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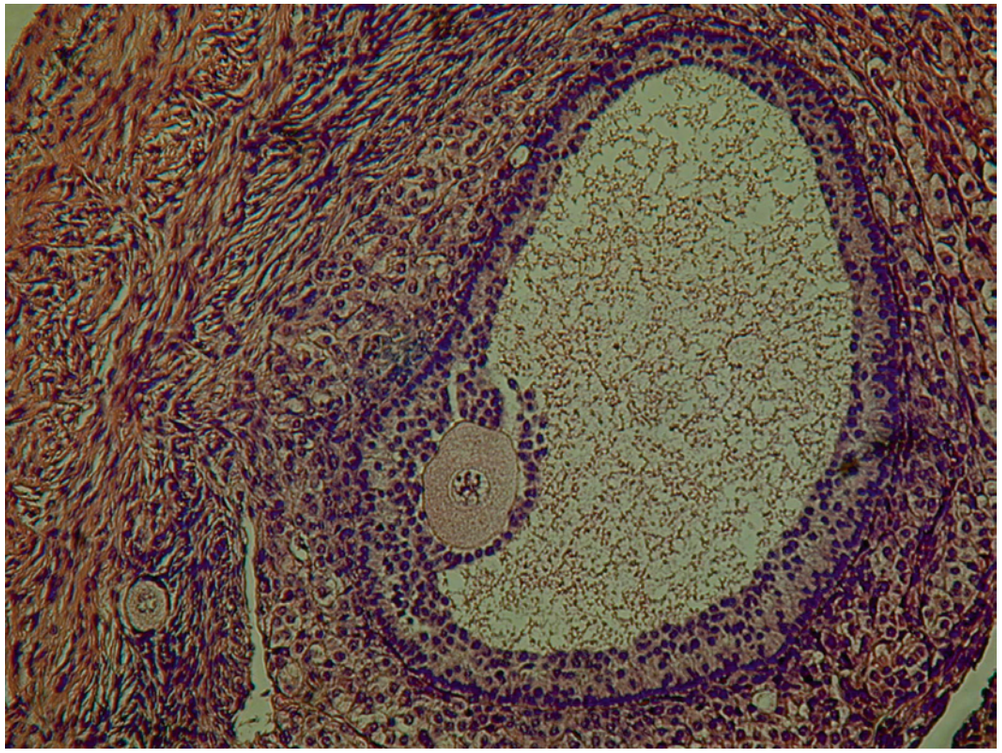

Inger Britt Carlsson

Karolinska Institutet

From the Department of Clinical Science, Intervention and Technology,
Division of Obstetrics and Gynecology,
Karolinska University Hospital Huddinge,
Karolinska Institutet, Stockholm, Sweden

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All we know is still infinitely less than all that remains unknown.
William Harvey

TO MY FAMILY, WITH LOVE

ABSTRACT

Cryopreservation of ovarian tissue containing immature oocytes is one approach to preserving the potential fertility of young women who risk losing their oocytes as a consequence of treatment with anti-cancer agents, or genetic disorders. This cryopreserved tissue can then be transplanted back into the ovary when the woman wants to have children. Our research group has also been developing an alternative procedure, namely maturation of ovarian follicles and their oocytes *in vitro*, for cancer patients that risk retransmission of the disease after transplantation. Live offspring can already be produced in mice from small antral follicles that have matured and been fertilized *in vitro*. In the case of women, it is much more challenging to obtain mature oocytes from ovarian tissue *in vitro,* due to the much longer period required for maturation and the dense structure of this tissue.

Employing ovarian biopsies obtained from volunteers, our research group has been actively optimizing conditions for culturing human ovarian tissue and we have shown that if structural and biochemical systems are kept intact (i.e., by culturing pieces of tissue instead of isolated follicles), primordial human follicles can develop into primary and even secondary follicles *in vitro*. However, fully mature oocytes have not yet been obtained and further optimization of this system is required. The mechanisms controlling the initiation of the growth of small ovarian follicles are not yet known in detail, although a number of factors produced in the ovary itself are known to be involved. These include the family of transforming growth factor beta, as well as other growth factors.

Our present findings can be summarized as follows: Ovarian cortical tissue should be cultured in the form of cubes on diluted Matrigel™ matrix and the composition of the extra cellular matrix (ECM) should be chosen on the basis of the goals of the study in question (Article I). Kit ligand (KL) mRNA and c-Kit mRNA and protein are expressed in follicles during all stages of development, from primary to antral stage, and the reduction in the survival of follicles in long-term culture caused by an antibody that blocks the c-Kit receptor indicates that signaling via KL/c-Kit plays an important role in the early development of human ovarian follicles (Article II). Moreover, anti-müllerian hormone (AMH) plays a key role in suppressing the entry of follicles into the growing pool, i.e., is one of the hormones involved in inhibiting the recruitment of primordial follicles (Article III). Finally, endogenous growth differentiation factor-9 (GDF-9) is an important regulator of the transition from primary to secondary follicles; BMPRII-Fc can suppress this transition; and, furthermore, the rhGDF-9 protein promotes the early development and growth of follicles, an effect which could be of clinical value.

Thus, we report here important new information concerning the early maturation of human oocytes and follicles in this culture system. This system provides a valuable tool for the identification of factors that promote or inhibit the recruitment of ovarian follicles and will aid in the improvement of procedures for assisted reproduction.

LIST OF ORIGINAL ARTICLES INCLUDED IN THIS THESIS

The present thesis is based on the following original articles, which are referred to in the text by their Roman numerals:

I: Scott JE, **Carlsson IB**, Bavister BD, Hovatta O. Human ovarian tissue cultures: extracellular matrix composition, coating density and tissue dimensions. Reprod Biomed Online. 2004 Sep;9(3):287-93.

II: **Carlsson IB**, Laitinen MP, Scott JE, Louhio H, Velentzis L, Tuuri T, Aaltonen J, Ritvos O, Winston RM, Hovatta O. Kit ligand and c-Kit are expressed during early human ovarian follicular development and their interaction is required for the survival of follicles in long-term culture. Reproduction. 2006 Apr;131(4):641-9.

III: **Carlsson IB**, Scott JE, Visser JA, Ritvos O, Themmen AP, Hovatta O. Anti-Müllerian hormone inhibits initiation of growth of human primordial ovarian follicles *in vitro*. Hum Reprod. 2006 Sep;21(9):2223-7.

IV: **Carlsson IB**, Lindeberg M, Pulkki MM, Pasternack A, Scott JE, Pettersson K, Myllymaa S, Laitinen MPE, Mottershead DG, Ritvos O and Hovatta O. Effects of Growth Differentiation Factor-9 Agonists and Antagonists on Early Human Ovarian Follicle Growth and Survival in Long-Term Culture. Submitted to JCEM 2008 for publication.

LIST OF ABBREVIATIONS

ACK2- Anti-c-Kit antibody
αMEM- Alpha-minimal essential medium
AMH- Anti-Müllerian hormone
ART- Assisted reproductive techniques
BMP- Bone morphogenetic protein
BMPRII- Bone morphogenetic protein receptor-Type II
cAMP- Adneosine 3',5'-cyclic monophosphate
cGMP- Guanosine 3',5'-cyclic monophosphate
CL- Corpus luteum
COC- Cumulus oocyte complex
ECM- Extracellular matrix
EGF- Epidermal growth factor
FSH- Follicle stimulating hormone
FSHR- Follicle stimulating hormone receptor
GC- Granulosa cell
GDF-9- Growth differentiation factor-9
GDF-9B- Growth differentiation factor-9B (also known as BMP-15)
GFR- Growth factor reduced
GH- Growth hormone
GHR- Growth hormone receptor
GL- Granulosa luteal cells
GnRH- Gonadotropin releasing hormone
GV- Germinal vesicle
Gy- Gray, absorbed dose of radiation
hCG- Human chorionic gonadotropin
HPO- Hypothalamus pituitary ovary Axis
HSA- Human serum albumin
IGF- Insulin-like growth factor
ITS- Insulin transferrin selinite
IVM- *In vitro* maturation
KL- Kit ligand (also known as stem cell factor, steel factor)
KO- Knock out
LD50- Lethal dose 50
LH- Luteinizing hormone
LIF- Leukemia inhibitory factor
MI- Metaphase I
MII- Metaphase II
OSE- Ovarian stromal epithelium
PCOS- Polycystic ovary syndrome
PGCs- Primordial germ cells
POF- Premature ovarian failure
SCF- Stem cell factor (also known as Steel Factor and Kit Ligand)
SCID- Severe combined immunodeficiency
TGF-ß- Transforming growth factor- beta

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Ex ovo omnia.
William Harvey

1 INTRODUCTION

Infertility is a growing worldwide problem today, affecting approximately 15% of all couples of fertile age. One major source of infertility are the side-effects of both chemo- and radiotherapy for cancer, which can cause the loss of gametes (Nicosia et al., 1985; Wallace et al., 1989; Byrne et al., 1992; McVie, 1999). Thus, as survival rates following anti-cancer treatment improve more and more patients are requiring assisted fertility.

Since the early 1970's, men have had the option of cryopreserving their sperm, a procedure that has also been applied for preservation of male fertility prior to cancer therapy. Today, cryopreservation is becoming more readily available as an option for women as well. Thus, current attempts to preserve a woman's fertility include cryopreservation of embryos; both mature and immature oocytes; and / or of ovarian tissue. This last approach is the only one that can be applied with prepubertal girls.

In this context, since immature ovarian follicles constitute most of the ovarian reserve, cryopreservation of cortical tissue is one of the most promising alternatives. This is also a good alternative for girls with Turner's syndrome (Hreinsson et al., 2002a), who experience a continuous decline in the number of their ovaian follicles. Our group and several others (Hovatta et al., 1996; Hovatta et al., 1997; Oktay et al., 1997b; Gook et al., 1999; Cortvrindt and Smitz, 2001; Gook et al., 2003; Schmidt et al., 2003) have achieved efficient survival of follicles in cryopreserved cortical tissue and autotransplantation of such tissue into mice (Mussett and Parrott, 1961; Gunasena et al., 1997; Sztein et al., 1998; Liu et al., 2001; Snow et al., 2002), sheep (Gosden et al., 1994; Salle et al., 2002), humans (Donnez et al., 2004; Meirow et al., 2005) and a number of other species (Candy et al., 1995; Aubard et al., 1998; Gunasena et al., 1998; Wang et al., 2002) has already produced offspring. In some cancer patients however, such autotranspantation entails a risk for retransmission of tumor cells (Shaw et al., 1996) and in these cases, the only safe alternative is reimplantation of *in vitro* fertilized oocytes from cryopreserved tissue that has been allowed to mature *in vitro*.

Look deep into nature, and then you will understand everything better.
Albert Einstein

2 REVIEW OF THE LITERATURE

2.1 The ovary and its structure

The oocyte-producing ovary in female mammals is functionally homologous to the testes in the male. The two ovaries located in the pelvis of every women, one on the left and the other on the right side, are each approximately 4 cm long and 2 cm wide, with a weight of 2 - 4 grams. Directly beneath the outermost layer of ovarian stromal epithelium (OSE) lies a layer of dense connective tissue known as the tunica albuginea. Together with surrounding fibroblasts and fibers of collagen and elastin the follicles form the ovarian cortex situated under the tunica albuginea. Finally, the ovarian medulla under this cortex contains blood and lymphatic vessels.

The oocyte and surrounding cells of the follicle constitute the fundamental reproductive unit of the ovary. The cortex of young girls consists primarily of such follicles, whereas in older women most of these follicles have been replaced by fibers as a consequence of ovulation and atresia. As the follicles grow in size they initially migrate deeper into the cortex and subsequently migrate to the surface as the time for ovulation approaches Thus, the surface of a young ovary appears smooth, while upon aging this surface becomes scarred from repeated ovulation.

The number of ovarian follicles is largest prior to birth, declines gradually throughout childhood and then drops rapidly at 37-38 years of age, ending in menopause when less than approximately 1000 follicles remain (Faddy et al., 1992). It is important to realize that not only do follicle numbers decrease with age, but the quality of the follicle and the oocyte it contains also declines, with an increasing frequency of structural damage and aneuploidy (de Bruin et al., 2004).

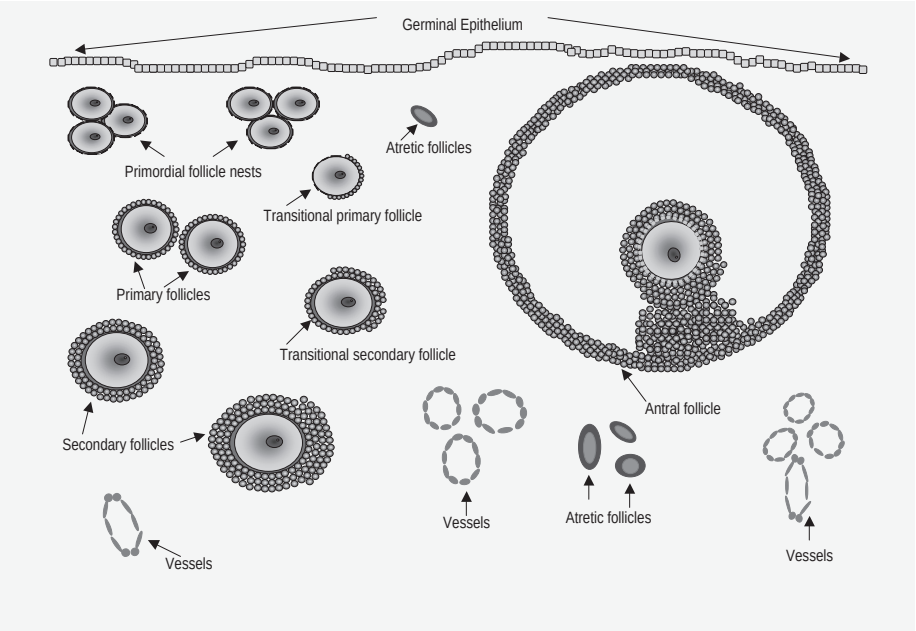


Figure 1. The basic morphological components of the ovarian cortex.

The several tasks performed by the ovary include housing and nurturing the oocytes, and the secretion of hormones that promote follicle maturation and the development of secondary sex characteristics. It is the follicle that provides a protective cover and a suitable environment for the oocyte. The primordial follicle is in turn, embedded in fibrous tissue (the stroma) and a growing number of granulosa cells (GCs) with the stroma being arranged itself around these GCs to form thecal layers. During the fertile period of a woman's life, only 400-500 follicles mature and ovulate, whereas the remainders become atretic.

2.2 Oogenesis

The development of oocytes, referred to as **oogenesis,** involves several processes, including oocytogenesis and ootidogenesis, with folliculogenesis being one aspect of the latter. The germ cells originate from the embryonic ectoderm and migrate early during gestation (5-6 weeks) to the genital ridge. At 11-12 weeks of gestation, the primordial oocytes continue to undergo mitosis (Faddy et al., 1992), but prior to birth they enter meiosis, become arrested in the prophase of the first meiotic division and are thus transformed into primary oocytes. At this point, the oocyte is surrounded by a single layer of GCs, which is in turn, surrounded by a basal membrane (Gosden and Bownes, 1995). For some follicles, this state is maintained from the onset of puberty to the onset of menopause. Following the onset of puberty 15 - 20 primary oocytes enter the growing

pool during each menstrual cycle, progressing through the first meiosis and becoming arrested in the metaphase of meiosis II. Meiosis II is only completed upon fertilization.

Oocytogenesis, i.e., the transformation of oogonia into primordial oocytes, is completed prior to birth in humans, with a maximum of approximately 7 million immature oocytes being produced by 20 weeks of gestation. Most of these undergo apoptosis, so that at birth, approximately 2 million remain, and by the time of puberty, only approximately 400,000 (Faddy et al., 1992; Faddy, 2000). The general belief is that these are all the oocytes a woman will ever have.

However, Johnson and co-workers have recently challenged this dogma, claiming that in the mouse new ovarian follicles can develop from germ line stem cells (Johnson et al., 2004; Johnson et al., 2005). Initially, these investigators proposed that the germ-line stem cells responsible for this neo-oogenesis are located in the ovarian stromal epithelium (OSE) (Johnson et al., 2004); but later they proposed that these stem cells are present in the bone marrow or circulation, rather than the OSE (Johnson et al., 2005). In either case this challenge to the traditional concept that female mammals are born with all of the oocytes they will ever have and this number declines with age has provoked intensive debate. For instance, Eggan and co-workers concluded that chemotherapy does not destroy all of the oocytes and that there is thus no evidence that the bone marrow or any circulating cells contribute to the formation of mature oocytes that undergo ovulation (Eggan et al., 2006). Furthermore, Liu and collaborators could detect no mRNA either specific for meiosis or associated with oogenesis in adult human ovaries and concluded that neo-oogenesis does not occur in this organ (Liu et al., 2007).

Ootidogenesis is the process by which a primordial oocyte develops into a primary oocyte and involves entry into meiosis and arrest at prophase I, called dictyate. The primary oocyte then remains in this state until menarche, when a few such oocytes are recruited into the growing pool and become secondary oocytes. This process involves disappearance of the nucleus, (also known as the germinal vesicle); completion of the first meiosis by extrusion of the first polar body; immediate entry into a second round of meiosis; and arrest in the metaphase of this meiosis II until fertilization occurs (if it ever does) and the second polar body is extruded.

Clearly, a detailed understanding of the development of ovarian follicles and their oocytes would be enormously helpful in connection with our attempts to produce *in vitro* matured oocytes capable of being fertilized.

2.2.1 Nuclear Maturation during meiosis

During meiotic cell divisions by which germ cells (oocytes and sperm) are produced, the genetic material contained in each daughter cell is halved completing two nuclear divisions, but only a single replication of the nuclear DNA. The first cell division associated with meiosis is divided into prophase I, metaphase I, anaphase I and telophase I; while the second cell division, which results in gamete formation, is divided

analogously into prophase II, metaphase II, anaphase II and telophase II. Prior to the first meiotic cell division, the oocyte itself is called a germinal vesicle (GV) oocyte as the nucleus it contains is also known as a germinal vesicle. The GV/nucleus disappears as the cell enters metaphase I (MI) and at metaphase II (MII) the first polar body is extruded. Final maturation of the oocyte, including extrusion of the second polar body, occurs immediately after fertilization. This entire process is referred to as **nuclear maturation**.

2.2.2 Cytoplasmic maturation

While nuclear maturation is easy to follow under the light microscope, the parallel process of **cytoplasmic maturation** cannot be seen in this way. Cytoplasmic maturation includes reorganization, accumulation of various species of mRNA and protein, and epigenetic modifications. Thus, during oocyte maturation, large amounts of RNA are stored in the cytoplasm (Neilson et al., 2000) and organelles are formed and reorganized, with the number of mitochondrial profiles increasing dramatically (Wassarman and Josefowicz, 1978). In addition, the oocyte stores lipids, granules and proteins required for the formation of new membranes in its cytoplasm post-fertilization (Picton et al., 1998; Obata and Kono, 2002).

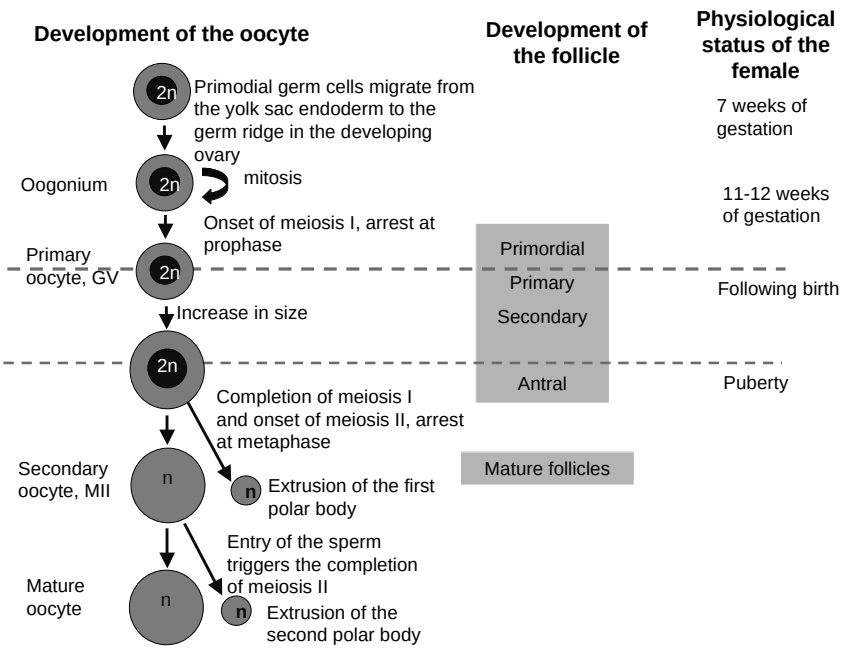


Figure 2. Temporal relationship between the development of the follicles and oocytes and the physiological status of the female

2.3 Folliculogenesis

The process by which a follicle matures from the primordial to the preovulatory stage, with many steps in-between is referred to as **folliculogenesis**. This development involves two major processes, i.e., recruitment of the follicle into the growing pool and the proliferation and differentiation of the granulosa and theca cells. The first of these processes is regulated by paracrine and autocrine signals produced in the ovary itself; while the second is controlled both by this internal signalling and by endocrine signals from outside the ovary.

The follicle begins as an oocyte surrounded by a single layer of GCs enclosed in turn, by a basement membrane. During development, the proliferating GCs provide nutrients and various molecular signals to the oocyte, which increases in size. Re-organization of the follicle and the differentiation and proliferation of the GCs results in the formation of an antrum prior to ovulation (Eppig, 1991). In these ways, the follicle supports the oocyte, both chemically and physically. During this developmental process the follicle migrates from the cortex to the medulla and then back again as ovulation approaches.

Gonadotropins, primarily follicle stimulating hormone (FSH), plays important roles in the growth of and sustained steroidogenesis by follicles. The "two cell, two gonadotropin theory" proposes that, the interstitial theca cells are stimulated by luteinizing hormone (LH) to produce aromatizable androgens and that these androgens are subsequently transported to the GCs, where they are converted into estrogens by aromatizing enzymes which are regulated by FSH (Hillier et al., 1994). When the follicle has reached a size of 200 μm, it becomes more dependent on FSH for growth and its rate of steroid production increases.

As the midpoint of the menstrual cycle approaches (after approximately 14 days), a dramatic increase in circulating levels of estrogen occurs, followed by a surge in LH which triggers the dominant follicle to ovulate (Yen et al., 1999). After ovulation, this follicle transforms into the corpus luteum, which is responsible for the production of progesterone and maintenance of the early phase of pregnancy. This cycle is repeated continuously (with interruptions during periods of gestation) until the pool of follicles is exhausted and the woman enters menopause. At any one time-point, the ovary of a fertile woman contains follicles in all stages of development.

2.3.1 Initiation and growth of follicles

For unknown reasons, certain resting follicles start growing, representing a continuous recruitment process that begins immediately after follicle formation and ends in most cases with atresia. In addition to gonadotropins, a complex network of cell-cell interactions regulates the transition of primordial to primary follicles. Unfortunately, the factors and hormones which stimulate or inhibit initiation of this process remain to be identified, although members of the transforming growth factor beta superfamily such as

AMH (Durlinger et al., 1999) have been implicated. Recently, Pten has been implicated in mouse studies and its role in the human remains to be elucidated (Reddy et al., 2008).

Of the more than 99% of follicles that undergo atresia, most (50-75%) degenerate at the antral stage. Only a few respond to the cyclic gonadotropin stimulation that occurs after puberty to reach the preovulatory stage. The preovulatory gonadotropin surges associated with each reproductive cycle cause the dominant preovulatory follicle to release its mature oocyte for potential fertilization.

2.3.2 Stages of follicular development

The quiescent **primordial follicles** consists of an immature oocyte surrounded by a single layer of flattened granulosa cells, all of which are separated from the surrounding somatic cells by a basement membrane (Gosden et al., 2002). A typical primordial follicle has a diameter of approximately 30 µm.

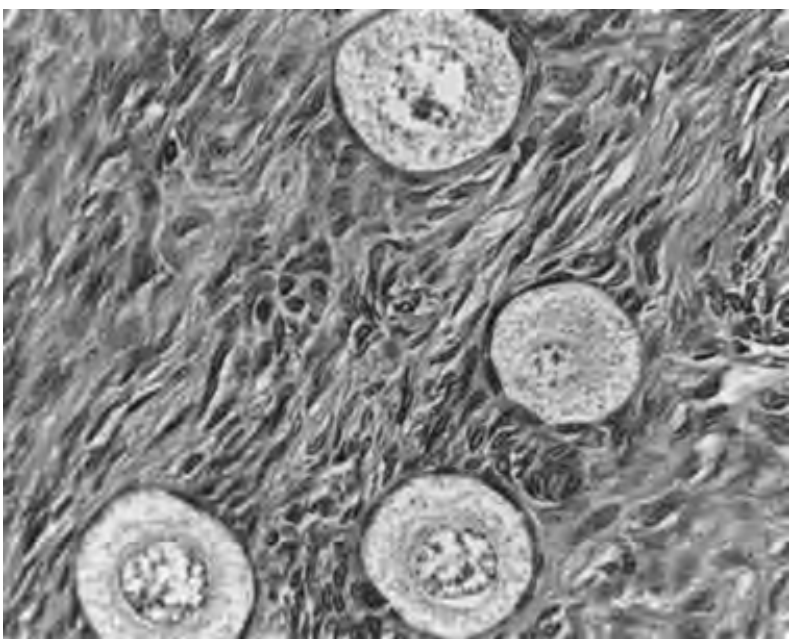
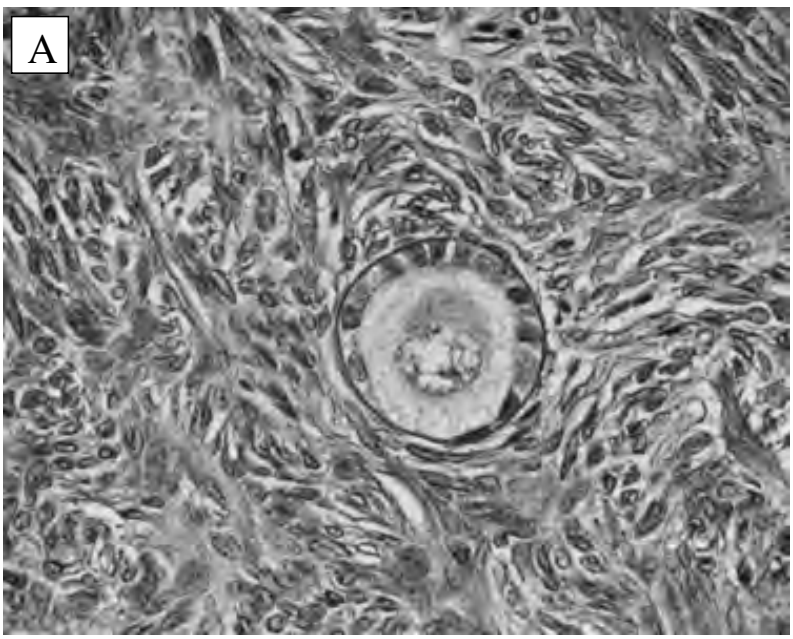


Figure 3. Light micrograph of several primordial follicles, (magnification: 400X)

As these follicles are recruited into the growing pool, the granulosa cells grow larger and become cuboidal, while continuing to surround the oocyte as a single layer. As the GCs begin to express markers for proliferation (Hirshfield, 1989; Wandji et al., 1996; Wandji et al., 1997), transcription of oocyte genes is activated, initiating paracrine signaling

between these two cell types that causes them both to start growing. At this stage expression of the FSH receptor begins.

The appearance of **primary follicles,** approximately 60 µm in diameter, is the first sign of activation and initial recruitment. The GCs secrete mucopolysacharides around the oocyte to form the zona pellucida, a thick layer of glycoproteins and acid proteoglycans situated between the oocyte and GCs themselves. It is interesting to note that while the origin of the ZP is controversial, ZP proteins have been detected in both the oocyte and GCs of primordial follicles (Gook et al., 2008). Gap junctions connecting the oocyte and surrounding GCs allow amino acids, nucleotides and lipid precursors to be transported into the oocyte. Moreover, the microvilli of the cumulus cells penetrate through the zona pellucida to form gap junctions with the oocyte plasma membrane (Li et al., 1995) for bidirectional transfer of nutrients, metabolic precursors and signal molecules, including growth factors and inhibitory and stimulatory meiotic signals (Eppig, 1991; 1992).



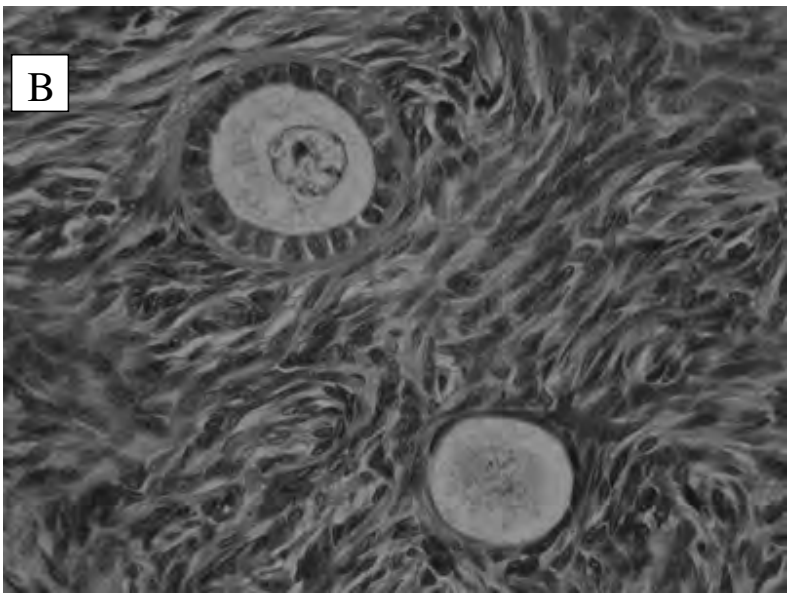


Figure 4. Light micrograph of A) a transitional follicle involved in the formation of a primary from a primordial follicle and B) a primary (upper) and primordial (lower) follicle (magnification: 400X)

In the case of **secondary and pre-antral follicles,** the granulosa cells proliferate and form multiple layers around the oocyte, which also becomes larger. In addition, stromal cells are recruited from outside the basement membrane to become the layers known as theca interna and theca externa, with capillaries between these two layers. Thus, at this point the follicle starts to receive a blood supply and will be exposed to circulating factors (Reynolds et al., 1992). The diameter of the follicle is now 100-200 µm approximately.

Once the oocyte has completed its growth, the granulosa cells proliferate further and arrange themselves into several layers, producing a primary multilaminar follicle. This developmental step is dependent on FSH, which both stimulates proliferation of the granulosa cells and increases the number of FSH receptors expressed at their surface, thereby magnifying its own effects. The differentiation of the theca cells surrounding the follicle into the theca interna and theca externa, is currently thought to be promoted by LH. The GCs present in a secondary follicle demonstrate very high mitotic activity as their number increases.

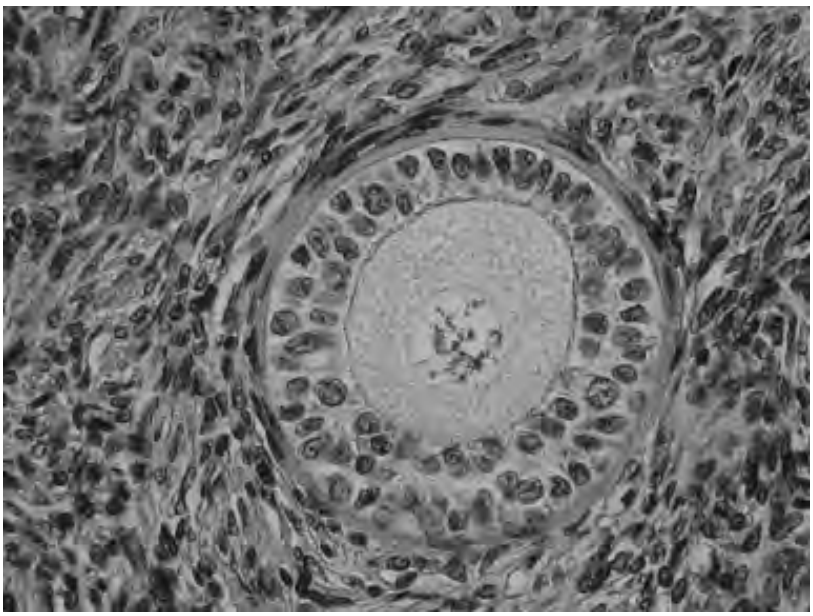


Figure 5. Light micrograph of a secondary follicle (magnification: 400X)

In their early phase, **antral follicles** contain a fluid-filled space called the antrum, the development of which is limited only by the level of FSH present. This follicular fluid consists of blood exudates and local secretions and contains metabolites produced locally At this point, the follicular diameter is approximately 500 µm..

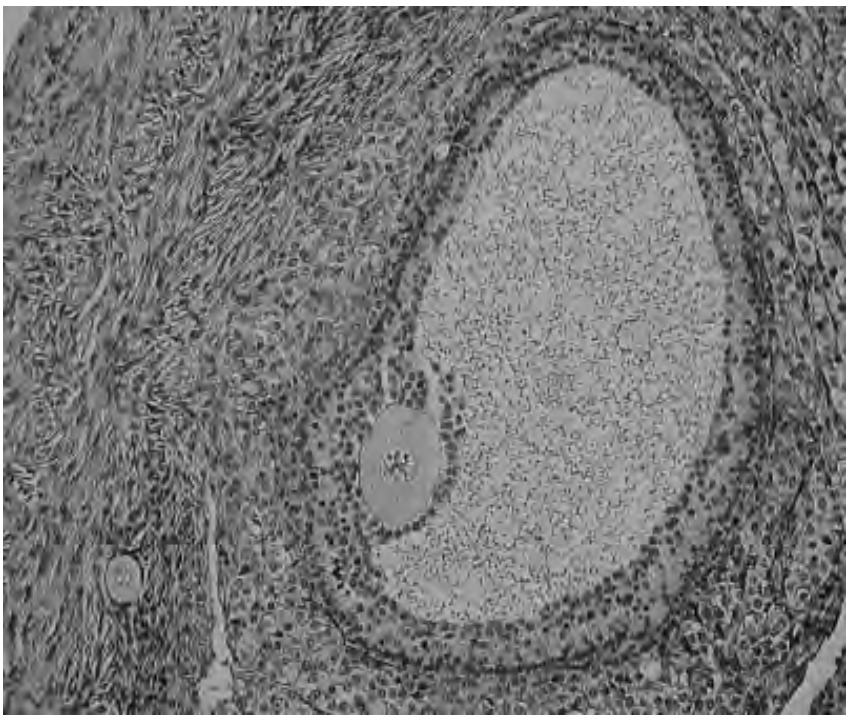


Figure 6. Light micrograph of an antral follicle, (magnification: 100X)

A large fluid-filled antrum is a characteristic feature of the mature follicle, also known as a Graafian or antral follicle. These follicles contain two types of granulosa cells: mural granulosa cells, forming a thin layer along the periphery of the follicle and cumulus granulosa cells, surrounding the oocyte. The basement membrane separates these granulosa cells from the theca interna containing cuboidal, steroid-secreting cells and the external theca externa consisting of vascularized, fibrous connective tissue. The interaction of LH with its receptor on the surface of the theca cells stimulates these cells to produce androgen, which is subsequently aromatized in the GC's to yield estrogen. The diameter of the follicle at this stage is approximately 20 mm.

Prior to reaching preovulatory stages, most follicles degenerate through atresia and those that do remain compete for available FSH. Since the estrogen and inhibin secreted by these follicles suppress the action of FSH, follicles expressing lower levels of the FSH receptor do not survive.

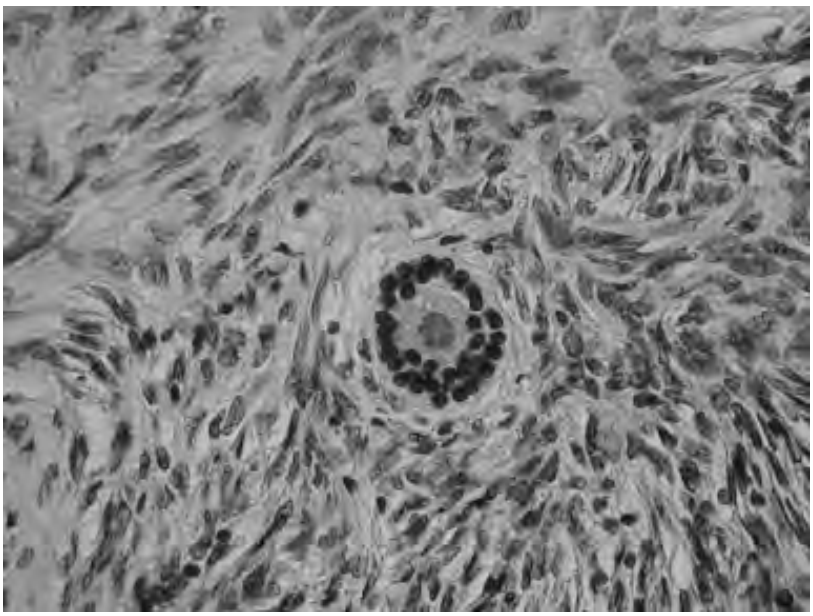


Figure 7. Light micrograph of an atretic follicle (magnification: 400X)

During a period of approximately 200 days the diameter of the oocyte expands from about 35 µm to its full size of 120 µm (Gougeon, 1986; 1996; Picton et al., 1998) and this cell matures in other ways as well, including the accumulation of both RNA and protein in the cytoplasm and nucleus. Synthesis of mRNA and proteins is rapid during the early phase of oocyte growth and slowly attenuates thereafter.

Atresia can occur at any time from gestation to menopause and is independent of the use of birth control pills, breast-feeding or pregnancy. Of the many follicles that start to grow, only one will be ovulated in the end. All of the others become atretic, which explains why the number of follicles in the ovary drops significantly with aging. Only approximately 400 follicles are ovulated during the fertile period of a woman's life (Gougeon, 1996), while more than 99% undergo atresia via apoptosis (Hsueh et al., 1994; Tilly, 1996), most often at the antral stage of development (Chun et al., 1994; Gougeon et al., 1994; Amsterdam et al., 2003). Both the oocyte and GCs undergo ultrastructural and morphological changes in connection with atresia (de Bruin et al., 2002).

2.3.3 Folliculogenesis during the menstrual cycle

The development of follicles is also controlled by the **menstrual cycle**, which consists of a **follicular (or proliferative) phase** and a **luteal (secretory) phase** separated by ovulation. During the proliferative period, (approximately days 1-14), follicle stimulating hormone (FSH) is involved in a complex interplay of hormones that triggers follicular

growth. Elevated levels of FSH recruit preantral follicles and the follicles that express higher levels of the FSH receptor, or are more responsive to this hormone for some other reason, will continue to grow, while the others undergo atresia.

During this same follicular phase, the follicles also produce more and more estradiol, which stimulates growth of the uteral endometrium. The dominant follicle secrets both estradiol and inhibin, which in a negative feedback loop, attenuates the release of gonadotropin releasing hormone (GnRH) from the hypothalamus and of FSH from the anterior pituitary. Maintenance of elevated levels of estradiol causes the pituitary to release a surge of LH, which promotes maturation of the dominant follicle. Circulating levels of LH peak after 8-26 hours, stimulating this dominant follicle to rupture and release the oocyte.

The oocyte then travels to the fallopian tube for potential fertilization and ovulation is followed by the luteal secretory phase of the menstrual cycle. The residual follicle that has released its oocyte rearranges to form a corpus luteum (CL). In the absence of pregnancy, the CL degenerates and circulating levels of steroids and inhibin are reduced, which, in turn, leads to enhanced secretion of FSH and the cycle is repeated (Browder et al., 1991).

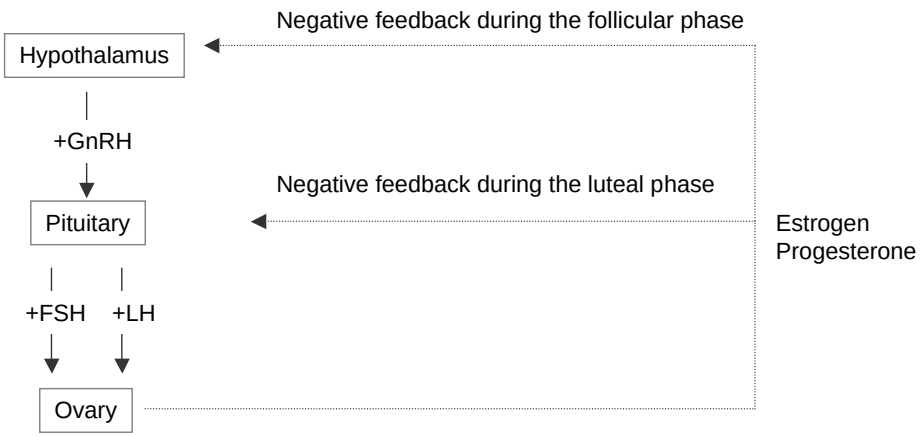


Figure 8. Simplified schematic illustration of the hypothalamus-pituitary-ovary axis

The hypothalamus-pituitary-ovary (or HPO) axis is one way of describing hormonal interactions between these three organs. In brief, the hypothalamus secretes pulses of GnRH when the level of estrogen is low, this GnRH stimulates the pituitary gland to produce FSH and LH, and these gonadotropins, then exert their actions on the ovary. Estrogen inhibits production of GnRH by the hypothalamus, giving rise to a negative feedback loop that prevents overproduction of estrogen.

Estrogen produced by the growing ovarian follicles promotes the development of secondary sex characteristics; while the progesterone, synthesized by the CL helps maintain the endometrium in a state that is appropriate for implantation of the embryo. The ovary continues to produce estrogen even after menopause, but in decreasing amounts. Unlike other mammals, fertile women experience no pauses in their estrous cycles, which are repeated continuously (approximately 13 28-day cycles each year).

2.4 Growth factors and hormones related to folliculogenensis

2.4.1 Transforming Growth Factor-ß superfamily

Among the many growth factors that influence the growth and development of follicles are members of the **Transforming Growth Factor-ß** (TGF- ß) superfamily (Erickson and Shimasaki, 2000). Almost all of the more than 40 protein hormones presently known to belong to this superfamily, exhibit similar structures including 7 cysteine residues, and are conserved between species. This family can be divided into three major groups, i.e., TGF-ß itself; activins and inhibins; and bone morphogenetic proteins (BMPs), the largest of the subgroups. TGF-ß signaling involves two sub-types of transmembrane serine/ threonine kinase receptors, both of which are required for signal transduction. The protein ligands interact with these cell surface receptors to generate intracellular signals through Smads.

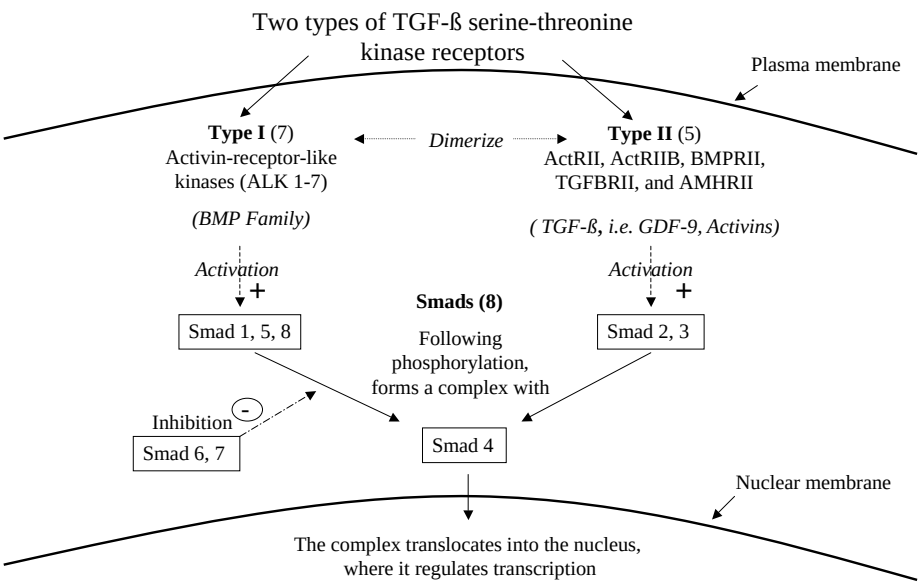


Figure 9. TGF- ß activated signalling pathways

2.4.1.1 Anti-Müllerian hormone

In contrast to the stimulatory actions of most other members of the transforming growth factor-β superfamily produced in the ovary itself (Rabinovici et al., 1990; Huang et al., 1993; Erickson and Shimasaki, 2000), **anti-Müllerian hormone** (AMH) is believed to inhibit the initiation and progression of early stages of follicular development (Durlinger et al., 1999). Originally identified in connection with its role in promoting male sex differentiation during embryonic development (Josso et al., 1993), AMH induces regression of the Müllerian ducts and is therefore also referred to as Müllerian-inhibiting substance (MIS). This hormone is produced by the granulosa cells of human females from the late antenatal stage until menopause (Rajpert-De Meyts et al., 1999).

Immunohistochemical analysis of the adult human ovary employing a monoclonal antibody against human AMH revealed no staining of primordial follicles while the granulosa cells of 74% of the primary follicles exhibited at least weak expression of this protein (Weenen et al., 2004). The most intense staining was present in pre-antral and small antral follicles, with virtually no staining of follicles larger than 5 mm. This pattern of expression is similar to that observed in rodents, where AMH is detected in all follicles until they reach the stage where selection occurs (Baarends et al., 1995; Durlinger et al., 2002).

On the basis of these findings, it has been proposed that the pool of growing follicles produces AMH to act as a paracrine feedback inhibitor of the recruitment of neighboring primordial follicles. Female AMH-knockout mice were originally believed to be reproductively normal (Behringer et al., 1994), but further investigation revealed that it plays a role in inhibiting follicular development (Durlinger et al., 1999). Thus, at 4 months of age, AMH-deficient mice demonstrate a reduced number of primordial follicles and an elevated number of growing follicles, with a consequent increase in ovarian weight. At 13 months of age, the primordial follicle pool in these same animals is almost completely depleted.

Furthermore, neonatal mouse ovaries cultured in the presence of AMH contain only 60% as many growing follicles as the control ovarian cultures (Durlinger et al., 2002), without any detectable effect on the levels of growth differentiation factor-9 or the mRNA encoding the AMH receptor-type II mRNA. In more developed, pre-antral follicles, AMH attenuates FSH-stimulated growth (Durlinger et al., 2001), suggesting that this hormone might play a role in determining responsiveness to FSH and, hence, in the cyclic recruitment and selection of follicles for ovulation. In the case of rats, however, AMH enhances rather than attenuating FSH-stimulated growth of pre-antral follicles (McGee et al., 2001).

2.4.1.2 Growth differentiation factor-9

Growth differentiation factor–9 (GDF-9) was identified in 1993 in connection with genomic screening for mouse TGF-ß (McPherron and Lee, 1993). In the human, this

factor is produced by oocytes and play(s) an essential role(s) during folliculogenesis (Mazerbourg and Hsueh, 2003). Both GDF-9 mRNA and the corresponding protein are present in oocytes located in primary follicles (Aaltonen et al., 1999). In humans, GDF-9 enhances the survival, growth rate and recruitment of follicles (Hreinsson et al., 2002b) and, moreover, mutations in the GDF-9 gene may be associated with premature ovarian failure (Kovanci et al., 2007) .

In most species, expression of GDF-9 is detected in non-growing primordial follicles (Bodensteiner et al., 1999; Eckery et al., 2002; Wang and Roy, 2004). However, in the case of sheep GDF-9 transcripts have been detected in fetal ovaries even prior to follicle formation (Mandon-Pepin et al., 2003), indicating possible functions for this factor at this earlier stage as well (Juengel et al., 2004). Furthermore, in ovine and bovine, GDF-9 mRNA is expressed at all stages of follicular development (Bodensteiner et al., 1999). Vitt and co-workers observed enhanced activation of primordial follicles in rats treated with GDF-9 (Vitt et al., 2000).

In mice deficient in GDF-9, the follicles are arrested in the primary stage, while the oocyte continues to grow in size until it degenerates (Dong et al., 1996; Bodensteiner et al., 1999). In addition, GDF-9 and GDF-9B/BMP-15 act synergistically to promote the growth and differentiation of granulosa cells and theca cells in mice which in turn supports oocyte maturation (Gilchrist et al., 2004).

Like other members of the TGF-ß superfamily, GDF-9 activates type I and II serine/threonine kinase receptors which results in phosphorylation of Smad proteins (Massague, 1998). Thus, GDF-9 has been shown to bind both to the activin receptor like kinase 5 (ALK5), a type I receptor, and the bone morphogenetic protein receptor II (BMPRII), a type II receptor on the GCs (Vitt et al., 2002; Kaivo-Oja et al., 2005; Mazerbourg and Hsueh, 2006) (see Figure 9). GDF-9B/BMP-15 also uses BMPRII as its type II receptor, but as a type I receptor it uses ALK6 or BMPRIB (Moore et al., 2003), The biological effect of both of these factors can be blocked by using a fusion protein composed of BMPRII ectodomain coupled to the Fc domain of human IgG (Vitt et al., 2002; Moore et al., 2003). For example, inhibition of the actions of GDF-9 and GDF-9B/BMP-15 partially attenuates the proliferation of GCs induced by oocytes (Vitt et al., 2002; Mazerbourg and Hsueh, 2006).

2.4.1.3 Growth differentiation factor -9B / Bone morphogenetic protein -15

Growth Differentiation Factor -9B / Bone Morphogenetic Protein -15 (GDF-9B/BMP-15) is a paracrine signaling molecule involved in oocyte and follicular development. It was discovered independently by two research groups in 1998 (Dube et al., 1998; Laitinen et al., 1998). It is expressed in the oocytes of primary follicles in sheep, humans, rats and mice (Dube et al., 1998; Laitinen et al., 1998; Aaltonen et al., 1999; Jaatinen et al., 1999; Galloway et al., 2000). Furthermore, Aaltonen et al. showed that GDF-9B/BP-15 is expressed in the late primary follicle stage. Therefore, the expression of GDF-9 mRNA is earlier than GDF-9B/BMP-15 in the human (Aaltonen et

al., 1999). GDF-9B/BMP-15 is 50 -55% homologous to GDF-9 in the human (Aaltonen et al., 1999), rodent (Jaatinen et al., 1999) and sheep (Galloway et al., 2000).

There are four sheep mutants with a dose dependent effect on fertility. In Inverdale and Hanna sheep GDF-9B/BMP15 mutant phenotypes (+/-) results in 3 offspring, (+/+) 1 offspring, and (-/-) no offspring (Davis et al., 1991; Davis et al., 1992; Galloway et al., 2000). With regard to Belclare / Cambridge sheep it has been shown that mutations in the genes of both GDF-9 and GDF-9B/BMP-15 have an additive effect and the sheep have greater ovulation rates if they have both of the mutations as opposed to a single mutation (Hanrahan et al., 2004). It has also been discoved in sheep that immunization against GDF-9B/BMP-15 results in a disturbance in folliculogenesis (McNatty et al., 2007). Mouse KOs have also been shown to be subfertile with decreased ovulation rates (Yan et al., 2001).

In summary, GDF-9 and GDF-9B/BMP-15 are essential for ovarian follicular development in sheep. And the ovulation rate in sheep is influenced by the dose of GDF-9 and GDF-9B/BMP-15. The effect of these two compounds in the human has yet to be discovered.

2.4.2 c-Kit and SCF / KIT / Kit Ligand

The c-Kit ligand (KL, also known as steel factor or stem cell factor, SCF), a ligand for the c-Kit proto-oncogene receptor tyrosine kinase, is a pluripotent growth factor involved in the differentiation and growth of hematopoietic stem cells, neuroblasts, melanoblasts and primordial germ cells (PGCs) (Galli et al., 1994). In the mouse ovary, KL is expressed in mouse granulosa cells, whereas the receptor has been detected only in oocytes and theca interna cells (Manova et al., 1990; Horie et al., 1991; Keshet et al., 1991; Motro et al., 1991; Manova et al., 1993; Motro and Bernstein, 1993). Human oocytes and granulosa cells both express c-Kit mRNA and the corresponding protein (Horie et al., 1993; Tanikawa et al., 1998).

KL promotes the survival of mouse PGCs in culture, both by itself and in combination with leukemia inhibitory factor (LIF) (Dolci et al., 1991; Godin et al., 1991; Matsui et al., 1991; Pesce et al., 1993). Moreover, Morita and co-workers have shown that KL can inhibit apoptosis in cultures of prenatal mouse oocytes, but only in combination with LIF (Morita et al., 1999). In cultures of fetal mouse ovarian tissue, KL initiates the growth of oocytes (Tisdall et al., 1997; Klinger and De Felici, 2002).

KL and c-Kit participate in follicle formation in the fetal mouse and sheep (Tisdall et al., 1997; McNatty et al., 2000). Female mice carrying a distinct mutant allele, (Sl pan), that is associated with reduced production of the KL protein, exhibit almost a normal number of germ cells, but their ovarian follicles do not develop beyond the primary stage with one-layer of granulosa cells (Huang et al., 1993). A similar phenotype is demonstrated by wild-type mice following the administration of ACK2, an antibody against c-Kit that blocks c-Kit/KL receptor (Yoshida et al., 1997). In addition, in mice heterozygous for the

Kit (W-lacZ) granulosa cell proliferation and oocyte growth in preantral follicles are altered (Reynaud et al., 2001). In cultures of preantral mouse follicles, maturation of the oocyte cytoplasm and production of testosterone by the follicle are enhanced by the addition of KL (50 ng/ml) to the medium whereas blocking the c-Kit receptor with an antibody attenuates oocyte survival (Reynaud et al., 2000).

Two members of the TGF-β family produced by oocytes, GDF-9 and GDF-9B/BMP-15, appear to regulate the expression of KL in a specific manner. Thus, GDF-9 suppresses this expression in mouse preantral granulosa cells, but leads to elevated levels of KL mRNA in cultures of bovine antral granulosa cells. Furthermore, GDF-9B/BMP-15 stimulates the expression of KL by rat antral granulosa cells, while KL down-regulates the level of GDF-9B/BMP-15 mRNA in a paracrine manner (Otsuka and Shimasaki, 2002).

All in all, little is presently known about the involvement of KL/c-Kit in the early development of human follicles.

2.4.3 Growth Hormone

Growth hormone (GH), which is known to play important roles in folliculogenesis and the maturation of oocytes is synthesized primarily in the pituitary gland. However, the presence of GH mRNA in bovine granulosa cells and oocytes indicates that this hormone can also be synthesized locally in the ovary, where it may act in both a paracrine and autocrine manner (Izadyar et al., 1999). Indeed, growth hormone receptors (GHR) have been detected in rat ovaries (Lobie et al., 1990; Tiong and Herington, 1991); in bovine GCs, cumulus cells and oocytes (Izadyar et al., 1997); and in the GCs of human antral follicles and the corpus luteum (Sharara and Nieman, 1994).

Knock-out mice lacking GHR exhibit delayed sexual maturation, give birth to abnormally small litters characterized by elevated mortality (Zhou et al., 1997). This phenotype appears to be due to defective functioning of the ovary, rather than of the pituitary gland. Furthermore, administration of GH to equine or bovine increases their numbers of small follicles (Cochran et al., 1999), or of antral follicles (Gong et al., 1991; Gong et al., 1993), respectively.

In *in vitro* studies GH stimulates the growth of murine preantral follicles and follicular cell proliferation (Liu et al., 1998; Kobayashi et al., 2000) and exerts a direct inhibitory effect on apoptosis in both bovine (Sirotkin and Makarevich, 1999) and rat follicles (Eisenhauer et al., 1995; Chun and Hsueh, 1998) at early stages of their development. In addition, over expression of bovine GH in a transgenic mouse strain reduces apoptosis in the mouse follicles of these animals (Danilovich et al., 2000). Since GH deficiency in rats is associated with attenuated responsiveness to LH (Advis et al., 1981), the enhanced follicular survival and proliferation promoted by GH may reflect its potentiation of the actions of LH. GH may also be involved in selection of the follicle destined to ovulate, since binding sites for GH are absent from atretic follicles in pigs (Quesnel, 1999) and in

bovine that lack the GHR, development of the dominant follicle is disrupted (Chase et al., 1998).

In humans, GH has been shown to stimulate the differentiation and proliferation of lutenized GCs (Ovesen et al., 1994), and the production of estrogen (Mason et al., 1990), as well as to up-regulate the expression of enzymes involved in steroid synthesis (Doldi et al., 1996), thereby enhancing steroid production in synergy with gonadotropins (Carlsson et al., 1992; Lanzone et al., 1992).

2.4.4 Insulin-like growth factor

The two insulin-like growth factors (IGF) presently known as IGF-I and IGF-II are polypeptides with a high degree of sequence homology to insulin. IGF-I is one of the most potent natural activators of the AKT signaling pathway, which promotes cell growth and proliferation, yet identified as well as being a potent inhibitor of programmed cell death. Insulin itself exerts a profound influence on numerous intracellular processes including gene transcription, protein synthesis, amino acid transport, and glucose homeostasis (Cheatham and Kahn, 1995) and for this reason is a common supplement to cell and tissue culture media.

Insulin receptors have been detected in the human ovary and, furthermore, IGF-I mRNA is expressed by the theca cells of small antral follicles and IGF-II has been identified in the theca cells of small antral follicles and the GCs of dominant follicles (el-Roeiy et al., 1993). Moreover, mRNA encoding the IGF-I receptor is present in the GCs of small antral and dominant follicles, with the corresponding protein being detected in the oocytes and GCs of primordial and preantral follicles (Qu et al., 2000).

Erickson and co-workers found that IGF-I can potentiate the effect of FSH on steroidogenesis by both GC and GL cells (Erickson et al., 1989). IGF-II may also be involved in this process, since the addition of an IGF-II antagonist to the culture of a preantral follicles inhibits estradiol production by their GCs (Yuan and Giudice, 1999). Thus, IGFs may play important roles in rendering both GCs and GL cells more responsive to FSH.

In addition, Louhio and collaborators observed that IGF-I and IGF-II both enhance the survival of early-stage follicles and that IGF-I may be involved in activating the meiotic cell cycle in GCs in culture of human ovarian cortical tissue (Louhio et al., 2000), an effect also exerted by Insulin. Significantly, IGF-I KO mice are infertile, since their follicles do not develop past the secondary stage and these animals thus do not ovulate (Baker et al., 1996).

2.4.5 Additional factors

The **extracellular matrix** (ECM) between cells consists of glycoproteins, proteoglycans, and hyaluronic acid and performs a number of functions, including the synthesis and maintenance of a structural framework; that also allows anchorage; segregation of tissues; and regulation of intracellular communication. Moreover, the ECM contains many growth factors essential for cell growth. *In vitro* the structural support of the ECM helps maintain the three-dimensional organization of follicles and improves both the growth and viability of follicles in cultures ovarian cortical tissue (Hovatta et al., 1997).

The Matrigel™ basement membrane matrix has been used successfully in the cultures of many different types of cells, tissues and organs from both animals and humans and is therefore presently employed as the ECM in the majority of such investigations. Matrigel™ is a solubilized preparation of the basement membrane of mouse Engelbroth-Holm-Swarm tumor and is rich in ECM proteins, including laminin, collagen IV, heparin sulphate proteoglycans, entactin and nidogen (Kleinman et al., 1982; Kleinman et al., 1986). In addition, Matrigel™ contains growth factors (Vukicevic et al., 1992) that may or may not influence the cultured cells and therefore, a growth factor reduced form of Matrigel™ matrix has also been developed (Taub et al., 1990). Ideally, the ECM utilized in ovarian tissue cultures should mimic the *in vivo* environment as closely as possible.

Follicle stimulating hormone (FSH) plays essential roles in female reproduction and primary follicles express receptors for this peptide (Oktay et al., 1997a). Women who are homozygous for an inactivating mutation in the FSHR gene exhibit hypergonadotrophic ovarian dysgenesis, amenorrhea and infertility. Moreover, Aittomaki and co-workers have reported that the ovarian follicles of these women rarely develop beyond the primary stages (Aittomaki et al., 1996). In addition, xenografts of human follicles require stimulation by FSH to proceed beyond early secondary stages (Oktay et al., 1998); in the human, FSH reduces atresia and significantly enhances follicle diameter (Wright et al., 1999). *In vitro* this hormone also prevents apoptosis in preantral and antral mouse follicles (Chun et al., 1996; Abir et al., 1997), attenuates atresia in human preantral follicles (Roy, 1993); and acts as a survival factor for and promotes the growth of early stage follicles in cultures of human ovarian tissue (Wright et al., 1999).

Insulin promotes the uptake of glucose and amino acids, as well as the synthesis of proteins and nucleic acids. By scavenging iron, **transferrin** might help reduce intracellular levels of toxic reactive species of oxygen. **Selenite** is also an antioxidant. Thus, supplementation with insulin transferrin selinite (ITS) may stimulate follicle growth and reduce atresia in comparison to culture media simply containing serum (Wright et al., 1999). Indeed, human GCs respond to insulin by increasing their estrogen and progesterone production (Erickson et al., 1990) and in the case of the rat both the GCs and theca cells respond to insulin in this same manner (Erickson and Shimasaki, 2000). Both transferrin and selenium may act as free radical scavengers in the media thus affecting growth and atresia (Roth, 1997). Insulin, IGF-I and IGF-II may act as a survival factor for early stage follicles in human ovarian tissue cultures (Louhio et al., 2000) (Please see 2.4.4 for further details).

Second messengers

Cyclic guanosine monophosphate (cGMP) acts as a second messenger in ovarian signaling pathways and has been shown to decrease apoptosis (Scott et al., 2004). The cGMP / nitrous oxide pathways are involved in various ovarian functions including responses to FSH (LaPolt et al., 2003). *In vitro* 8-br-cGMP enhances the development of secondary follicles, and synthesis of estrogen and attenuated atresia (Scott et al., 2004) while cGMP suppresses apaptosis and inceases the proliferation of GCs in cultured preantral ovarian follicles (McGee et al., 1997). **Cyclic adenosine monphosphate** (cAMP) also reduces atresia in secondary follicles in culture (Zhang et al., 2004).

2.5 Which factors influence fertility?

Worldwide, 1-2% of all women are affected by ovarian disorders such as premature ovarian failure (POF). Some of the genetic causes of such failure include Turner's syndrome, fragile X syndrome and mutations that inactivate the FSHR (Aittomaki et al., 1995) and several more. In addition, POF may arise as a detrimental side-effect of radio- and/or chemotherapy on the ovary.

2.5.1 Radio- / Chemotherapy

Approximately 60,000 North American women under the age of 40 are diagnosed with invasive cancer each year and radio and chemotherapy are used in the treatment of most of these cancers, as well as of other disorders such as autoimmune diseases (e.g., Systemic Lupus Erythematosis). The commonly used chemotherapeutic agent cyclophosphamide causes a defect in DNA with regard to methylation, as well as epigenetic changes, and demonstrates pronounced selectivity on the primordial follicles in the mouse ovary (Meirow et al., 1999). Such risks are greatest with alkylating agents.

In the case of radiotherapy, ovarian cells at different developmental stages are differentially sensitive, with animal studies indicating that primordial follicles are most sensitive to the toxic effects of radiation (Gosden et al., 1997). In this context, the lethal dose 50 (LD50) i.e., the dose of radiation that kills half of the follicles is 0.15 gy, in the mouse, 50 gy in the monkey and 2 gy in humans (Wallace et al., 2003) which can be compared to the typical dose of 10-12 gy used in radiotherapy (Meirow and Nugent, 2001; Wallace et al., 2003).

Oocytes do not reproduce and once they are damaged by chemo- and or radiotherapy, they are lost forever. Of course, younger patients have more follicles and thus a much greater chance of going through such treatment as a child and still experiencing normal puberty and menarche. However, even these patients will probably enter menopause sooner than if they had not been exposed to such gonadotoxic treatments. The population of girls and women who are survivors of cancer and cancer treatment is growing

constantly and development of improved approaches to the preservation of fertility is therefore of great urgency.

2.5.2 Turner's syndrome and other causes of premature ovarian failure

Turner's syndrome results from the deletion of an X chromosome (45, X) and is present in approximately 10% of all aborted fetuses. This syndrome can cause gonadal dysgenesis, cardiac abnormalities and enlargement of blood vessels. Moreover, in individuals with Turner's syndrome, the oogonia do not undergo meiosis and oocytes are lost at an accelerated rate at an early age, sometimes at such an extent that the ovary is seen as only a streak. Various mosaics and chromosomal translocations give rise to different degrees of this syndrome (Singh and Carr, 1966). Thus, some affected girls exhibit very small ovaries at an early age due to the accelerated loss of oocytes, while others demonstrate more normal ovarian function (Hreinsson et al., 2002a) and even become pregnant (Pasquino et al., 1997).

Other genetic conditions that can give rise to POF include fragile X syndrome (Marozzi et al., 2000), X- chromsomal abnormalities (Devi and Benn, 1999) and mutations in the FSHR gene (Aittomaki et al., 1996).

2.6 Utilization of cryopreserved tissue

2.6.1 Transplantation

2.6.1.1 Xenografting

Xenografting of ovarian tissue from one species into another is most commonly investigated employing the immunocompromized mouse as a recipient. The ovarian tissue can be transplanted either subcutaneously or under the kidney capsule (Oktay et al., 2000; Gook et al., 2001; Abir et al., 2003; Gook et al., 2003) and such xenotransplantations have resulted in successful pregnancies in mice (Snow et al., 2002). Although xenotransplantation is an excellent tool for examining the viability of cryopreseved ovarian tissue after thawing, this approach however is not yet a clinical option because of the possible transmission of animal pathogens (Kim et al., 2002).

2.6.1.2 Heterotopic transplants

Heterotopic transplantation involves transplanting tissue from one location in the body to another part of the same body. Oktay et al. reported that heterotopic transplants of ovarian tissue to the forearm (Oktay et al., 2001), or abdomen (Oktay et al., 2004), of women resulted in temporary restoral of function. The forearm transplant led to the resumption of menstrual cycling, decreased circulating levels of FSH and LH and increased serum estradiol, and developed growing follicles for a period of three years. The abdominal transplant elevated circulating levels of estradiol and underwent follicular development. Although IVF employing oocytes recovered from either of these

transplants did not result in pregnancy, the women involved resumed normal menstrual cycles and could thus avoid hormone replacement therapy.

2.6.1.3 Orthotopic transplants

Orthotopic transplantation involves re-implantation of tissue at its site of origin. Several laboratories have succeeded in utilizing this approach to obtain 3-7 months of post-grafting ovarian function (Oktay 2000; Callejo 2001; Radford 2001). More recently, successful cryopreservation and orthotopic transplantation of whole ovaries has been achieved in sheep and in this case reattachment of the ovarian vasculature was found to prevent ischemic follicular loss of follicles (Arav et al., 2005). In 2004, the first live primate birth following orthotopic transplantation of fresh tissue was obtained (Lee et al., 2004). Also, in 2004, Donnez et al., achieved the first live human birth after orthotopic transplantation of cryopreserved ovarian tissue (Donnez et al., 2004). Moreover, Meirow and co-workers employed orthotopic transplantation of cryopreserved ovarian tissue to achieve pregnancy in a patient who experienced ovarian failure due to chemotherapy (Meirow et al., 2005). These successes confirm that transfer of cryopreserved ovarian tissue back into the patient cannot only restore normal hormonal homeostasis, but also can result in pregnancies.

Despite these promising breakthroughs, an important concern remains, namely is the transplanted tissue healthy and in particular free from cancer cells? Investigations on mice with severe combined immunodeficiency (SCID) have produced contradictory answers to this question. One such study found no transmission of cancer (Kim et al., 2001) while in another retransmission of the disease did occur (Shaw et al., 1996). According to Sonmezer and Oktay (Sonmezer and Oktay, 2004), the risk for ovarian metastisis is high in patients with leukemia, neuroblastoma or Burkitt's lymphoma; moderate in connection with adenocarcinoma, colon cancer or breast cancer at stage IV and with infiltrative lobular histology; and low in those diagnosed with Wilm's tumor, Hodgkin's lymphoma, non-Hodgkin's lymphoma, breast cancer in stages I-III or osteogenic sarcoma.

2.6.2 Culturing ovarian cortical tissue

Achieving maturation of ovarian follicles and their oocytes *in vitro* is a long and complicated process that is dependent on a thorough understanding of the needs of these cells at all stages of development. Human ovaries have been cultured for some time now. When Martinovich tried to culture the whole ovary in 1938 the tissue became necrotic, since the medulla did not receive an adequate supply of nutrients and catabolites were not removed.

Subsequently, a number of chemical and mechanical procedures designed to isolate components of the ovary for culturing have been developed. Initially, collagenase was used successfully to remove the basement membrane and surrounding cells in order to

isolate ovarian follicles from mice (Eppig and Schroeder, 1989; Demeestere et al., 2002). Isolation of preantral follicles requires a longer period of such enzymatic treatment (Roy, 1993) than does isolation of primordial and primary follicles (Oktay et al., 1997b). One problem associated with enzymatic isolation is that the basement membrane is damaged (Gosden et al., 2002) whereas with mechanical, through the use of needles, the surrounding cells can be removed without disrupting the basement membrane.

Abir and co-workers found that culturing follicles isolated at an early stage of development resulted in massive degeneration within 24 hours, with no follicles surviving beyond 48 hours (Abir et al., 1999). In this same year, Hovatta and collaborators showed that the survival of follicles within pieces of ovarian tissue in culture is better than that of isolated follicles (Hovatta et al., 1999), perhaps due to the damage of the basement membrane incurred during isolation of the follicles. Thus, culturing ovarian cortical tissue in the form of small tissue pieces is now considered to be optimal. This causes less potential damage to supporting cells and their three dimensional organization and interactions that are critical for normal follicular development are kept. The potential for synchronized cytoplasmic and nuclear maturation should be much higher with tissue pieces, an important consideration in light of the increasing numbers of imprinting defects being discovered in oocytes that have not matured properly. In addition, in 1997, Hovatta and co-workers reported that the exchange of gas and nutrients is improved and necrosis attenuated by using small ovarian cortical tissue pieces supported in a matrix (Hovatta et al., 1997). Furthermore, as stated earlier, they showed that isolation of the follicles does not improve follicle growth and that cultures of tissue pieces had larger numbers of follicles (Hovatta et al., 1999).

In vitro cultures have also been utilized to investigate the process of meiosis. For example, Hartshorne and co-workers demonstrated that in cultures of oocytes obtained from human fetal ovaries, meiosis not only continues but is also initiated *in vitro* (Hartshorne et al., 1999).

In the mouse the promise of ovarian tissue culture has been dramatically demonstrated by the live birth of “Eggbert” (Eppig and O'Brien, 1996). Eggbert was conceived by first culturing primordial follicles in tissue for 8 days then isolating the follicles enzymatically and culturing them for an additional 8 plus 6 days respectively in two different types of media; and finally, removing the oocyte from the complex and culture using IVM for a further 17 hours. However, Eggbert did not develop normally and died prematurely. Seven years later, in 2003, the same investigators modified their procedure slightly by adding and removing various factors at different time points of culture to achieve the birth of 59 normal pups (O'Brien et al., 2003). This achievement demonstrates clearly that even minor changes can have a large impact on such a complex system.

Culturing biological material *in vitro* is, indeed, a complex procedure and although the progression from primordial follicles to live offspring has been achieved in mice, in the case of cultures of human ovarian tissue only secondary and early antral follicles have been obtained (Hovatta et al., 1997; Hovatta et al., 1999; Wright et al., 1999; Louhio et al., 2000; Hreinsson et al., 2002b).

At present it is not possible to obtain a large proportion of human antral follicles with this approach. One possible explanation for this failure is that, on the basis of a small number of archived human ovaries, follicular development from initiation to ovulation has been hypothesized to take approximately 200 days in women (Gougeon, 1986).

One major advantage of this *in vitro* approach is that it allows us to investigate the hormones and other factors involved in the recruitment and growth of human ovarian follicles. In culture, the majority of human follicles become activated within one week, with many reaching the secondary stage after only a few weeks (Hovatta et al., 1999). Investigations on bovine and baboons indicate that a release of the inhibition of ovarian tissue *in vitro* allows the primordial follicles to enter the growing pool (Wandji et al., 1996; Wandji et al., 1997; Fortune et al., 2000). This is of particular interest, since if all of the follicles develop at the same time, the supply of follicles will be exhausted too rapidly. On the other hand, if the tissue itself is not transplanted back into the patient, entry of all of the follicles into the growing pool can be advantageous, allowing harvest of the COCs, performance of IVM and subsequent cryopreservation of the embryos. IVM has already been shown to be a viable and valuable clinical procedure by collecting oocytes through aspiration in a non- stimulated cycle and maturing them to the MII stage (Hreinsson et al., 2003).

2.7 Some reflections

The ultimate goal of these *in vitro* approaches is to achieve effective preservation of fertility for women and young girls who are not able to have their ovarian tissue re-implanted due to the associated risk of reintroducing their disease. This goal is especially important for women treated for blood-born malignancies. Potentially culturing *in vitro* could provide a number of oocytes for harvesting at a given time-point rather than the single oocyte produced by the dominant follicle. The numbers of patients who are offered and choose tissue cryopreservation is ever increasing. In addition to this clinical significance, *in vitro* culture offers, as mentioned, an excellent system for elucidating the physiology of follicular recruitment and development.

With such a rapidly growing field come rapidly growing responsibilities.

I hear and I forget. I see and I remember. I do and I understand.
Confucius

3 AIMS OF THE PRESENT STUDIES

These studies were designed to accomplish the following:

1. To examine the effects of the size of the tissue samples, coating density, the matrix composition of the matrix, and various additions to the media, (i.e. rhSCF, anti-c-kit antibody, rrAMH, mGDF-9, antibody directed against GDF-9, BMPRII, rrAMH and rhGDF-9) on cultures of human ovarian tissues
2. To identify factors that play critical roles in the recruitment and early growth of follicles; and
3. To employ these factors to develop a medium that effectively promotes the maturation of ovarian tissue and oocytes *in vitro*.

The important thing is not to stop questioning.
Albert Einstein

4 METHODOLOGICAL CONSIDERATIONS

Please refer to the individual manuscripts for more detailed protocols.

4.1 SAMPLES OF HUMAN OVARIAN TISSUE

A total of 186 women, 19 – 44 (mean 33) years of age, acted as donors of ovarian tissue in connection with the four investigations included in this thesis (see Table 1 for additional information).

Informed consent was obtained from all of these women prior to collection of the biopsy, and the Ethics Committee of the Karolinska Institutet, pre-approved all of the studies (project number: 2006/966-32, 401/99). All of the biopsies utilized in articles I, III and IV, were collected at the Karolinska University Hospital Huddinge, while in the case of article II collections were performed at Karolinska University Hospital Huddinge, Hammersmith Hospital and Helsinki University Central Hospital. In the latter case, ethical approval was obtained from the Imperial College School of Medicine at Hammersmith Hospital, UK, and the Department of Obstetrics and Gynecology, Helsinki University Central Hospital and the Family Federation of Finland, Finland respectively.

4.2 ESTABLISHING CULTURES OF HUMAN GRANULOSA LUTEAL CELLS

(Article II)
Human granulosa luteal (GL) cells were obtained from follicular aspirates of women who were menstruating regularly and undergoing oocyte retrieval for IVF necessitated either by tubal obstruction or male infertility. In this connection the ovaries were stimulated by administration of a combination of an analogue of gonadotropin-releasing hormone (Suprecur; Hoechst, Frankfurt am Main, Germany) and human menopausal gonadotropin (Pergonal; Serono Nordic, Vantaa, Finland; or Humegon; Organon, Oss, The Netherlands). Oocyte retrieval was performed 36 – 37 hours following subsequent treatment with human chorionic gonadotropin 10,000 IU (hCG) (Profasi; Serono; or Pregnyl; Organon).

The GL cells were pooled, dispersed by enzymatic digestion with 0.1% hyaluronidase (Sigma Aldrich) in Ham's F-10 medium (Gibco, NY, USA) for 30 minutes at 37° C, and then separated from erythrocytes by centrifugation on a Ficoll- Paque gradient for 15 minutes at 1000g (Pharmacia, Uppsala, Sweden) (Jalkanen et al., 1987). Thereafter, the cells were either immediately subjected to RNA extraction or else plated at a density of 2 – 5 X 10^5 cells/well on 35 mm six-well dishes (Costar, Cambridge, MA, USA) and cultured in Dulbecco's Modified Eagle's Medium supplemented with 10% fetal calf

serum, 2 mM L -glutamine and antibiotics (Gibco) at 37° C under a humidified atmosphere containing in 5% CO_2 for six days prior to the extraction of RNA. During this period the culture medium was changed every other day.

4.3 CULTURING HUMAN OVARIAN TISSUE

(Articles I-IV)

In artcles I, III, and IV, the biopsy specimens were placed following collection in pre-equilibrated HEPES buffered oocyte collection medium: articles I, III and IV;(Gamete-100; Vitrolife, Kungsbacka, Sweden containing 10% human serum albumin (HSA Octapharma, Stockholm, Sweden) or Flushing Media; MediCult Jyllinge, Denmark) and article II; HEPES buffered culture medium (MEM; Gibco Invitrogen, Carlsbad, CA, USA). The tissue was then transferred immediately to the culture laboratory. Ovarian tissue samples were cut into designate pieces, using a needle and scalpel under sterile conditions. The tissue pieces were then either directly fixed for histological analyses (uncultured control at 0 days) or treated according to protocols for the separate investigations (Table 1).

In all of the articles the basic set up was similar, using an organ culture method described earlier (Hovatta et al., 1997; Hovatta et al., 1999). Organ cultures were performed in 24-well plates (Nunclon, Roskilde, Denmark or Falcon; Becton Dickinson MA, USA) fitted with Millicell CM inserts (12 mm diameter, 0.4 mm pore size; Millipore, MA, USA) coated with 100 µl of designate extracellular matrix (Matrigel, Growth Factor Reduced Matrigel or Laminin; Becton Dickinson) before use. In most of the studies the extra cellular matrix was diluted 1:3 with the base media being used without the supplements. Base media was αMEM in article I, III and IV. The media was supplemented with 10% human serum albumin (HSA; Pharmacia Upjohn, Sweden or Octapharma, Stockholm, Sweden). Further supplements were recombinant human FSH (0.5 IU/ml, Gonal-F, Serono, Zurich, Switzerland) (Wright et al., 1999) 8-bromoguanosine 3′,5′-cyclic monophosphate (8-br-c-GMP, 1.1 mg/ml, Sigma-Aldrich, MO, USA) (Scott et al., 2004), 1% ITS-G (Gibco Invitrogen) (with a final concentration of 10 µg insulin/ml, 5.5 µg transferrin/ml and 6.7 ng/ml of sodium selenite in the media) and 0.5% antibiotic/antimycotic solution (50 IU/ml penicillin, 50 µg/ml of streptomycin sulphate, 0.125 µg/ml of amphotericin B; Gibco Invitrogen). In article II the culture medium was EBSS supplemented with either 10% human serum albumin (HSA; Pharmacia Upjohn, Sweden) or inactivated human serum (5%) obtained from women undergoing pituitary desensitization for IVF treatment. Further supplements were FSH (0.11 U/ml; Metrodin or Gonal-F; Serono), and 0.5% antibiotic/antimycotic solution. The cultures were performed at 37° C in a 95% air 5% CO_2 humidified environment. Culture medium (500 µl) was added to each well; 100 µl were pipetted into the insert and 400 µl into the well outside the insert. Every second day, 100 µl of culture medium were removed and 100µl of fresh medium added to the well with 3 drops into the insert.

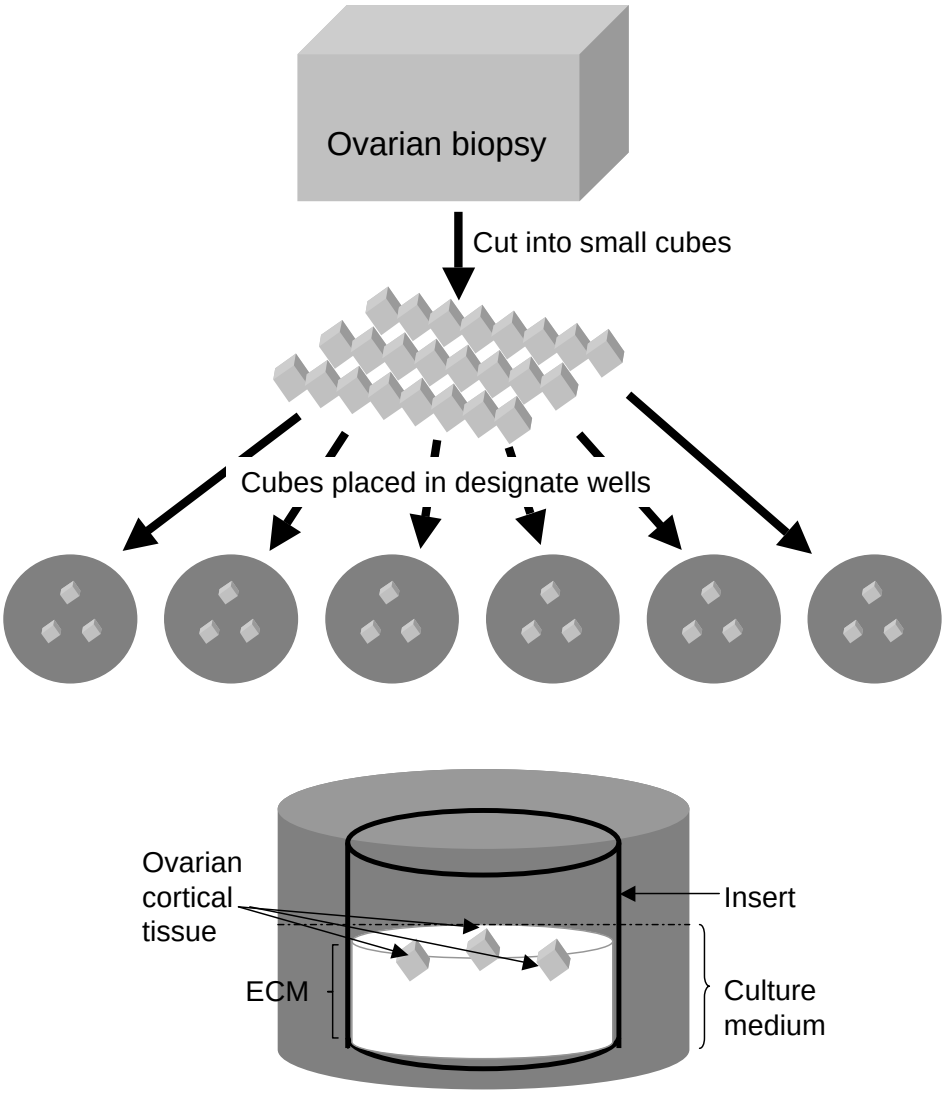


Figure 10. Simplified diagram of the culturing method used.

Table 1. Study designs

Article # / Study #	# of Patients (age)	Supplement	Concentration of Supplement	Days in Culture	Culture Plate Type	Tissue Shape	Coating Density	Extracellular Matrix
I / 1 tissue dimensions	10	none	N/A	0, 7 and 14	Nunclon	Slices vs Cubes	Diluted	Matrigel™
I / 2 coating density	14	none	N/A	0, 7 and 14	Falcon	Cubes	Diluted vs Thick vs Thin	Matrigel™
I / 3 matrix composition	9	none	N/A	0, 7 and 14	Falcon	Cubes	Diluted	Matrigel™ vs Growth Factor Reduced Matrigel™ vs Laminin
II	55 (19-44)	rhSCF or anti-c-Kit antibody	0, 1, 10, 100ng/ml or 800ng/ml	0, 7 and 14	Nunclon and Falcon	Slices	Diluted	Matrigel™
III	15 (6-41)	rrAMH	0, 10, 30, 100 and 300ng/ml	0 and 7	Falcon	Cubes	Diluted	Growth Factor Reduced Matrigel™
IV / 1	10 (29-37)	mGDF-9	Control and 200ng/ml	0, 14, 28 and 42	Falcon	Cubes	Diluted	Growth Factor Reduced Matrigel™
IV / 2	15 (25-37)	mGDF-9	Control, vehicle control, 200, 600 and 2000ng/ml	0 and 7	Falcon	Cubes	Diluted	Growth Factor Reduced Matrigel™
IV / 3	18 (24-40)	rhGDF-9	Control and 200ng/ml	0, 14, 28 and 42	Falcon	Cubes	Diluted	Growth Factor Reduced Matrigel™
IV / 4	15 (27-39)	mGDF-9Ab	Control, vehicle control, 100, 300, 1000 and 3000ng/ml	0 and 7	Falcon	Cubes	Diluted	Growth Factor Reduced Matrigel™
IV / 5	25 (26-40)	BMPRII ecdFChis6	Control, vehicle control, 100 and 1000ng/ml	0 and 21	Falcon	Cubes	Diluted	Growth Factor Reduced Matrigel™

4.4 HISTOLOGICAL ANALYSIS

(Article I-IV)

Fresh uncultured ovarian biopsy material (day 0) and cultured specimens were fixed in Bouin's solution (Sigma-Aldrich) for 4-5 hours at room temperature, dehydrated in 70% ethanol in the refrigerator, where it was stored until it was ready for embedding in paraffin and serially sectioned at a thickness of 4µm. To prevent double counting of follicles, eight sections were omitted between sections mounted on the slide. Following staining with haematoxylin and eosin, follicles were counted and their developmental stages recorded according to the classifications of Gougeon (Gougeon, 1986). Briefly, those follicles containing a single layer of flattened granulosa cells were regarded as primordial, those having one or more cuboidal granulosa cells were classified as primary, and follicles having two or more layers of cuboidal granulosa cells around all or part of the oocyte were identified as secondary. Atretic follicles were identified by oocyte fragmentation, eosinophilia of the cytoplasm, pyknotic GCs and or clumping of the chromatin. A digital imaging analysis system (Easy Image Mätning; Tekno Optik, Sweden) was used to measure the area of the tissue pieces, from which the volume was calculated by multiplying the area of the tissue piece by the known section thickness of 4 µm. The density of the follicles was then determined as the total number of follicles per cubic millimeter of ovarian tissue. The system was also used to measure the diameter of the oocytes and follicles in the tissue.

4.5 IMMUNOHISTOCHEMICAL ANALYSIS OF C-KIT

(Article II)
For the purposes of immunohistochemical analysis, the paraffin embedded sections were incubated at 60° C for 15 minutes, rehydrated in a series of etanol solutions containing increasing amounts of water and then placed in a water bath at 37° C. Following inhibition of endogenous peroxidase activity by incubation for 5 minutes with peroxidase and subsequent incubation for 30 minutes with anti-c-kit antibody (ACK2) (4 mg/ml diluted in PBS), the sections were incubated for 30 minutes with peroxidase-conjugated goat anti-mouse IgG, as the secondary antibody (DAKO EnVision + System; Dako Corporation, Carpinteria, CA, USA). Following the incubation they were washed twice with PBS. Finally, bound antibodies were visualized utilizing the chromogenic peroxidase substrate 3,30 -diaminobenzidine (Dako), followed by a counterstaining was with Erlich's hematoxylin for 1 minute. Immunostaining with PBS + 0.1% BSA (Sigma) and the peroxidase-labeled secondary antibody was used as a negative control as well as an antibody against myelin basic protein. All of the incubations were performed at room temperature unless otherwise specified.

4.6 NORTHERN AND *IN SITU* HYBRIDIZATION

(Article II)
The total RNA prepared from freshly isolated GL cells and cytoplasmic RNA from cultured GL cells were extracted with the guanidine isothiocyanate – cesium chloride method (Chirgwin et al., 1979) and modified NP-40 lysis procedures (Ritvos and Eramaa, 1991)and therafter subjected to Northern blotting.

After fractionation on the basis of size on an agarose gel and transfer to a membrane filter (Laitinen et al., 1997) each RNA sample was hybridized with single or double-stranded cDNAs labeled with [α-32P]deoxy-CTP.

For the preparation of the cDNA probe, commercial c-Kit cDNA was first cloned in the pGEM7 vector (Promega, Madison, WI, USA) and then cut out with EcoRI. The 546 bp (representing nucleotides 704-1249), single-stranded c-Kit cDNA probe was then obtained by PCR amplification for 40 cycles utilizing a primer with a sequence 5' – TAAATCCACTGTGATATCTTA-3', which is complimentary to that of SP6 promoter sequence) (Laitinen et al., 1997). Double-stranded c-DNA encoding rat glyceraldehyde-6-phosphate dehydrogenase (GAPDH) (Laitinen et al., 1997) served as a control for even loading of the hybridization filters. This cDNA was labeled with [α-32P]deoxy-CTP employing the Prime-a-gene kit (Promega).

For *in situ* hybridization analyses, the [α-33P]UTP-labeled antisense cRNA probes were transcribed *in vitro* from EcoRI-linearized plasmids containing a 905 bp human KL cDNA (Laitinen et al., 1995). Thereafter, *in situ* analyses of KL mRNA were carried out on 9 µm thick cryostat sections as described previously (Heikinheimo et al., 1997). Finally, the slides were dipped in an NTB-2 emulsion (Eastman Kodak, New Haven, CT,

USA) and exposed for up to 57 days.

4.7 AGONISTS AND ANTAGONISTS OF GDF-9

(Article IV)

Agonists

To stimulate GDF-9 effects in ovarian tissue culture, we first used mouse GDF-9 (mGDF-9) and then recombinant human GDF-9 (rhGDF-9). The mouse GDF-9 was a whole pro mature complex of the GDF-9 protein; produced using conditioned media from 293T cells (Kaivo-Oja et al., 2003)**.** This conditioned medium was used in both a dose response study and a long-term culture study (experiments 1 and 2). The rhGDF-9 was the purified untagged mature region, that we have previously characterized *in vitro* (experiment 3) (Mottershead et al., 2008).

Antagonists

To inhibit GDF-9 action in tissue culture, we used GDF-9Ab-37 (experiment 4) and BMPRII-Fc (experiment 5). GDF-9Ab-37 neutralizes mouse GDF-9 in mouse GC cultures and it recognizes the human GDF-9 sequence epitope LSVLTIEPDGSIAY (Gilchrist et al., 2004). The BMPRII-Fc protein was produced and purified (details in Myllymaa S. et al., manuscript in preparation) in our laboratory and is designed such that the ecto-domain of the BMP type II receptor (which is utilized by both GDF-9 and GDF9-B/BMP-15) is N-terminal to the Fc domain of human IgG1. This protein effectively neutralizes recombinant rat or mouse GDF-9 (Vitt et al., 2002; Gilchrist et al., 2006).

4.8 STATISTICAL ANALYSIS

(Article I)

Analysis was performed using one-way analysis of variance (ANOVA) followed by post-hoc test. Before the analysis was performed, data was tested for normality and homogeneity of variance with logarithmic transformation, where necessary.

(Article II)

The data were analyzed using Chi-square and Mann-Whitney U-tests. $P<0.05$ was considered significant.

(Article III)

Statistical comparisons between groups for all data were carried out using one-way analysis of variance (ANOVA) followed by Fisher's calculation of differences. The data were tested for normality and homogeneity of variance. Significance was reported at the $P<0.05$ level. Cultures containing 300ng/ml of AMH were excluded from statistical analysis owing to the small number of samples (n=5).

(Article IV)
Statistical comparisons between groups for all data were determined using a one-way analysis of variance non-parametric, which is a one-way ANOVA. Significance is reported at the 0.05 level.

A man should look for what is, and not for what he thinks should be.
Albert Einstein

5 RESULTS AND DISCUSSION

The recruitment and growth of follicles is a complex process, involving a multitude of factors with both positive and negative effects. In order to help elucidate these processes we performed the following studies.

Article I:

Article I consists of three studies designed to optimize ovarian cortical tissue culture *in vitro*. Study 1 was designed to look at the effect of using strips versus cubes of tissue, study 2 was designed to look at different coating densities of the extracellular matrix and finally study 3 was designed to look at the composition of the matrix itself and the effect it has on the tissue in culture.

In the first study, looking at tissue dimensions, we found that there was no significant difference between cubes and slices when compared to one another. However, when compared to the uncultured control tissue, the cubes cultured for seven days were the only group without a significant decrease in viability. The same pattern was observed when looking at the density, where again the cubes after seven days of culture were the only treatment group not significantly different from the uncultured control. Therefore, we decided that cubes were better than slices of tissue. We concluded that the reason for this is the superior surface area-to-volume ratio, giving it more contact with the surrounding media and better exchange of nutrients, gases and metabolites. It is important to note that although the increased viability of cubes was not seen after 14 days of culture, it is still important, in that if the initial stages of culture are not ideal, there may be compounded problems observed later.

The second set of experiments revealed that the activation of the follicles was independent of the thickness of the coating with extracellular matrix. The reduction in the number of primordial follicles was accompanied by a proportional increase in the combined numbers of primary and secondary follicles (with the exception of 14 days of culture on a thick coat of Matrigel™) and there was no difference in this pattern or in the overall density of follicles between the various cultures at any time. The viability of all of the cultured tissue samples was lower than that of uncultured control tissue with the exception of the 7 days of culture on diluted Matrigel™). Thus, since diluted Matrigel™ supports follicle survival, and in addition is much simpler to work with and less expensive than the other coating conditions tested, we conclude that this provides the most advantageous coating conditions.

Finally, we found no differences in qualities of ovarian tissue cultured on matrices of different compositions. This observation was particularly interesting in the case of growth factor reduced (GFR) Matrigel™, which might be expected to support follicle growth and development less effectively. Regardless of the matrix employed, follicle

activation occurred in all cultures and all cultures exhibited similar follicle densities, as well as an attenuated level of viability in comparison to uncultured control tissue samples. For these reasons, we conclude that the matrices tested here in themselves are equally effective in supporting cultures of human ovarian tissue. The choice of extracellular matrix must however, take into account other factors as well, e.g. the possible binding of growth factors of interest in a particular study to different types of matrices.

In summary, these findings indicate that ovarian cortical tissue should be cultured on diluted Matrigel™ in the form of cubes, and that the composition of the ECM should be chosen on the basis of the aims of the study.

Article II:

Since little was known about the roles of KL and c-Kit in the early development of human follicles we examined the expression of these proteins in human ovarian tissue. In addition, we tested the effects of exogenous KL and of an antibody against c-Kit receptor on the growth and viability of ovarian tissue in culture.

In situ hybridization revealed the expression of KL mRNA in the GCs of primary follicles; while immunohistochemical analysis demonstrated that the c-Kit protein is expressed in the GCs of both primary and secondary follicles, but not in the theca cells of preantral and antral follicles. This latter finding was further confirmed by northern blotting of freshly isolated GCs.

Culturing ovarian tissue in the presence of different concentrations of exogenous KL had no influence on follicular development, diameter or viability. Such effects may have been masked by the presence of endogenous KL and/or the high rate of spontaneous maturation that occurs even in control cultures. We were able to exclude serum as having a masking effect as we performed further cultures that were serum free.

Follicle development in cultures where anti-c-Kit antibody (ACK2) was added to the medium was similar to that in the controls following 7 or 14 days of culture, demonstrating a species difference with respect to the rat and mouse (Yoshida et al., 1997; Parrott and Skinner, 1999). However, ACK2 did cause a significant elevation in the numbers of atretic follicles at both of these time points. Thus, after 7 days, ACK2 reduced viability from 49% to 28% and after 14 days in culture from 62% to 38%, even at the primordial stage of development. This clearly indicates, KL/c-Kit is an important factor in connection with early human follicular development.

In summary, this investigation revealed that KL mRNA and c-Kit mRNA and protein are expressed, in follicles from the primary to antral stage of development. Moreover, the reduction in survival caused by an antibody that blocks the c-Kit receptor demonstrates that KL/c-Kit signaling plays an important role in the early development of human ovarian follicles.

Article III:
These experiments were designed to elucidate the influence of AMH on the initiation and regulation of growth of human primordial follicles in the ovary. Since we focused primarily on potential inhibition of the recruitment of these primordial follicles, a 7 day period of culture was employed. The mean proportion of the developmental stages of the follicles increased significantly during the seven-day culture period in most groups when compared to the uncultured control. In the uncultured control tissue, 56% of the follicles where primordial whereas, after seven days of culture, the proportion of primordial follicles had decreased to between 14 and 26%. The exception to this was the culture containing 100ng/ml of AMH, where the primordial follicle proportion was 40% and did not differ significantly from the uncultured control. There was no significant influence on follicle viability and density between culture groups.

Furthermore, we found that the addition of recombinant rat AMH (rrAMH) (100 ng/ml) suppresses the initiation of primordial follicle recruitment into the growing pool and/or their subsequent activation without exerting any detrimental effect on the viability. In the other treatment groups, with a lower concentration, the proportion of various developmental stages of the follicles changed in a similar manner to our earlier studies (Hovatta et al., 1997; Hovatta et al., 1999; Hreinsson et al., 2002b; Scott et al., 2004) In those cultures, the proportion of primordial follicles was not significantly different from that of the uncultured control tissue and furthermore, it was significantly greater than that in the vehicle control and in 10ng/ml cultures, indicating the inhibition of growth initiation. This was in accordance to the results shown in mouse cultures where treatment with AMH appeared to inhibit follicular development (Durlinger et al., 2002). These results also support the idea that AMH is necessary to slow or stop the initiation and growth of primordial follicles (Durlinger et al., 1999). Therefore, strengthening the idea that AMH is produced by the pool of growing follicles as a negative paracrine feedback signal (Visser and Themmen, 2005).

When Schmidt and co-workers (Schmidt et al., 2005) cultured ovarian slices from 6 women for 4 weeks in the presence of recombinant human AMH (rhAMH) at 300 ng/ ml, with and without testosterone, the results showed that AMH, alone and with testosterone, advanced the presence of primary and secondary follicles, although the diameter of the oocytes did not increase. As stated earlier, our study design was quite different in that we cultured for only seven days as we wished to look at initiation of primordial follicles into the growing pool.

In summary, the findings documented in article III indicate that AMH plays a crucial role in the suppression of follicles from entering the growing pool and is one of the hormones involved in inhibition of primordial follicle recruitment.

Article IV:
In article IV we explored the effects of two factors that stimulate and two factors that suppress follicular growth (for further details please see section 4.7). In a previous investigation (Hreinsson et al., 2002b) we showed that rat GDF-9 has a beneficial effect on human ovarian tissue culture, promoting follicle development and improving viability.

In two of the studies included in this article we were able to demonstrate that under defined conditions, not only can we culture for up to 42 days and still have viable follicles, but also we can improve upon our viability by culturing with recombinant human GDF-9 (rhGDF-9).

Furthermore, we performed a dose response study using mouse GDF-9 (mGDF-9). Unfortunately, despite nice bioactivity in both human and rodent GCs (Kaivo-Oja et al., 2003; Gilchrist et al., 2004; McNatty et al., 2005), we did not observe anything significant in this 7-day dose response study. This carried forward into the 42 days study using mGDF-9 as well. The bioactivity was good but it was not effective in culture. This study emphasizes the importance of using pure recombinant GDF-9 in ovarian tissues to obtain an optimal effect. Our hypothesis is that the mouse GDF-9 used in these two studies is a complex of biologically active mature region and the pro region needed in the synthesis of the molecule. There are no indications that the mouse GDF-9 would be inhibited by its pro region, but we recently found that the human GDF-9 protein is inactive as expressed in 293T conditioned media where the pro and mature regions exist in complex (Mottershead et al., 2008). There are two possible explanations as to why. First of all, the pro region might prevent the mature region from accessing the receptor and secondly, the ECM may further restrict the access of the complex to the receptors by binding the complex. By purifying and isolating the human GDF-9 mature region from the pro region, and other possible inhibitory factors, we were able to obtain a pure bioactive ligand that has 5 times higher activity than the unpurified mouse. Unfortunately, when we began this study only the mouse protein was commercially available; but luckily, human GDF-9 came just two years later.

The rhGDF-9 had a significant effect on early follicle growth, development and survival. All treatment groups had significantly fewer primordial follicles as the follicles had started to enter the growing pool, but the cultures with rhGDF-9 at 14, 28 and 42 days had significantly fewer primary follicles and in turn, significantly greater numbers of secondary follicles than those that did not contain rhGDF-9. The 14 day cultures with rhGDF-9 had significantly more viable follicles than either the 14 or the 28 day culture without. Further, there was a significant increase in oocyte size in the 28 and 42 day cultures containing rhGDF-9.

In the last two studies we looked at inhibiting the effects of endogenous GDF-9. First of all, we looked at the effect of a validated GDF-9 neutralizing antibody. Secondly, we studied the effects of the soluble receptor BMPRII-Fc, which has been shown to be effective in neutralizing the biological effect of both GDF-9 (McNatty et al., 2005; Mottershead et al., 2008) and GDF-9B/BMP-15 (Kaivo-Oja et al., 2003). The neutralizing antibody showed that it might suppress initiation of primordial follicles at a dose of 3000 ng/ml. From this we conclude that even though they were able to block the endogenous activity of GDF-9 in mouse (Gilchrist et al., 2004) GC mitogenesis assays, high concentrations were required for optimal suppression. As we used it in an untested setting (organ culture) we found that this does not operate effectively in this setting. From this we conclude that the antibody is not optimal for our system. Using BMPRII-Fc, we were able to show that the treated group with 1000 ng/ml had significantly more primary

follicles than the other groups, indicating that it prevented them from progressing to the secondary stage. Furthermore, the treatments containing 100 and 1000 ng/ml BMPRII-Fc had significantly smaller follicles when compared to the rest. When looking at the BMPRII-Fc we showed that, when administered at a very high dose (1000 ng/ml), we were able to successfully suppress the effect of locally produced GDF-9 and GDF-9B/BMP-15 high affinity ligands (Gilchrist et al., 2006; Edwards et al., 2008).

In summary, the findings documented in article IV demonstrate that endogenous GDF-9 plays an important role in the transition from primary to secondary follicles and that BMPRII-Fc can suppress this transition. Furthermore, the rhGDF-9 protein promotes the development and early growth of follicles, an effect that could be useful a clinical setting.

Success is going from failure to failure without losing enthusiasm.
Winston Churchill

6 SUMMARY AND CONCLUDING REMARKS

Over the course of many years, we have characterized the involvement of various factors in folliculogenesis in human ovaries. We have been successful in, among other things, improving upon the methodology for such characterization (Hovatta et al., 1997; Hovatta et al., 1999; Hreinsson et al., 2002b; Scott et al., 2004; Zhang et al., 2004). The findings described here contribute further to improving the system used to culture biopsies of developing ovary. Thus, ovarian cortical tissue should be cultured in the form of cubes on diluted Matrigel™ matrix and the choice of the composition of the ECM should be based on the goals of the study in question (Article I). KL mRNA and c-Kit mRNA and protein are expressed in follicles at all stages of development, from the primary to antral, and the reduction in the survival of follicles in long-term organ culture caused by an antibody that blocks the c-Kit receptor indicates that signaling via KL/c-Kit plays an important role in the early development of human ovarian follicles (Article II). Moreover, AMH is a key factor involved in suppressing the entry of follicles into the growing pool, i.e., is one of the hormones that inhibit the recruitment of primordial follicles (Article III). Finally, we found that endogenous GDF-9 participates in the transition from primary to secondary follicles; BMPRII-Fc can suppress this transition; and that the rhGDF-9 protein promotes the development and early growth of follicles, an observation that could have valuable clinical applications (Article IV).

The cortical tissue of the ovaries contains the vast majority of the follicles and their oocytes. For women treated for malignancy or certain other forms of disease, culturing this tissue under conditions that promote optimal viability and least risk for retransmission of disease is important for preservation of fertility. In the case of the mouse this approach has been employed to obtain normal offspring, but in women, where the developmental process takes much longer, this level of success has not yet been attained. Furthermore, we must be certain that there are no genetic abnormalities in the cultured oocytes before fertilizing them. For all of these reasons, the culture system must be developed and optimized even further before oocytes from cultured primordial follicles can be used clinically.

Science never solves a problem without creating ten more.
George Bernard Shaw

7 FUTURE PERSPECTIVES

The relatively long period of culture required for clinical application remains a barrier that must be overcome. At present, it appears that, as in the case of the mouse, a sequential culturing of human ovarian tissue will be required, with changes in the culture conditions as the needs of the oocyte and follicle alter. As the follicle grows in size, access to nutrients and gas and waste exchange may become inadequate, a problem that may be solved by isolating the follicle once both the theca interna and externa have developed and interactions with and support from the other somatic cells in the tissue are no longer required. Furthermore, we still do not know the details of how the oocyte aquires nuclear and cytoplasmic competence, maturation processes which are difficult to monitor and require the development of novel non-invasive techniques. There are many areas being investigated at this time. I believe that sequential culturing is necessary and have been performing experiments along these lines that are not included here.

Once viable antral follicles have been achieved in tissue culture, we would like to aspirate the COCs and obtain maturation of the oocytes *in vitro*. To this end, we are performing studies designed to identify factors that promote such maturation, e.g., the potential involvement of GH and EGF. Furthermore, we are investigating the involvement of both MMPs and TIMPS in ovulation. Other possible improvements in the conditions employed for culturing cortical ovarian tissue, including a completely new approach, are also under investigation in our laboratory. Of course, the progress of such experimentation is limited by the availability of human cortical ovarian tissue.

Ultimately, the goal of my research is to develop optimal conditions for culturing human ovarian tissue, including development of a sequential long-term culturing system that successfully provides the follicle with what it requires.

If we knew what it was we were doing, it would not be called research, would it?
Albert Einstein

8 POPULAR SCIENCE DESCRIPTION

If we can culture ovarian tissue to mature and produce normal eggs outside of the body, we should be able to help women suffering from premature ovarian failure, cancer, genetic disorders, or other diseases to have their own children. The ovary contains a large number of eggs, each of which is supported by surrounding cells in a unit called the follicle. These supporting cells, which are of several different types, produce sex hormones (estrogen and progesterone), as well as a number of other growth factors and hormones necessary for reproduction. Large numbers of immature follicles are present in the ovary, providing a nearly inexhaustible supply of potentially fertilizable eggs.

These follicles have been grown successfully within their surrounding tissue in the laboratory, but it is not yet possible to get them to mature under these conditions. Of particular importance in this context is the early development of the follicles. Although this development is poorly understood, a family of hormones called transforming growth factor-beta (TGF-β) appears to be involved.

For example, growth differentiation factor-9 (GDF-9) is known to be necessary for follicles to mature. We need to understand the roles played by TGF-β hormones and other factors in the early stages of the development of human eggs. In addition to improving our knowledge of this fundamental biological process, my studies were designed to eventually help develop new approaches that allow women who, for whatever reason, have difficulties getting pregnant to bear their own children.

If you want to go quickly, go alone.
If you want to go far, go together.
African Proverb

9 ACKNOWLEDGEMENTS

Life is a collaboration and I certainly did not get here on my own. Accordingly, I have many people whom I would like to thank:

First of all, my sincere gratitude goes to the women who allowed us to use biopsies of their ovaries for these studies. Without this help, it would have been impossible to achieve the new knowledge described here which will hopefully lead to discoveries that will help countless women and girls in the future.

My principal advisor, **Professor Outi Hovatta:** first of all, thank you for allowing me to join your group and giving me the opportunity to perform this research. Thank you also for your invaluable support and guidance during these years. It has been a real pleasure getting the opportunity to work for you and with you. Your adventurous spirit is inspiring! And I wish you many more exciting adventures both in science and life. Kiitos!

My assistant supervisor, **Dr. Olli Ritvos**: thank you for your continuous support, encouragement and your amazing memory, (if only I could remember 1/3 of what you know…) Both you and the other members of your group have made invaluable contributions to both my project and to me. Kiitos!

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A journey of a thousand miles begins with a single step.
Confucius

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