUE, ARTICLES II & III**

After collection and initial processing of the ovarian tissue, the tissue pieces were equilibrated with cryoprotectant. Our decision to use PrOH and sucrose during slow cooling was based on previous results (Hovatta *et al.*, 1996).

First the tissue was immersed in the basic buffer solution for 5 minutes, transferred to the first cryo-solution (1.5M PrOH) for 10 minutes and to the second cryo-solution (1.5M PrOH with 0.1M sucrose) for 5-15 minutes. All steps were carried out at room

temperature. The tissue pieces were transferred in 1ml of the second cryo-solution to 1.8ml Nunc cryovials and placed in a programmable freezer (CryoLogic, Australia). The samples were cooled from room temperature to -6.5 °C at a rate of 2.0 °C/min, seeding was performed with liquid nitrogen-cooled forceps during a 10min holding period, then cooled to -35 °C at a rate of 0.3 °C/min and plunged into liquid nitrogen during a free fall from -35 °C.

The tissue was stored in liquid nitrogen for 5–7 months (article III). The cryopreserved tissue from patients reported in article II is kept in liquid nitrogen storage until the patients express their wish to utilise it.

In article III, cryopreservation solutions with HSA were obtained commercially (Freeze-Kit 1<sup>®</sup>, Vitrolife), and consisted of a phosphate-buffered salt solution (PBS) supplemented with HSA at 25mg/ml. Cryopreservation solutions with serum consisted of PBS supplemented with pooled heat-inactivated serum (20% v/v) obtained from women undergoing pituitary de-sensitisation preceding fertility treatment at the Fertility Unit. These basic solutions were supplemented with PrOH and sucrose as previously described.

The cryopreservation solutions used in article II consisted of PBS with autologous serum at 20%. Otherwise the cryopreservation method was the same.

The tissue was thawed (article III) using thawing solutions containing HSA (Thaw Kit 1<sup>®</sup>, Vitrolife) or serum, corresponding to the cryopreservation procedure.

All thawing steps were performed at room temperature. The cryovials were removed from the liquid nitrogen, air-thawed for 30 s and thawed in a water bath until the ice had melted. The tissue pieces were incubated as follows: 1.0M PrOH and 0.2M sucrose for 5 minutes, 0.5M PrOH and 0.2M sucrose for 5 minutes, 0.2M sucrose for 10 minutes and finally in PBS for 10 minutes.

Two to three tissue pieces from six subjects were fixed directly after thawing for light microscopy analysis. All other tissue pieces were cultured in pre-equilibrated  $\alpha$ MEM (Invitrogen) supplemented with HSA (10%, Pharmacia, Stockholm) and ascorbic acid (50  $\mu$ g/ml, Sigma-Aldrich, Steinheim, Germany) (Thomas *et al.* 2001) for four hours at 37 °C and 5% CO<sub>2</sub> to see if normality of the tissue was restored.

#### **4.3 CULTURE OF FOLLICLES, ARTICLE I**

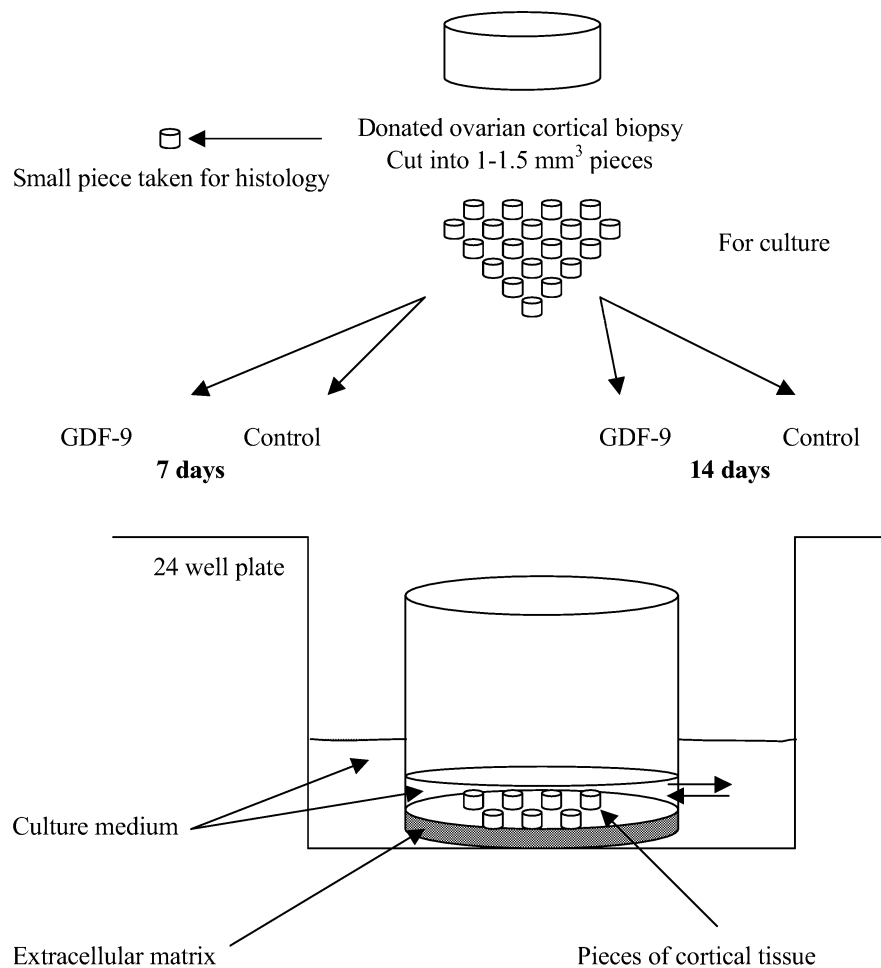
The culture medium consisted of  $\alpha$ MEM supplemented with 10% HSA or 5% foetal bovine serum (FBS). ITS-mix (1%, Invitrogen) was added to the culture medium giving effective concentrations of 10 mg insulin/ml, 5.5 mg transferrin/ml and 6.7 ng sodium selenite/ml. Antibiotic/antimycotic solution (0.5%, Invitrogen) was used giving 50 IU penicillin G/ml, 50 mg streptomycin sulphate/ml and 0.125 mg amphotericin B/ml. To minimize apoptosis, 2.5 mM 8-br-cGMP (Sigma) was added as previously described (McGee *et al.*, 1997), and recombinant FSH (Gonal-F<sup>®</sup>, Serono Nordic, Stockholm) at 5 IU/ml was added as a survival factor (Wright *et al.*, 1999).

To test the effects of GDF-9, we added recombinant rat GDF-9 to the culture medium (200 ng/ml) of one of two parallel cultures from the same biopsy specimen. Either N-tagged biologically inactive recombinant rat GDF-9 or culture medium without GDF-9 were used as the negative control (Hayashi *et al.*, 1999).

Tissue pieces were cultured at 37 °C in 5% CO<sub>2</sub> in a humidified incubator for 7 and 14 days in Millicell culture well inserts (Millipore, Bedford, MA, USA) fitted into 24-well

Nunc plates. The inserts had been coated with 100  $\mu$ l extracellular matrix and contained 100  $\mu$ l culture medium with a further 400  $\mu$ l culture medium in the surrounding well (Figure 5). Culture media were sampled and replenished every second day. Three combinations of serum supplements and extracellular matrix were used. Tissue from the first 19 biopsies was cultured with 10% HSA on culture plate inserts coated with human extracellular matrix (ECM) (Becton Dickinson, Bedford MA). The following 9 cultures contained 5% FBS (Invitrogen) with ECM, and in the final 6 cultures we used 5% FBS with Matrigel matrix (Becton Dickinson).

**Figure 5**



Culture system for ovarian cortical biopsies.  $\alpha$ MEM was supplemented with 10% human serum albumin or 5% fetal bovine serum, ITS-mix, antibiotics, cGMP and FSH. 200 ng/ml GDF-9 was added, control cultures were performed without GDF-9.

#### **4.4 FIXATION AND HISTOLOGY FOR LIGHT MICROSCOPY, ARTICLES I, II & III**

Tissue was fixed in Bouin's solution, a mixture of Picric Acid, Formalin and Acetic Acid, for 24 hrs at 2-8 °C and then dehydrated in 70% ethanol. Following paraffin embedding (Paraplast, Sherwood medical, St. Louis, USA) the tissue pieces were cut into 4 µm sections. To avoid double counting of follicles, at least ten sections were discarded before the next one was mounted onto the slide, after which staining was performed with haematoxylin and eosin for histological examination.

Primordial follicles were defined as containing only flat follicular cells, primary follicles having one or more cuboidal follicular cells, and secondary follicles with at least part of the follicle with two or more layers of cuboidal granulosa cells. Atretic follicles were defined as those with pyknotic follicular cells, eosinophilia of the ooplasm and clumping of the chromatin material.

To measure oocyte and follicle diameter and the area of the tissue pieces, we used a digital image analysis system (Easy Image Mätning, Tekno Optik, Stockholm) connected to an inverted microscope. The volume of the section was then calculated by multiplying the area of the tissue piece by the known thickness, 4 µm.

#### **4.5 TRANSMISSION ELECTRON MICROSCOPY, ARTICLE III**

The tissue samples taken for TEM were first incubated for 2 hrs at room temperature in 2% glutaraldehyde and 0.5% paraformaldehyde in 0.1 M sodium cacodylate buffer and 0.1M sucrose at pH 7.4. The tissue was stored in the fixative at 4 °C for 3-4 weeks before further processing. The tissue blocks were embedded in LX-112, cut into 0.5 µm sections and stained with toluidine blue. Light microscopy was used to select sections with follicles for further examination. Thin sections, approximately 50 nm, were cut and stained with 2% uranyl acetate, followed by lead citrate and examined in a transmission electron microscope (Tecnai, Fei, Eindhoven, The Netherlands) at 80 kV. Micrographs were taken at low magnification (1400-2000 x) and printed copies at a final magnification of 3200-4500 x were used for evaluation.

Oocytes, granulosa cells and stromal cells were scored separately, both in the freshly dissected tissue and in the two test groups. An independent observer mixed all the micrographs and they were then evaluated in a blind fashion by two experienced electron microscopists. The ultrastructure of the different cell types was evaluated by using a scoring system. The nuclear content and membrane integrity, cristae of the mitochondria and their density, the density of the cytoplasm, the content of membrane vesicles and attachment to the granulosa cells were the criteria used for evaluation of the oocytes. In granulosa cells, the same parameters were judged except for attachment. The stromal cells were evaluated only by their nuclear content and the integrity of the extracellular matrix. The individual score of each cell type was calculated and divided by the maximum score, thus giving an index of morphological preservation. The mean and standard deviation of the different cell types in each group were calculated.

Normal structures were scored as 2, small changes as 1 and severe damage as 0. The final total score of the individual cell types was divided by the maximum possible score of all evaluated cells to give a ratio reflecting the preservation of the cells. A perfectly preserved cell group would thus obtain a ratio of 1.0 (100%) and the results are presented as a cumulative proportion of maximum possible score for all groups.

#### **4.6 LIVE / DEAD ASSAY, ARTICLE III**

Ovarian tissue was digested with 1.5 mg/ml Collagenase Type II (Invitrogen) in pre-equilibrated  $\alpha$ MEM with 10% pooled human serum (same as for cryopreservation) at 37 °C and 5% CO<sub>2</sub> in a humidified incubator. After 1 hr the presence of isolated follicles was checked. If there were follicles floating in the medium, all the medium and tissue was filtered (100  $\mu$ m mesh size, Gelman). Tissue blocks on the filter were collected and the flow through medium was centrifuged at 100 g for 5 minutes. Most of the supernatant was removed and the pellet with a small amount of medium was checked under an inverted microscope for follicle identification. Follicles were stored in culture medium in the incubator for later analysis. This procedure was repeated every 30 minutes for as long as follicles presented in the medium. Usually after 2–3 hrs digestion a clear view of the follicles in the stroma was possible and they could be stained along with fully isolated follicles. At this point the pellet together with the individual follicles was resuspended in Live/Dead working solution (Live/Dead Viability/Cytotoxicity Kit (L-3224), Molecular Probes, Eugene, Oregon USA). The working solution contained 2 $\mu$ M Calcein AM which is converted to calcein by intracellular esterases and produces an intense green fluorescence in live cells, and 4 $\mu$ M Ethidium Homodimer-1 (EthD-1) which enters cells with damaged membranes and produces bright red fluorescence upon binding to nucleic acids. EthD-1 is excluded by the intact plasma membrane of live cells.

After 20–30 minutes incubation at room temperature, the cell suspension was briefly centrifuged again (100 g for 1 minute) and most of the supernatant was removed. Partly and fully isolated follicles were mounted onto glass slides. After the cover slides were mounted, the viability of the follicles was assessed under a fluorescence microscope. Only follicles showing exclusive esterase activity (green colour) were counted as viable in the assay and follicles showing red colour in either the oocyte or surrounding pre-granulosa cells were counted as dead.

#### **4.7 HORMONE ASSAYS, ARTICLE II**

The assays in Sweden were performed as follows: Unconjugated estradiol-17 $\beta$  in serum was measured by a solid-state radioimmunoassay coated tube kit (ESTR-US-CT, Orion Diagnostica, Espoo, Finland) for direct quantitative determination. The detection limit for the assay was 5 pmol/l, with an intra-assay coefficient of variation (CV) of 13% at a mean value of 22.6 pmol/l. FSH and LH in serum were measured by immunometric assays (Immulite, Diagnostic Products Corporation, Los Angeles, California). The analytical sensitivity of the FSH assay was 0.1 IU/l, with intra-assay CVs of 5.4% at a mean of 7.8 IU/l, 6.1% at 19.7 IU/l and 7.7% at 42.5 IU/l. For the LH assay the analytical sensitivity was 0.1 IU/l, with an intra-assay CV of 4.8–6.5%.

The assays in Finland were performed as follows: Serum estradiol-17 $\beta$  concentrations were determined by a modified radio immunoassay (RIA) using coated tube technology (Spectria E2, Orion Diagnostica, Espoo, Finland) after diethylether extraction. The detection limit of the assay was 6 pmol/l. Serum LH and FSH concentrations were measured in single samples by time-resolved immunofluorometric assays (Wallac, Inc., Turku, Finland). The sensitivity of the LH and FSH assays was 0.05 IU/l (Wickman and Dunkel, 2001).



#### 4.8 IN-VITRO MATURATION OF OOCYTES, ARTICLE IV

Follicle priming for the women was achieved by 37.5 IU of recombinant FSH (Gonal F<sup>®</sup>) on cycle days (CDs) 2–6 after spontaneous or Gestapuran<sup>®</sup> induced bleeding (10mg per day for 10 days; Leo Pharma, Malmö). In 14 cases the FSH injections were continued for an additional 2–11 days to obtain follicles suitable for aspiration and one woman received a daily dose of 75 IU of recombinant FSH on CDs 2–8. Ultrasound (US) monitoring was performed initially on CD 3–6 to check for cysts, then on CD 6–8 for measurement of endometrial thickness and follicular size. Thereafter, US monitoring was performed every two to three days and oocyte retrieval was performed when at least one follicle had reached a diameter of 10–14 mm and the endometrium was at least 5 mm in thickness. The follicles were aspirated using a single lumen needle (Swemed Lab International AB, Billdal) and low-level continuous vacuum. No flushing was carried out. No hCG was administered to any of the women before OR.

In the individually tailored parallel group, there were 16 FSH-primed cycles; these were preferred for those women who did not have regular menstruations. In 12 cases the women received 37.5 IU/day of recombinant FSH on CDs 2–8, two received 75 IU/day on CDs 2–6, one received 50 IU/day on CDs 2–6 and in one case 200 IU/day on CDs 2–6 with one additional day of 100 IU. Twenty two were natural cycles without FSH stimulation. No hCG was administered before OR during these cycles. Otherwise, clinical management was the same for the study groups and the parallel group.

The follicle aspirates were collected in 1–1.5 ml HEPES-buffered medium (Gamete-100<sup>®</sup>, Vitrolife). The aspirates were filtered through a Falcon cell strainer of 70 µm mesh size (Becton-Dickinson) keeping the filter immersed to prevent the oocytes from drying out and the oocytes were then washed in the same medium. They were transferred to IVF medium (IVF-100<sup>®</sup>, Vitrolife) until final preparation of the maturation medium was finished.

The maturation medium consisted of Tissue Culture Medium 199 (TCM-199, Invitrogen) supplemented (10%) with serum collected from the subject involved on the morning of OR, 0.3 mM pyruvate (Sigma-Aldrich), recombinant FSH (0.075 IU/ml; Gonal-F<sup>®</sup>), Penicillin-G (0.05 mg/ml; Sigma-Aldrich) and Streptomycin Sulphate (0.075 mg/ml; Sigma-Aldrich). For the randomised study, either recombinant hCG (0.5 IU/ml; Ovitrelle<sup>®</sup>, Serono) or recombinant LH (0.5 IU/ml; Luveris<sup>®</sup>, Serono) was added to the medium. For the non-randomised parallel treatments, hCG (0.5 IU/ml; Profasi<sup>®</sup>, Serono) was added to the culture medium; otherwise the laboratory procedures were identical for the study groups and the parallel group. Finally the medium was filtered through a 0.2 µm sterile filter (Gelman). Oocytes were transferred to this medium after it had equilibrated to 37°C and pH 7.3. The time for in vitro maturation was 32–36 hrs. All oocytes were cultured in maturation medium, irrespective of morphology at OR.

After the maturation period the cumulus cells were removed by incubation with hyaluronidase (80 IU/ml; Hyase<sup>®</sup>, Vitrolife) for up to one minute and denudation by pipetting. After washing, the oocytes were transferred to IVF medium in 20 µl droplets under oil. End point of maturation in this study was defined as nuclear maturation from GV to MII stage identified by extrusion of the first polar body. MII oocytes were inseminated by microinjection (ICSI). Fertilized oocytes displaying two pronuclei 16–18 hrs after microinjection were selected for further culture until embryo transfer (ET)

on day 2–3 after ICSI. Embryo selection for transfer or cryopreservation was performed according to previously published criteria (Fridström *et al.* 1999).

One hour after OR the women started oral estradiol supplementation (Progynon, Schering AG, Berlin, Germany), 6 mg daily. The day after OR vaginal progesterone (Progesteron MIC, Apoteket) was added, 400 mg in the evening and thereafter 400 mg three times daily. A maximum of two embryos was transferred at a time, using a soft catheter and ultrasonographic guidance.

Estradiol and progesterone substitution was continued until the time of the pregnancy test. If the result was positive, the hormonal substitution was continued until the ninth gestational week. Pregnancy testing was performed by urinary assay 14–16 days after ET. Negative results were confirmed by registering menstrual bleeding. Positive results were confirmed by abdominal ultrasound examination 4 and 6 weeks after OR. Only clinical pregnancies confirmed by ultrasonography were recorded.

#### **4.9 STATISTICAL ANALYSIS**

The Chi square test and Fisher's exact test were used to test differences between groups in articles I, III and IV as regards differential proportions and frequency results. The statistical program Statistica (Stat Soft Inc., Tulsa, USA) was used for more advanced statistical analysis. In article I we used Student's t-test for statistical analysis of differences in the diameters of follicles and oocytes, and analysis of variance (ANOVA) for analysis of follicular density. The Mann-Whitney U-test was performed to analyse differences in the oocyte:follicle ratio in article I and for analysis of differences in TEM scoring in article III.

$P < 0.05$  was considered statistically significant.

## 5 RESULTS AND DISCUSSION

In all the studies included in this thesis, the majority of the follicles in freshly dissected tissue were in the latent primordial stage. In article I, 55% of the follicles were primordial, 34% were at the primary stage and 11% were secondary. In article III, 65% of the follicles were primordial, 28% were primary and 7% were secondary. The relatively high proportion of follicles at the primary stage may be explained by the age of some of the patients donating biopsies, ranging up to 41. As follicles are depleted from the ovaries with age, a larger proportion of the follicles will be in the growing stages (Gougeon *et al.*, 1994). The proportion of secondary follicles in the donated biopsies compares well with earlier data (Wright *et al.*, 1999) with growth to secondary stage being the end-point in article I. Viability of the follicles in freshly dissected tissue was 95% in article I and 99% in article III, as determined by light microscopy.

### 5.1 ARTICLE I

A total of 900 follicles from 34 women were analysed. The average age (+/-SD) of the patients was 32.8 +/- 5.0 (range 19-41) years. In-vitro culture caused initiation of growth in all groups. The main results were that a significantly higher proportion of secondary follicles (53% vs. 31%,  $p < 0.01$ ) and a significantly lower proportion of primordial follicles (22% vs. 47%,  $p < 0.001$ ) was seen after seven days in culture containing HSA with GDF-9 compared with controls. This difference was not seen in the culture containing FBS, possibly due to a masking effect by other growth factors present in the serum.

The mean diameter of the oocytes in freshly dissected tissue was 39.1 +/- 8.2 micrometers (range 28-77) and of the follicles 52.9 +/- 26 (SD) micrometers (range 32-158). The follicles and oocytes grew significantly in size during culture in medium supplemented with HSA ( $p < 0.01$ ), with the exception of oocytes cultured with GDF-9 for 7 days. Increase in diameter was not observed when culturing with FBS, after 7 days the oocyte diameter was significantly smaller than in uncultured tissue. The significance of this is not clear and interpretation is made difficult by the large variation within the groups.

The proliferative effects of GDF-9 on the granulosa cells were confirmed by our analysis of diameters of the follicles and oocytes and the ratios between the two. A significant decrease in the ratio between the diameters of the oocytes and follicles was found in medium containing HSA between cultures with GDF-9 and controls, confirming the results of the developmental stages regarding the proliferative effect of GDF-9.

The proportion of viable follicles was reduced during culture in all groups. A positive effect of GDF-9 on viability was seen in culture medium containing both HSA and FBS. Significantly improved viability compared with controls was observed after seven days in the FBS group (76% vs. 48%,  $p < 0.01$ ) and after 14 days in the HSA group (72% vs. 48%,  $p < 0.001$ ). A reduction in the density of follicles was also seen during culture, as the final stage of follicular atresia is resorption by the stromal cells. This reduction was significantly less pronounced in culture with GDF-9 and the difference was statistically significant after 14 days of culture in medium containing HSA ( $p = 0.02$ ) and FBS ( $p = 0.01$ ). GDF-9 may rescue the follicles in the biopsies from

becoming atretic during culture by stimulating the growth of a large proportion of these follicles.

We have shown that GDF-9 is an important promoter of early granulosa cell growth and acts as a survival factor of human ovarian follicles in-vitro. GDF-9 is important during all stages of follicle growth; it promotes granulosa cell proliferation during the early pre-antral stages and induces expansion and mucification of cumulus cells prior to ovulation (Elvin *et al.*, 1999). When designing culture systems for completing follicle growth and maturation in-vitro these positive effects must be taken into consideration. Other factors, which also have been shown to stimulate follicle growth and promote survival in-vitro include FSH (Wright *et al.*, 1999), IGF I, IGF II and insulin (Louhio *et al.*, 2000). Stage specific culture systems should also be considered (O'Brien *et al.*, 2003).

Our results regarding GDF-9 are confirmed by the in-vivo results in rodents (Vitt *et al.*, 2000b), which also show a positive effect of GDF-9 on progression of follicles to secondary stages.

## 5.2 ARTICLE II

We found follicles in ovarian cortical tissue from eight out of nine subjects from whom ovarian tissue was taken. One girl had only streak ovaries and no ovarian tissue was obtained.

All these girls had received growth hormone (GH) therapy and estradiol substitution when necessary, depending on individual subjects. Six of the girls had had spontaneous onset of puberty and development at least up to Tanner stage 4 (Tanner, 1978), four of them were on HRT at the time of biopsy.

Three of the girls, aged 13, 15 and 19 years, already had high serum concentrations of follicle-stimulating hormone (FSH; 67, 94 and 69 IU/l respectively); the others had slightly elevated or normal FSH levels (taken without HRT). Four of the girls had non-mosaic Turner's syndrome, with all of the analysed cells displaying karyotype 45X; the others had varying degrees of mosaicism. One 13-year-old girl had karyotype 45X/46XXp+, in which p+ depicts uncharacterised chromosomal material, and another 13-year-old had karyotype 45X/46XX/47XXX.

Follicular density in the biopsies was 1.5 - 499 follicles per mm<sup>3</sup>, the highest numbers were observed in tissue from the youngest girls. A correlation was seen between the FSH levels and follicular density, as well as between karyotype and follicular density. Individuals with low FSH levels had high follicular densities and the highest follicle counts were observed in the girls with the lowest degree of mosaicism (Figure 6).

Girls with non-mosaic Turner's syndrome had follicles in their ovaries, although the counts were lower than in the mosaic subjects. We were not able to see a clear correlation between follicular density and age, perhaps due to the small number of patients included in the study.

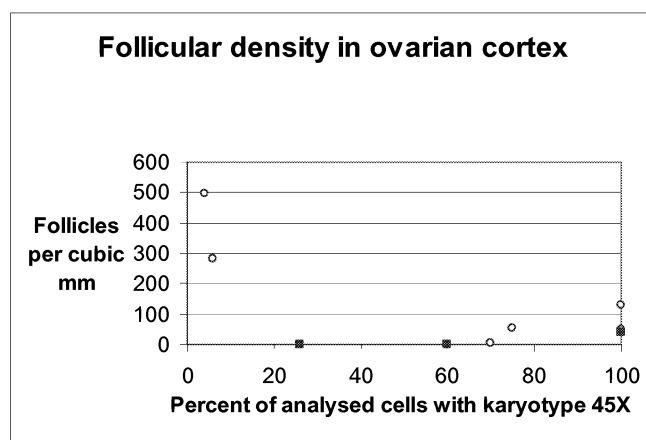
Primordial and primary follicles were seen in the ovaries of one 12-year-old and one 13-year-old non-mosaic subject, and also in three of the mosaic subjects. Three of the older girls had only growing follicles at secondary stages, consistent with the situation in the ovary when menopause is imminent (Faddy and Gosden, 1996). In one 19-year-old girl a single secondary follicle was found and the oocyte displayed abnormal staining patterns. Two 15-year-old girls had, in addition to normal follicles, clearly atretic primordial and primary follicles. These atretic structures are hardly ever seen in

normal ovarian tissue but may be encountered in organ culture of ovarian cortical tissue. Normal early antral follicles were seen in the tissue from one 17-year-old girl with high follicular density in her ovaries.

Girls with Turner's syndrome may be considered a new group who can benefit from preservation of fertility through cryopreservation of ovarian tissue. The finding that these girls still have follicles in their ovaries runs contrary to previous assumptions that they, in most cases, have no hope of having their own genetic offspring. Even though a clear correlation between follicle density and age was not observed in this study, the results indicate that the disappearance of follicles from their ovaries occurs later than previously thought. Ovarian tissue should be obtained at a young age to maximize the number of follicles available for preservation.

Concern has been raised regarding chromosomal disorders in the children of women with Turner's syndrome. This originates mostly from case reports, which may be biased toward a higher frequency of aberrations. In a recent study, six of twenty-five children of women with Turner's syndrome had chromosomal aberrations, no case of Down's syndrome was present and only two were diagnosed with malformations (Birkebaek *et al.*, 2002). Normality of the oocytes in the ovaries of girls with Turner's syndrome has not been studied.

**Figure 6**



Follicular density in the ovarian cortex of the nine girls with Turner's syndrome included in the study. Black boxes represent those receiving HRT at the time of biopsy, open circles those who were not receiving HRT.

### 5.3 ARTICLE III

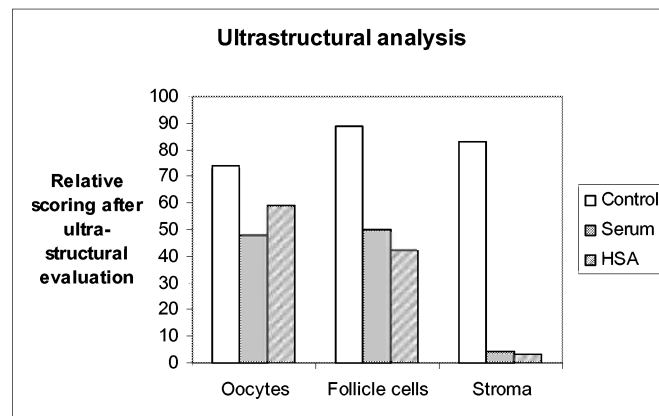
We used three methods to compare the use of serum and HSA in the cryopreservation of follicles in ovarian cortical tissue: Histology by light microscopy on 693 follicles, Transmission Electron Microscopy (TEM) on 66 follicles and Live/Dead (L/D) assay

on 559 follicles. The main end point was the viability of the follicles and in the case of TEM a detailed ultrastructural analysis.

A significant reduction in viability ( $p < 0.01$ ) was seen after cryopreservation and thawing when evaluated by light microscopy. Using cryopreservation solutions with serum, 65% of the follicles and 75% of the oocytes were judged to be viable. When using HSA, 70% of the follicles and 74% of the oocytes were judged to be viable. The differences between serum and HSA were not significant. However, tissue cryopreserved with serum showed a reduction in viability after 4 hrs culture whereas this was not observed when cryopreserved with HSA.

Reduced viability after cryopreservation and thawing was seen in the TEM analysis as shown by the significantly higher score of oocytes, granulosa cells and stromal cells in the freshly dissected tissue. The most noticeable reduction in score compared with controls was observed in stromal tissue (Figure 7). The relative score obtained by quantifying the TEM analysis is not directly comparable to the other viability estimates used in this study. The relatively low scoring of the fresh control samples may be considered to show the strict scoring applied and the results after cryopreservation and thawing may be considered from this perspective. As far as we know, this is the first time systemised and blinded TEM scoring has been used to evaluate ultrastructural parameters of ovarian follicles. No significant differences were observed between cryopreservation in medium containing serum or HSA.

**Figure 7**



TEM analysis before (control) and after cryopreservation and thawing.

A morphometric analysis of the TEM images showed a vacuolisation of 13 % of all oocytes in freshly dissected tissue. After cryopreservation and thawing the vacuolisation was 18 % with both serum and HSA. The number of pyknotic cell nuclei in granulosa cells in freshly dissected tissue was 1/161 (<1 %). With serum this ratio was 16/91 (17.6 %) and with HSA 13/50 (26.0 %, n.s.).

The viability of the isolated follicles in the L/D assay was 93 % in the control tissue and this was reduced to 82 % in both groups after thawing ( $p < 0.01$ ). It is possible that the

higher proportion of live follicles after thawing in the L/D assay compared with light microscopic analysis is due to some extent to follicle loss during isolation for the L/D assay, especially secondary follicles were lost. However, we estimate that this possible bias was limited since most follicles were partly isolated. Staining in tissue slides as described by Cortvriend and Smits (2001) might eliminate this potential loss.

Our results are comparable with those published by other authors in terms of L/D assay of thawed follicles (Oktay *et al.*, 1997) and the extent of vacuolisation (Gook *et al.*, 1999). The relatively poor survival of stromal cells after cryopreservation and thawing has been noted by other authors (Gook *et al.*, 1999) and a discrepancy between survival of the oocytes and the granulosa cells has also been observed (Siebzehnruhl *et al.*, 2000). The most challenging problem regarding cryopreservation of ovarian cortical tissue may be the simultaneous preservation of the oocytes, granulosa cells and the stroma surrounding the follicles. Improvement regarding the preservation of stromal tissue surrounding the follicles remains a challenge.

#### **5.4 ARTICLE IV**

A total of 710 immature oocytes for culture and maturation in-vitro were collected from 111 women. Of these, 228 oocytes were randomised for maturation in culture medium containing recombinant hCG and 256 oocytes for maturation with recombinant LH. Mean numbers of 6.3 oocytes (hCG) and 6.9 oocytes (LH) were obtained per OR.

In culture, 55% of the oocytes matured to metaphase II (MII) with recombinant hCG and 56% matured with LH (n.s.). No significant differences were observed regarding fertilization rate or embryo cleavage between the groups. Most of the subjects coming for OR reached embryo transfer, 78% with hCG and 68% with LH. Three clinical pregnancies were achieved in the hCG-maturation group and one in the LH group. These differences were not statistically significant.

In the parallel treatments, not included in the randomised study, we retrieved 226 oocytes from 38 ORs, 59% of the oocytes matured, and 82% of the cycles progressed to ET. The pregnancy rate in this set of treatments was higher than in the study groups, 23% per ET. Two thirds of these cycles were repeated cycles for patients previously included in the study and previous experience of monitoring these patients might have contributed to more successful treatments.

Failed maturation was observed in four cases in the study group, two of these patients had one oocyte, one had two oocytes and one had eight oocytes. Two in the parallel group had failed maturation, one patient with one oocyte and one with three oocytes.

Cryopreservation of embryos was performed in one case in the study group and in three cases among the subjects not included in the study. No pregnancies have yet been achieved after thawing of these embryos.

The four pregnancies resulting from the randomised treatments proceeded uneventfully, whereas two of the seven pregnancies in the parallel group ended in miscarriage, one in the first trimester and the other in the second trimester. One pregnancy in the randomised group of treatments showed two implantation sacs, one of which was viable; all other pregnancies were singletons. Nine healthy infants have been born, six girls and three boys, the mean (+/- SD) birth weight was 3383 g +/- 650 g, ranging from 2465 g to 4630 g.

When analysing the quality parameters of the embryos transferred in the IVM treatments, lower embryo scoring and slower progression of cleavage was seen compared with the regular IVF/ICSI-programme where most patients get at least one embryo of good quality and good cleavage progression for embryo transfer. Asynchrony between nuclear and cytoplasmic maturation (Combelles *et al.*, 2002) may be one cause for this observation. Ten of the women included in our study had a previous history of a poor response after conventional IVF/ICSI, or previous failed IVF/ICSI cycles, which may also partially explain the modest pregnancy rates observed. Selection criteria that might have reflected diminished ovarian reserve, such as elevated FSH levels or decreased inhibin B levels in serum were not used to select patients for our study. Endometrial development may also affect implantation rates, our inclusion criteria for endometrial thickness was 5 mm or more at the time when oocyte aspiration was decided. This is lower than commonly seen in IVF/ICSI treatments and may also contribute to a lower pregnancy rate in our study.

After the conclusion of the projects presented in this thesis, IVM treatments have been continued at Huddinge University Hospital. For regularly menstruating women, natural cycles are used without any exogenous gonadotrophins. Anovulatory patients receive low-dose FSH priming which has previously been shown to be beneficial for these women (Mikkelsen and Lindenberg, 2001).

Performing clinical IVM treatments requires training and expertise both in the laboratory and in the clinical management of the patients. We have improved our results during the years we have performed these treatments, both due to training and improved patient selection. Table 1 shows the overall results from IVM treatments at Huddinge University Hospital.

**Table 1**

Year	Oocyte aspirations	Embryo transfers	Pregnancies	Pregnancies per ET
2001	68	50	3	6 %
2002	56	46	10	22 %
January to August 2003	30	20	7	35 %

Results from IVM treatments at Huddinge University Hospital 2001 - 2003.



## 6 CONCLUSIONS

Various aspects of in-vitro maturation and culture of human ovarian follicles and oocytes have been studied. Growth and development of human ovarian follicles was attenuated in culture by supplementing the culture medium with GDF-9. Positive effects of GDF-9 on the proliferation of follicular cells were seen, which resulted in an increased progression of follicles to the secondary stage when cultured for seven days in medium containing HSA. This progression was confirmed by analysing the ratio of oocyte/follicle diameter, which decreased significantly during culture. Increased viability of the follicles was also seen in culture with GDF-9, both through histological analysis and by comparing follicular densities in ovarian cortical tissue cultured with or without GDF-9. Follicular density was consistently and significantly higher when cultured with GDF-9. In conclusion, GDF-9 is seen to be an important promoter of follicle growth in in-vitro culture systems, and that the positive effects of GDF-9 on follicle viability are exerted through initiation of growth of the follicles. Modulating other aspects, such as composition of the extracellular matrix, combination with other growth factors and sequential culture systems should be studied further.

New results regarding the final stages of oocyte maturation in culture were obtained. Recombinant hCG was shown to be equally effective as recombinant LH in promoting maturation of oocytes in IVM treatment. Embryological and clinical parameters were studied and no difference was discernible in the maturation of the oocytes, embryonic development or pregnancy rates between treatment with these two gonadotrophins. A functioning IVM programme was established during the process of these studies. Optimising IVM for various groups of patients was shown to be necessary since normally ovulating patients, treated without any FSH in the parallel group of patients had higher pregnancy rates than were observed among the study groups. These were, however, comparable with the pregnancy rates of the FSH-primed patients in the parallel group.

We see comparable results in our IVM programme after conclusion of the study and pregnancy rates have been improving with increasing experience. The overall results from the study were promising, in our hands a relatively good pregnancy rate of 23% per ET, could be achieved after IVM. In 2003, so far, the pregnancy rate in IVM has been 35% per ET, approaching conventional stimulated IVF/ICSI cycles. The rate of cycles that did not reach embryo transfer in the study, 18-32 %, was higher than is observed in stimulated cycles, which reduces the overall pregnancy rate.

IVM can be considered to be an alternative to regular IVF/ICSI treatments for selected groups of patients and studies regarding the impact of endometrial development in IVM treatments are planned.

A group of patients was identified who may benefit from fertility preservation through cryopreservation of ovarian cortical tissue. Follicles were found in adolescent girls with Turner's syndrome and even girls with non-mosaic Turner's syndrome may have follicles in their ovaries. This is an important finding as regards the preservation of fertility since the follicles seem to prevail in the ovaries longer than previously has been thought. Further studies are needed to determine if hormonal parameters, such as elevated FSH levels, or other criteria may be of value to determine which subjects can

be recommended to undergo ovarian biopsy for this purpose. Degree of mosaicism does not appear to be of great assistance in this evaluation.

A new study has been initiated where ovarian cortical tissue from adolescent girls with Turner's syndrome is obtained for cryopreservation and the chromosomal constitution of the oocytes is analysed by fluorescent in-situ hybridization in a part of the biopsy. At the same time information will be gathered regarding which criteria could be of assistance in selecting subjects for the procedure.

The risk associated with laparoscopy has to be evaluated when these girls are counselled, however the final conclusion is that cryopreservation of ovarian cortical tissue should be offered to these girls.

The methods for cryopreservation need further study and optimisation. Analysing a total of 1318 follicles, we showed that HSA is equally effective as serum for cryopreservation of follicles in ovarian tissue. Light microscopic analysis, transmission electron microscopy (TEM) and fluorescent viability markers were used to compare HSA and serum in this respect. Serum, traditionally used because of protection against zona hardening during cryopreservation and thawing and because of the protecting effects of certain macromolecules, was shown not to confer any additional protective effect on the follicles when compared with HSA. This was not unexpected since the primordial follicles have not yet developed a zona pellucida. Good viability of the follicles after cryopreservation and thawing was shown in all types of analysis. Vacuolisation of oocytes in TEM analysis has been observed previously, one possible explanation for this may be the poor preservation of lipid droplets during fixation and dehydration. The need for preservation of stromal tissue was highlighted by the TEM analysis.

The aspects of fertility preservation studied in this thesis highlight the necessity for further development of the current methods for preservation and in-vitro culture of oocytes and follicles in ovarian cortical tissue. Increased awareness of patients regarding the options available in this field exerts pressure to enhance understanding of these systems through basic research and to develop methods to increase their in-vitro survival and function. The results obtained in these projects give impetus to clinicians and scientists to utilise all options available for fertility preservation for patients at risk of premature ovarian failure.

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