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Ju mer man tänker, ju mer inser man att det inte finns något enkelt svar.

Källa: Nalle Puh A. A. Milne

Till min familj

ABSTRACT OF THESIS

Infertility is a problem that affects about 15 percent of all couples. Many can be helped by assisted reproductive techniques, but still today many couples are treated without a successful outcome. The reason for infertility is sometimes known, but there are also many cases of unexplained infertility. Therefore, basic knowledge about follicle development, maturation of oocytes and preimplantation embryo development needs to be explored further.

In **article I** we investigated the possibility to quantify gene expression in single embryos and blastomeres to detect the expected increase occurring at the time for the embryonic gene activation (EGA). We found that real-time RT-PCR was sensitive enough for this purpose, as we could detect an increased gene expression of the translation initiation factor 1A (eIF-1A) at the expected time in mouse and human preimlantation embryos and in mouse blastomeres. Possibly, with further improvements such measurements could be useful when evaluating the developmental capacity of early embryos.

In **article II** we utilized the unique opportunity to study non-luteinized granulosa cells (GC's) from *in vitro* maturation (IVM) treatments. We studied the effect of gonadotrophins (FSH and hCG) on the non-luteinized GCs by measuring secretion of estrogen and progesterone and the expression levels of the enzymes $p450_{aromatase}$ and $p450_{scc}$ (side chain cleavage enzyme) before and after treatment. These measurements were compared to luteinized GCs from conventional IVF-treated patients. Furthermore, we studied how the receptors for LH and FSH reacted to the different treatments in non-luteinized GC's. We observed increased or unchanged hormone production and gene expression of the enzymes involved in their synthesis in the non-luteinized GC's, while in the luteinized GC's the hormone production and enzyme expression was decreased or unchanged in response to the different treatments. In IVM GC's the LH receptor expression increased with FSH treatment but the expression of the FSH receptor was unaffected. We concluded that the more reactive non-luteinized GC's are more interesting to study as they are still in a stage where they can influence the oocyte.

The effect of growth differentiation factor -9 (GDF-9) on follicular development was investigated in **article III.** This was studied in ovarian tissue cultures using different GDF-9 agonists and antagonists. We found that the addition of exogenous rhGDF-9 stimulates growth of early follicles and promotes the transition from the primary to the secondary stage. We also saw that this transition could be inhibited by blocking endogenous GDF-9 using the soluble receptor BMPRII-Fc. These findings can be very useful trying to mature follicles from cryopreserved tissue *in vitro*.

Finally, in **article IV** we have adapted a recently developed research instrument, the Cell-IQ®, for studies of oocyte maturation *in vitro*. In this instrument the incubator and the imaging equipment is integrated and we can follow the different maturation events in detail without disturbing the culture conditions. This offers an excellent opportunity to optimize the culture conditions in order to improve the clinical IVM procedure.

Our findings provide new information regarding the complex regulation of folliculogenesis and oocyte maturation and we also provide helpful tools for further investigations aiming at improving assisted reproductive techniques.

LIST OF PUBLICATIONS

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

I. **Lindeberg M**, Hovatta O, Ahrlund-Richter L.
Real-time reverse transcription-polymerase chain reaction analysis of translation initiation factor 1A (eIF-1A) in human and mouse preimplantation embryos.
Reprod Biomed Online. 2004 Mar;8(3):338-43.

II. **Lindeberg M**, Carlstrom K, Ritvos O, Hovatta O.
Gonadotrophin stimulation of non-luteinized granulosa cells increases steroid production and the expression of enzymes involved in estrogen and progesterone synthesis.
Hum Reprod. 2007 Feb;22(2):401-6. Epub 2006 Nov 10.

III. 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 for publication to JCEM)

IV. **Lindeberg M**, Carlsson IB, Tarvainen J, Korpinen J and Hovatta O
A real-time monitoring system for maturation of human oocytes *in vitro* using Cell-IQ® equipment.
(Submitted for publication to Reproduction)

LIST OF ABBREVIATIONS

ActR	Activin receptor
ALK	Activin like kinase
AMH	Anti müllerian hormone
AR	Androgen receptor
Areg	Amphiregulin
ART	Assisted reproductive techniques
BERKO	Estrogen receptor beta knock-out
ERKO	Estrogen receptor alpha knock-out
BMP	Bone morphogenetic protein
BMPRII	Bone morphogenetic protein receptor type II
Btc	Betacellulin
cAMP	Adenosine 3',5'-cyclic monophosphate
CC	Cumulus cells
COC	Cumulus oocyte complex
Cx	Connexin
EGA	Embryonic gene activation
EGF	Epidermal growth factor
EGFR	Epidermal growth factor receptor
ER	Estrogen receptor
Ereg	Epiregulin
FSH	Follicle stimulating hormone
FSHR	Follicle stimulating hormone receptor
GC	Granulosa cell
GDF-9	Growth differentiating factor 9
GDF-9B	Growth differentiation factor 9B (also known as BMP15)
GH	Growth hormone
GHR	Growth hormone receptor
GnRH	Gonadotrophin releasing hormone
GVBD	Germinal vesicle break down
GV	Germinal vesicle
hCG	Human chorionic gonadotrophin
HPO	Hypothalamus pituitary ovary
ICSI	Intracytoplasmatic sperm injection
IGF	Insulin-like growth factor
IVF	*In vitro* fertilization
IVM	*In vitro* maturation
KL	Kit ligand (also known as stem cell factor (SCF))
LH	Luteinizing hormone
LHR	Luteinizing hormone receptor
MII	Metaphase II
MI	Metaphase I
MPF	M-phase promoting factor/Maturation promoting factor
OHSS	Ovarian hyper stimulation syndrome
PCOS	Polycystic ovary syndrome

PGC	Primordial germ cell
PI3K	Phosphatidyl inositol 3 kinase
PKA	Protein kinase A
POF	Premature ovarian failure
PR	Progesterone Receptor
TGFβ	Transforming growth factor beta
ZP	Zona pellucida

TABLE OF CONTENTS

1. INTRODUCTION

1.1 Female Infertility/fertility

Infertility is a worldwide problem that affects approximately 15% of all couples of fertile age. Female infertility can be caused by a variety of reasons i.e. hypothalamic dysfunction, ovarian factors such as polycystic ovarian syndrome (PCOS), anovulation, premature menopause or tubal factors such as tubal occlusion or tubal dysfunction. Many of the infertile couples can be helped through the continued development of assisted reproductive techniques (ART). However, there is still a need for improvements of the existing techniques and development of new methods for further progress in the field of ART.

Female fertility requires a functioning ovary to produce mature oocytes. The functional unit of the ovary is the follicle that contains the oocyte surrounded by granulosa cells and theca cells. Maturation of the oocyte depends on the production of hormones, androgens estrogen and progesterone that are produced in the somatic cells which surround the oocyte in the follicle. There are also a number paracrine and autocrine growth and differentiation factors produced by the oocyte, the other follicular cells or factors produced elsewhere that act in an endocrine fashion and are of importance for the maturation process.

1.2 Human Ovary

1.2.1 Ovarian reserve

The female reproductive system contains a non-renewable reserve of germ cells.
However this dogma has been contradicted in a report by Johnson where it was suggested that there are germ stem cells present in the surface epithelium of the ovary (Johnson, et al., 2004). Later the same group claimed that those stem cells originated from the circulation and bone marrow cells (Johnson, et al., 2005). This hypothesis has however not been confirmed by others since then and there is some controversy in the field concerning this idea.

According to the traditional standpoint the human ovary hold a decreasing reserve of oocytes from fetal life until the woman enters menopause. The oocyte quantity reaches its peak already before birth around 20 weeks of gestation, with approximately seven million follicles. At birth the number has decreased to around one to two million and at the onset of puberty only 300,000 to 400,000 follicles are left. When the woman enters menopause at an average age of 51 years approximately 1000 follicles remain in the ovary (Faddy, 2000). During the reproductive life of a woman, approximately 400 oocytes ovulate and at the same time hundreds of thousands of oocytes go through atresia and degenerate (Gougeon, 1996).

1.2.2 Folliculogenesis

During the maturation process the follicle grows and goes through the primordial, primary, secondary and preantral stage before it acquires an antral cavity. At the antral stage most follicles go through atresia, but a few of them reach the pre-ovulatory phase under the influence of gonadotrophins. Following the preovulatory gonadotrophin surge, one dominant follicle will then release a mature oocyte ready for fertilization(Gougeon, 1996) (McGee and Hsueh, 2000). While the follicle is still resting it is in the primordial stage and one layer of flat granulosa cells and a thin basal lamina surrounds the oocyte. The factor/s that trigger/s the transformation of the follicle from the primordial to primary stage remains unknown. Studies in bovine, baboon and in human indicate that *in vitro* culture of ovarian tissue relieve the inhibition present in vivo and push the primordial follicles into the growing pool (Hovatta, et al., 1997; Wandji, et al., 1997; Wandji, et al., 1996). Once it gets recruited into the growing phase, it transforms to a primary follicle and the granulosa cells change shape and become cuboidal. Production of proliferating cell nuclear antigen PCNA indicates that proliferation of the granulosa cells has started.

The secondary follicle is surrounded by two or more layers of granulosa cells. When there are three to six layers of cells the theca cells are recruited from the surrounding stroma cells forming theca interna and theca externa with vessels in-between the two layers. At this stage the follicle starts to gain a blood supply and can thereby be exposed to any factor circulating in the blood (Bassett, 1943; Reynolds, et al., 1992). When epithelioid cells appear in the theca interna the follicle enters the preantral stage and a fluid filled antrum starts to develop. Under the influence of gonadotrophins and growth factors the follicle grows and the granulosa cells proliferate and differentiate to mural GC's in the periphery of the follicle and cumulus cells (CCs) closest to the oocyte and finally the pre-ovulatory follicle is formed. The communication between the oocyte and its surrounding granulosa cells results in maturation of a fertilizable oocyte.

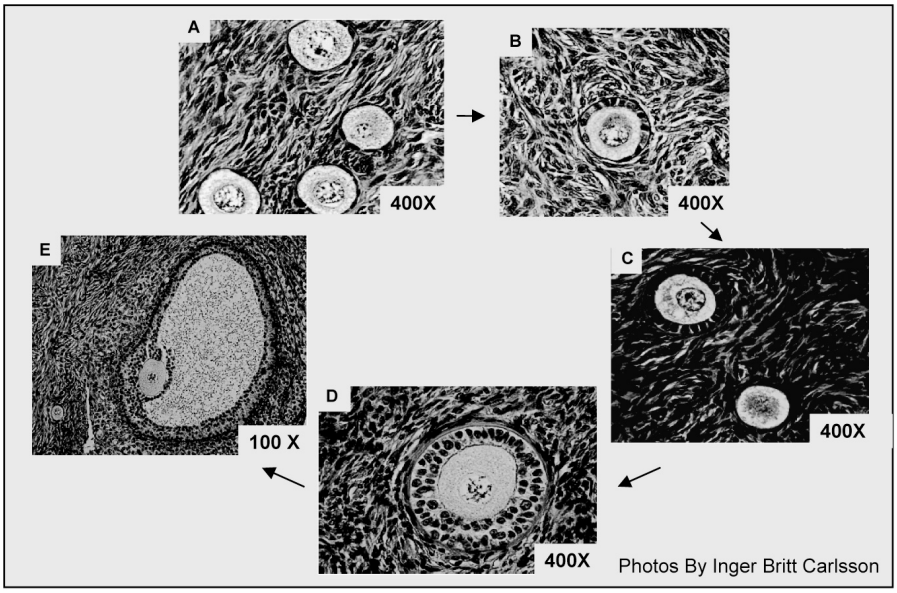

Figure 1. Light micrographs of the different follicular stages A: Primordial follicles B: A transitional follicle from the primordial to the primary stage. C: Primary follicle (upper). D: Secondary follicle E: Antral follicle.

1.3 Oogenesis and Oocyte maturation

1.3.1 Oogenesis

Oogenesis starts in embryonic life with the formation of primordial germ cells in the embryonic epiblast. Under the influence of cytokines such as *kit*-ligand (KL) and transforming growth factor β (TGFβ) they migrate to the genital ridge where they stay and develop into oogonia. Here they expand through mitotic cell division and pass through one last round of DNA replication before they enter meiosis and become oocytes. Coinciding with the initiation of meiosis the oocyte become enclosed in one layer of pregranulosa cells surrounded by a basal membrane and this unit is now referred to as a primordial follicle (Gosden, 1995). As the follicle grows the oocyte grows and matures from a primordial germ cell to a fertilizable MII oocyte. This is a long process which according to one old estimate lasts for over 200 days (Gougeon, 1986) and during this time the diameter of the oocyte increases from 35-120µm and the volume increases a 100-fold (Gougeon, 1996; Picton, et al., 1998). There are two components of oocyte maturation, the long imperceptible cytoplasmic maturation and the short, dramatic and discernible nuclear maturation; both are crucial for the achievement of a fully mature and competent oocyte.

1.3.2 Cytoplasmic maturation

Cytoplasmic maturation involves several events necessary to prepare the oocyte for fertilization and embryo development. It includes accumulation of mRNAs and proteins, cytoplasmic reorganization and epigenetic modification.

During oocyte maturation huge amounts of RNA is stored in the cytoplasm. The total RNA content is 0.6 ng in a mouse GV oocyte (Sternlicht and Schultz, 1981) and 2.0 ng in a human GV oocyte (Neilson, et al., 2000). Approximately 8% of the total RNA is mRNA which is more than in a somatic cell (Gosden, 2002). A large amount of the mRNA are degraded during the maturation process and in mouse an MII oocyte contains 19% less mRNA than a GV oocyte (Bachvarova, et al., 1985). Similarly, in human oocytes the mRNA content in the MII oocyte is 40% less than in the GV oocyte (Dobson, et al., 2004). The rate of RNA accumulation varies throughout the oocyte maturation and a sophisticated system for control of synthesis and expression of the mRNA has evolved. Different polyadenylation patterns time the translation of different mRNAs depending on when during the maturation process they are needed (Bachvarova, 1992). Two methods of adenylation exist in the oocytes. MRNAs with a polyA tail around 150 A-residues are transcribed immediately while mRNAs with polyA tails shorter than 90 A-residues are masked and stored for later use. The masked mRNAs contain an adenylation control element (ACE) (Fox, et al., 1989; Verrotti, et al., 1996). This element regulates the expression of the stored mRNAs by inducing elongation of the polyA tail of mRNAs translated later in the maturing oocyte.

During oocyte growth an extensive production and reorganization of cytoplasmic organelles takes place. Replication of mitochondria with its DNA molecules must be done with great accuracy as the cytoplasmic inheritance to the zygote almost exclusively comes from the egg. During the growth phase the number of mitochondria increases dramatically and become more vacuolated which is a sign of lower activity (Wassarman and Josefowicz, 1978). The number of ribosomes also increases which is of importance for the accumulation of mRNAs and proteins. As the oocyte grows larger the organelles migrate towards the periphery of the oocyte. The golgi apparatus enlarges and becomes active in secreting proteins for the formation of zona pellucida (Mehlmann, et al., 1995) and the endoplasmatic reticulum form a more cortical distribution where it may facilitate the exocytosis of cortical granules by releasing calcium. When the oocyte approaches maturity, pores appear in the nuclear membrane, and chromatin accumulates around the nucleolus. At the end of the growth period the characteristic large pale nucleus of the oocyte, the germinal vesicle (GV) is formed (Picton, et al., 1998). During this growth period the oocyte builds up storage of glycogen granules, lipid droplets and proteins, which are likely to provide energy and substrate for the formation of new membranes after fertilization (Picton, et al., 1998).

As the oocyte matures, the zona pellucida (ZP) is formed. It is composed of three proteins ZP1, ZP2 and ZP3 which combine and form a 15 μm thick layer that surrounds the oocyte (Cohen, et al., 1992). The structure of the zona matrix is formed by long filaments of ZP2 and ZP3 dimeres cross linked non-covalently by ZP1 dimeres (Wassarman, et al.,

1996). ZP3 is the protein that acts as the primary sperm receptor by binding the sperm head and inducing the acrosome reaction (Wassarman, 1988). Then ZP2 facilitates the sperm penetration by acting as a secondary receptor. Proteins released from the cortical granules inhibit polyspermic penetration.

Epigenetic modification or imprinting of the genome is another component of cytoplasmic maturation that has to be completed before the oocyte is fully mature. This modification of the genome results in a parent specific expression of the imprinted gene. The most studied form of imprinting is DNA methylation and in the female mouse it has been suggested that the imprinting is acquired during the postnatal growth phase of oogenesis (Obata and Kono, 2002).

1.3.3 Nuclear maturation

Nuclear maturation is characterized by the germinal vesicle break down (GVBD) when the GV oocyte turns into the metaphase I (MI) stage oocyte. Thereafter follows the first meiotic cell division i.e. the polar body extrusion which results in the formation of a mature metaphase II (MII) oocyte.

In the GV oocyte the cytoplasm has reached maturity but the nucleus is still immature. Nuclear maturation of the mammalian oocytes is obtained with the completion of the first meiosis, which is mediated by the activation of the M-phase promoting factor or maturation-promoting factor (MPF). This is a heterodimer that consists of the catalytic subunit p34 ^{cdc2} and the regulatory subunit cyclinB (Dunphy, et al., 1988; Gautier and Maller, 1991; Gautier, et al., 1988; Pines and Hunter, 1989). This complex is thought to be involved in nuclear envelope break down, chromosome condensation, cytoskeletal rearrangements and arrest in transcriptional activity (Moreno and Nurse, 1990).

The MPF activity is low in the GV oocyte and increases during the first meiosis with a peak at metaphase I. Then it decreases during the first meiotic cell division and increases again and remains high during the MII arrest (Fulka, et al., 1992; Wu, et al., 1997).

Synthesis and relocation of cyclinB from the cytoplasm to the nucleus and dephosphorylation of the catalytic subunit p34 ^{cdc2} leads to progression of the oocyte from G2 to M-phase (Naito, et al., 1995). The meiotc arrest at the GV stage is maintained by high concentrations of cAMP that is produced by the GC's and transported to the oocyte via gap junctions. Cyclic AMP dependent protein kinase A (PKA) phosphorylates the p34 ^{cdc2} subunit of MPF and keeps it inactive (Rime, et al., 1992). The LH surge causes a decline in cAMP, first by disruption of the gap junctions between the oocyte and GC's (Heikinheimo and Gibbons, 1998; Racowsky, et al., 1989) and also by decreasing cAMP production in GC's via the *Insl3-Lgr8* system (Leidig insulin-like 3, leucin-rich repeat containing G protein coupled receptor 8) (Kawamura, et al., 2004). The decline in cAMP allows dephosphorylation and thereby activation of the MPF complex by cdc25-phosphatase (Gautier and Maller, 1991; Millar and Russell, 1992). This is likely mediated by polo-like kinase 1(plk1)(Anger, et al., 2004). The MPF can in turn activate cdc25-phosphatase and form an auto regulatory loop, this mechanism may be the reason for the

very rapid activation of the MPF at the initiation of oocyte maturation (Galaktionov and Beach, 1991; Hoffmann, et al., 1993). Another important regulator of the MPF is the cellular equivalent to the viral oncogene *mos* (*c-mos*) (Sagata, et al., 1989a; Sagata, et al., 1989b). *C-mos* kinase has been suggested to enhance MPF activity in several ways. Indirect via phosphorylation of MAP kinase (Nebreda and Hunt, 1993; Roy, et al., 1996) and direct by inhibiting proteolytic degradation of cyclinB between meiosis I and II (O'Keefe, et al., 1991) and thereby maintaining a high MPF activity during metaphase arrest (Kubiak, et al., 1993).

1.3.4 Cumulus-Oocyte Interactions

As soon as the follicle enters the growing pool the oocyte starts to grow and communicate with its surrounding GC's. The GC's communicate with each other and with the oocyte through gap junctions mainly composed by connexins (Cx)(Anderson and Albertini, 1976; Eppig, 1982). In the ovary Cx43 and Cx37 are the most important. Disruption of the Cx43 gene causes arrest of follicular growth at early stages and due to defective development of the GC's the oocyte fails to undergo meiotic maturation (Ackert, et al., 2001; Juneja, et al., 1999). Absence of Cx37 is also detrimental for fertility as formation of a mature Graafian follicle is prevented and premature luteinization occurs, oocyte growth is impaired and meiotic competence is not achieved (Simon, et al., 1997).

A bi-directional flow of metabolites, nutrients and paracrine factors between the oocyte and the GC's drives the maturation process towards a fertilizable oocyte. Candidates suggested to recruit the follicle into the growing pool includes k*it*-ligand (KL) that is produced by the GC's and binds to its receptor c-kit on the oocyte (Packer, et al., 1994). Another one is the retinoblastoma (Rb) protein that is involved in initial cell differentiation and its expression peaks in preantral human oocytes (Bukovsky, et al., 1995). Further follicular development is partly controlled by oocyte derived growth factors from the transforming growth factor β (TGFβ) family such as growth differentiation factor-9 (GDF-9), GDF-9B also known as bone morphogenetic factor 15 (BMP15), anti müllerian hormone (AMH) activin and inhibins. Other growth factors that are of importance for follicular development and oocyte maturation are growth hormone (GH), insulin growth factor-I (IGF-I) and members of the epidermal growth factor (EGF)-family. The gonadotrophins and hormones are, of course, also major players in this intrinsic cross-talk between follicular cells. Some of these growth and differentiation factors will be described in more detail below in this literature review.

1.4 Preimplantation embryo development and EGA

Accurate and complete oocyte maturation is crucial for fertilization and early embryo development. Maternal factors stored in the egg control the first cell divisions. The transition from maternal to embryonic control, referred to as the embryonic or zygotic gene activation (E/ZGA), is a gradual process and varies in different species. In the mouse the major EGA happens at the 2-cell stage, whereas in pig and cow, it occurs at

the 8-cell stage (Telford, et al., 1990). In order to time the onset of embryonic transcription in human pre-implantation embryos several different studies have been performed. Embryos of different stages have been treated with transcription inhibitors (Braude, et al., 1988), while another approach has been to incorporate radio labeled nucleotides or amino acids to newly synthesized RNAs or proteins (Braude, et al., 1988; Tesarik, et al., 1986). Moreover, quantitative measurements of specific mRNA molecules (Heikinheimo, et al., 1995) as well as analysis of the occurrence of parentally imprinted mRNAs were performed (Lighten, et al., 1997). These studies all indicate that the EGA in the human occurs between the 4- and the 8-cell stage.

There are several mechanisms that regulate delayed genome activation in pre-implantation embryos. First, the high concentrations of histones that bind to the DNA molecules and make them inaccessible for the transcription machinery have to be diluted (Prioleau, et al., 1994). Next, DNA replication is necessary to reprogram the DNA molecules that are arranged into a repressed stage (Nothias, et al., 1995). The disruption of nucleosomes is necessary for the maternally derived transcription machinery to gain access to the promoters and initiate embryonic transcription (Wolffe and Hayes, 1999). Furthermore, the chromatin structure influences the EGA, as genes of paternal origin have a higher transcription level than genes from the maternal pronuclei (Aoki, et al., 1997). One reason for this is the protamine-histone exchange that occurs in the paternal pronuclei, but not in the female: this exchange may provide a window for the transcription factors to access the promoter sequences (Nonchev and Tsanev, 1990). There is also a higher degree of acetylation of histones associated with the paternal chromatin compared with the histones of the maternal chromatin and this contributes to the difference in transcription activity between the parental genomes (Adenot, et al., 1997; Ura, et al., 1997; Wade, et al., 1997). Demethylation also occurs faster in the paternal than the maternal pronuclei, which may be facilitated by the protamine-histone exchange and therefore contribute to the higher paternal gene expression (Mayer, et al., 2000; Santos, et al., 2002). Another factor that influences EGA is the length of the cell cycle; short cell cycles are suppressive for transcription (Memili and First, 2000) and at the onset of EGA the cell cycles become longer.

At the onset of EGA the maternally derived mRNAs gradually disappear, this process is initiated by shortening of the poly-A tail (Decker and Parker, 1994). This is a gradual process and in human embryos the maternally inherited *c-mos* mRNA decreases following fertilization and can not be detected after the 6-cell stage (Heikinheimo, et al., 1995).

1.5 Gonadotophins and other hormones

1.5.1 FSH and LH

The later part of follicular development depends on the gonadotrophins: follicle stimulating hormone (FSH) and lutinizing hormone (LH). They are both glycoproteins produced by the anterior pituitary and released after stimulation by gonadotrophin

releasing hormone (GnRH) produced by the hypothalamus. The first stages of follicle development are FSH independent but from the late secondary to early antral stage further development depends on the gonadotrophins. The first evidence of FSH binding to its receptor (FSHR) in rat GC's was shown in 1974 (Zeleznik, et al., 1974) and receptor expression in GC's was later confirmed when cDNA clones were isolated (Camp, et al., 1991; Sprengel, et al., 1990). In human ovaries, the FSHR is expressed in 33% of the primary and two-layer secondary follicles and in all multi layer follicles (Oktay, et al., 1997a). In the ovaries of women with a mutation in the FSHR, follicles beyond the primary stage are rarely seen (Aittomaki, et al., 1996). Furthermore, female mice deficient in FSH are infertile as the follicle development is blocked at the secondary stage (Kumar, et al., 1997).

Binding sites for LH was revealed on rat theca cells using labeled hCG as a substitute (as it binds to the same receptor as LH) (Zeleznik, et al., 1974).They also showed that the binding increased by FSH. Following the cloning of the LHR (McFarland, et al., 1989) expression was confirmed in ovarian cells by Segaloff et al. (Segaloff, et al., 1990). In immature follicles the LHR location is restricted to the thecal cells, but in antral follicles it is expressed also in GC's (Camp, et al., 1991).

From the preantral stage, the follicle starts to depend on FSH stimulation from the pituitary, the estimated time for a follicle to grow from the preantral to ovulatory stage is approximately 85 days (Gougeon, 1986). During this growth phase the follicles depend on the gonadotrophin fluctuations of the menstrual cycle. LH and FSH are both essential for the production of steroid hormones according to the generally accepted two-cell two-gonadotrophin system (Hillier, et al., 1994). According to this well accepted theory LH binds to the LHR on the theca interna cells and activates cAMP that in turn induces the conversion of cholesterol to androgens. The androgens then cross the basement membrane and enter the GC's. In the GC's FSH binds to the FSH receptors and activates cAMP to induce p450 aromatase that converts androgens to estrogens. The estrogen is then released into the follicular fluid and the circulation to participate in the further regulation of follicular maturation.

1.5.2 Estrogen

When the gonadotrophins act on the theca and granulosa cells the production of estrogen, mainly estradiol-17β (E2) (Erickson and Shimasaki, 2001), starts to increase and reaches its maximal level in the preovulatory follicle. One of the main actions of estrogen is, of course, the negative feed-back mechanism on the hypothalamus-pituitary-ovarian (HPO) axis, by down regulating the release of GnRH from the hypothalamus the plasma gonadotrophin levels are reduced.

Estrogen performs several actions directly in the ovary. In combination with FSH and IGF-1 it increases growth and differentiation of GC's (Adashi, et al., 1985; Zhou, et al., 1997a). Furthermore, estrogen in combination with FSH induces LHR on GC's (Wang and Greenwald, 1993), decrease apoptosis of GC's (Kaipia and Hsueh, 1997) and inhibit androgen synthesis in the theca cells (Leung and Armstrong, 1979).

Estrogen signals through two different receptors (ERs), ERα and ERβ (Jensen and DeSombre, 1973; Kuiper, et al., 1996). They are both expressed in the ovary with a predominance of ERβ in the GC's. Both receptors have been knocked out in mice, the ERα knock-out ERKO are acyclic, infertile and have hyperemic ovaries devoid of corpora lutea (Couse and Korach, 1999)). The ERβ knock-out mice (BERKO) have small ovaries, partially arrested follicular development, increased numbers of primordial follicles but significantly fewer numbers of primary and large antral follicles and corpora lutea. They have compromised fertility with reduced litter sizes (Krege, et al., 1998). The ERKO mice have a block in folliculogenesis at the early antral stage, before the increased GC proliferation starts (Hirshfield, 1991), while the follicles in the BERKO mice develop to the antral stage and can ovulate (Krege, et al., 1998). It is therefore hypothesized that the proliferative actions of E2 require ERα, whereas the differentiation and the anti proliferative effect required for reaching the antral stage is mediated by ERβ (Britt and Findlay, 2002).

1.5.3 Androgens

Production of androstenedione and testosterone in the theca interna cells is induced by LH. They signal through the androgen receptor (AR) localized on GC's, theca cells and stromal cells of the human ovary (Horie, et al., 1992). AR expression fluctuates during the different stages of the folliculogenesis and it is presumed to be under the control of oocyte derived factors (Drummond, 2006; Tetsuka, et al., 1995). Androgens have been shown to promote follicular growth in the earlier stages of folliculogenesis in mouse *in vitro* culture systems (Murray, et al., 1998; Wang, et al., 2001). Furthermore, activated ARs stimulate proliferation of porcine GC's by enhancing the growth-promoting action of GDF-9 (Hickey, et al., 2005). In contrast to these growth promoting actions it has also been shown that androgens can cause follicular atresia in immature rats primed with pregnant mares' serum (PMSG) (Conway, et al., 1990) and it has also been shown that apoptosis of rat GC's was induced by androgens (Billig, et al., 1993).

1.5.4 Progesterone

Progesterone is important for ovulation, implantation and maintenance of pregnancy. Production of progesterone takes place in the GC's and starts just prior to the gonadotrophin surge and increases in the corpus luteum, it is suggested to be crucial for ovulation (Zalanyi, 2001). This is supported by the fact that progesterone increases the production of proteolytic enzymes critical for ovulation (Iwamasa, et al., 1992). There are two different isoforms of the progesterone receptor, PR-A and PR-B. PR-A knock-out mice fail to ovulate and are infertile, while PR-B knock-outs are ovulatory and produce viable offspring (Lydon, et al., 1995; Mulac-Jericevic, et al., 2000). The receptors start to be expressed in large follicles of most species and expression is increased by the LH surge (Drummond, 2006). Progesterone enhances the effect of FSH on GC's by increasing cAMP (Goff, et al., 1979) and inhibits FSH induced estradiol production (Fortune and Vincent, 1983; Schreiber, et al., 1981). Moreover, it inhibits the transition from primordial to primary follicles in newborn rats (Kezele and Skinner, 2003) and

inhibits insulin dependent granulosa cell mitosis (Luciano and Peluso, 1995; Peluso, et al., 1995).

1.6 Growth Factors

1.6.1 Transforming growth factor beta (TGFβ)-superfamily

The TGFβ-superfamily is a large group of proteins that share common structural motifs (Chang, et al., 2002). Many of these proteins are produced in the ovary and are important as local regulators of follicular development and oocyte maturation. The members of the TGFβ-superfamily are all synthesized as prepropeptides with one signalpeptide, a large proregion and a smaller biologically active mature region (Chang, et al., 2002; Massague, 1990). To create the active protein the proregion is cleaved from the mature region by, so far, unknown proteases. The mature region contains a region of seven or nine cysteins of which six form a cystein knot characteristic for this family of proteins (Chang, et al., 2002; Vitt, et al., 2001). Another characteristic that is important for the activity of these proteins is their ability to form homo or heterodimers. These dimers can be covalently linked through a conserved cystein residue (Chang, et al., 2002), but also, in some cases such as GDF-9 and GDF-9B/BMP15, linked by non-covalent interactions (Liao, et al., 2003; Liao, et al., 2004).

The TGFβ-superfamily signals through membrane bound receptors that form hetero tetrameric complexes that contain two types of serine-threonine-kinases (Chang, et al., 2002; Massague and Wotton, 2000; Miyazawa, et al., 2002). In mammals currently seven type I receptors activin like kinases (ALK1-7) and five typeII receptors (Act RII, ActRIIB, BMPRII, TGFβRII and AMHRII) are known. (de Caestecker, 2004; ten Dijke and Hill, 2004) When a ligand binds, the kinase of the type II receptor phosphorylates and thereby activates the kinase of the type I receptor (de Caestecker, 2004; Shi and Massague, 2003; ten Dijke, et al., 2003). The activated type I receptor will then activate the intracellular signalling molecules called Smads. There are three families of Smads; receptor regulated Smads (Smad 1,2,3,5,8), collaborating Smads (Smad 4) and inhibitory Smads (Smad 6,7) (Shi and Massague, 2003). There are basically two main pathways, the TGFβ/activin pathway where the ligand has a higher affinity for the type II receptors and the BMP pathway with ligands that have a higher affinity for the type I receptors (de Caestecker, 2004; Shi and Massague, 2003; ten Dijke, et al., 2003). After dimerisation the TGFβ/activin pathway activates Smad 2/3 and the BMP pathway activates Smad 1/5/8 both pathways then lead to association of the activated receptor regulated Smad and the coSmad Smad 4 (Miyazawa, et al., 2002; Zhang and Derynck, 1999). This complex then translocates into the nucleus and activates transcription of specific genes (Zhang and Derynck, 1999). The inhibitory Smads 6 and 7 can inhibit the Smad 1/5/8 pathway but only Smad 6 can inhibit Smad 2 and 3 from being activated (Massague, 1998; Miyazawa, et al., 2002).

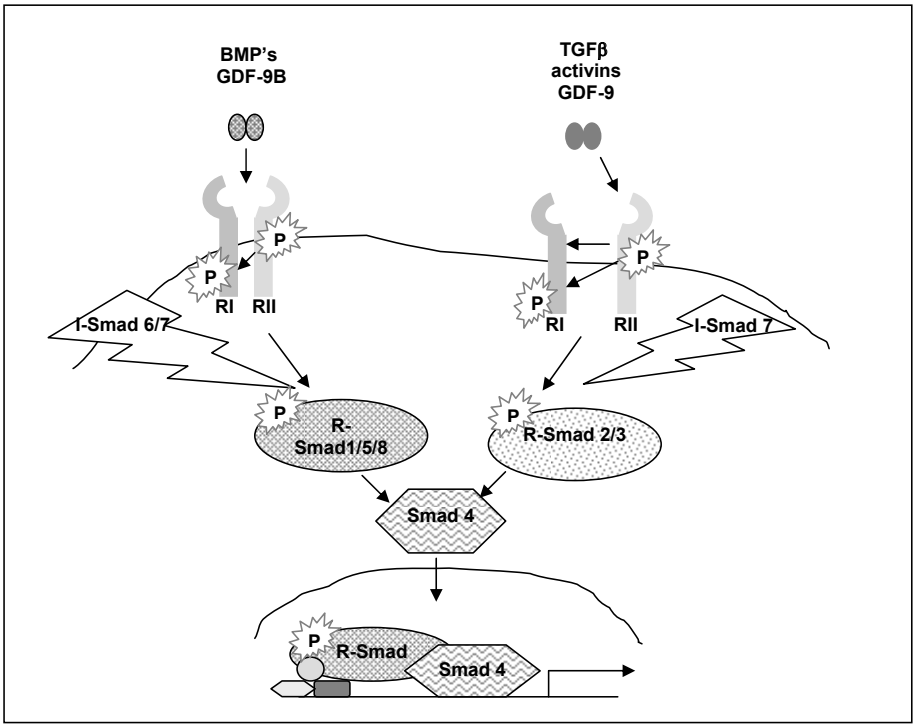

Figure 2. The TGFβ signalling pathways

1.6.1.1 Growth Differentiation Factor-9 (GDF-9) and GDF-9B/BMP15

Growth differentiation factor 9 (GDF-9) was discovered in 1993(McPherron and Lee, 1993). The expression was first localized to the oocyte (McGrath, et al., 1995), but later also to testis, hypothalamus, pituitary, uterus and bone marrow (Fitzpatrick, et al., 1998). Growth differentiation factor 9B (GDF-9B), also known as BMP15 was discovered independently by two groups in 1998 (Aaltonen, et al., 1999; Dube, et al., 1998; Laitinen, et al., 1998). GDF-9B is expressed in oocytes from the primary stage and progressively throughout folliculogenesis and is a potent regulator of follicle growth. Both these oocyte secreted factors are necessary for female fertility in some species. Homozygous mutations of GDF-9 in mouse and sheep (Dong, et al., 1996; Hanrahan, et al., 2004) and of GDF-9B in sheep (Braw-Tal, et al., 1993; Galloway, et al., 2000) result in infertility due to a block in follicular development at the primary stage, however mice that completely lack GDF-9B expression are fertile (Yan, et al., 2001). Heterozygous loss of these two genes has a different effect in different species. In sheep both GDF-9 and GDF-9B heterozygosity results in increased fertility (Hanrahan, et al., 2004; Montgomery, et al., 2001), but in mice it has no effect on the fertility (Dong, et al., 1996; Yan, et al., 2001). More data is emerging that indicate an important role for these factors also in human female fertility. A decrease in GDF-9 expression was observed in the oocytes

from women suffering from polycystic ovarian syndrome (PCOS) (Teixeira Filho, et al., 2002). Furthermore, a mutation in the proregion of GDF-9B causes hypergonadotrophic ovarian failure (Di Pasquale, et al., 2004), similar effects have also been reported due to other rare mutations in the GDF-9 and GDF-9B genes (Dixit, et al., 2006; Laissue, et al., 2006).

The oocyte secreted GDF-9 signals to its surrounding GC's by an unusual receptor combination, it binds to the BMPRII (Vitt, et al., 2002) then it uses the TGFβ type I receptor ALK5 (Kaivo-Oja, et al., 2005; Mazerbourg, et al., 2004) which leads to activation of the Smad 2/3 pathway (Kaivo-Oja, et al., 2003; Kaivo-Oja, et al., 2005; Mazerbourg, et al., 2004; Roh, et al., 2003). GDF-9B uses the more conventional pathway by binding the BMPRII and ALK6 receptors and thereby activating the Smad 2/5/8 intracellular pathway (Moore, et al., 2003; Shimasaki, et al., 2004). By activating the two different Smad signaling pathways the simultaneous secretion of these two proteins is likely to result in a synergistic effect (Gilchrist, et al., 2008).

New knowledge is emerging about the main effects of GDF-9 and GDF-9B on its surrounding GC's as more research tools such as recombinant proteins and antagonists become available. Both proteins have a mitogenic effect on both theca cells and GC´s (Di Pasquale, et al., 2004; Hayashi, et al., 1999; Hreinsson, et al., 2002; McNatty, et al., 2005a; McNatty, et al., 2005b; Nilsson and Skinner, 2002; Otsuka, et al., 2000; Vitt, et al., 2000a; Vitt, et al., 2000b). Another common function of GDF-9 and GDF-9B is the ability to inhibit gonadotrophin stimulated progesterone production from granulosa cells (Otsuka, et al., 2000; Vitt, et al., 2000a; Yamamoto, et al., 2002). Furthermore, they decrease the FSH induced p450 scc and StAR expression (Otsuka, et al., 2000; Yamamoto, et al., 2002). In coherence with this GDF-9 has been shown to inhibit hCG binding (Vitt, et al., 2000a) and expression of the LHR on GC's (Elvin, et al., 1999). In addition GDF-9B suppresses FSHR mRNA and thereby also the expression of LHR mRNA (Otsuka, et al., 2001b). It also suppresses FSH stimulated progesterone production without affecting the estradiol secretion (Otsuka, et al., 2000). However, GDF-9 has a suppressive effect on p450 aromatase expression in human GC's indicating a negative effect on estradiol secretion (Yamamoto, et al., 2002).

The oocyte secreted factors GDF-9 and GDF-9B influence the preantral to the antral transition when the GC's start to differentiate. The cells surrounding the oocyte turn into cumulus cells CC's and in the periphery of the follicle mural granulosa cells MGC's are formed. This differentiation is obtained by the fact that the oocyte secreted factors form a gradient within the follicle. The identity of the factors inducing the cumulus expansion is not completely clear but the combination of GDF-9 and GDF-9B seems to be involved (Dragovic, et al., 2005; Gui and Joyce, 2005; Yoshino, et al., 2006). The cumulus expansion is important for the final maturation of the oocyte and requires both oocyte and somatic signaling. This process is characterized by increased expression of genes controlling the synthesis of hyaluronan and prostaglandins (Richards, 2005).
Recently it has been shown in the mouse, that cumulus expansion requires simultaneous activation of the Smad 2/3 pathway (Dragovic, et al., 2007) and activation of the FSH /EGF pathway via mitogen activated protein kinase MAPK (Diaz, et al., 2006; Su, et al.,

2003). Another important task for factors secreted from the oocyte is to prevent FSH induced luteinization of the cumulus mass (Diaz, et al., 2007).

Factors secreted from the oocyte are also thought to have an anti-apoptotic effect on the cumulus cells. These factors create a gradient in the surrounding cumulus mass and the incidence of apoptotic cells increase at a larger distance from the oocyte (Hussein, et al., 2005).

Finally, recent studies have shown that GDF-9 and GDF-9B both have a beneficial effect on oocyte quality in *in vitro* matured oocytes. In bovine both these factors resulted in an increased blastocyst formation (Hussein, et al., 2006), and in mouse addition of GDF-9 to the culture media resulted in almost double rate of fetal survival (Yeo, et al., 2008).

1.6.1.2 Inhibins and Activins

Inhibins and activins are two other members of the TGFβ family which takes part in the follicular development and oocyte maturation. Inhibins are heterodimers that contains one α-subunit and one β-subunit and activins are homo or hetero dimeres comprised of the two different inhibin-β subunits (βA and βB) that are covalently linked (de Kretser, et al., 2002). In the ovary inhibins and activins are produced by the GC's. Inhibin originally got its name because of its negative effect on FSH secretion. Inhibin B secretion is high during early follicular phase and drops after the LH surge (Groome, et al., 1996). Inhibin can be used as a marker for the ovarian reserve as it is hypothesised that the reproductive aging results in lower amounts of circulating inhibin B and thereby in increased FSH levels (Welt, et al., 1999).

Activin A is the most common isoform of the activins and is important in folliculogenesis (Findlay, 1993). Production of activin A takes place in the granulosa cells. Both the two receptor types ActRI and ActRII and the Smads necessary for signaling are present in granulosa cells and oocytes in rodents and humans (Sidis, et al., 1998). This indicates paracrine signaling between granulosa cells and the oocyte. Activin induce proliferation in rat granulosa cells (Li, et al., 1995; Miro and Hillier, 1996), and in human lutein granulosa cells (Rabinovici, et al., 1990)}. It also promotes FSH receptor expression in rat granulosa cells (Hasegawa, et al., 1988; Xiao, et al., 1992), which may explain the transition of the follicle from the gonadotrophin-independent preantral stage to the gonadotrophin-dependent early antral stage. Activin A has also been suggested to have an anti-luteinizing role by increasing aromatase activity and oestradiol levels while suppressing progesterone synthesis (Miro and Hillier, 1992; Shukovski, et al., 1993; Smitz, et al., 1998). Furthermore, it has been observed *in vitro*, that activin A stimulates the meiotic maturation of the oocytes in human, rat and rhesus monkey (Alak, et al., 1998; Alak, et al., 1996; Itoh, et al., 1990; Sadatsuki, et al., 1993).

Activin is regulated by follistatin a single chain polypeptide that is unrelated to the TGFβ–superfamily. Follistatin is produced by the GC's and binds to the β subunit and thereby irreversibly inhibits its activity (Shintani, et al., 1997).

1.6.1.3 Anti-Müllerian Hormone (AMH)

Anti-Müllerian hormone (AMH) originally named Müllerian inhibitory substance (MIS) because of its role in male fetal sex differentiation (Josso, et al., 1993), is in contrast to the other members of the TGFβ-superfamily suggested to have an inhibitory role in follicular recruitment (Durlinger, et al., 1999). It is produced by the GC's from after birth and expression is first observed at the primary stage of follicle development and expression persists until the antral stage and disappears in the dominant follicle. The same expression pattern has been observed in human, mice and rat ovaries (Baarends, et al., 1995; Durlinger, et al., 2002; Weenen, et al., 2004). This expression pattern indicates that AMH produced by the growing pool of follicles acts as a negative regulator of follicle recruitment in a paracrine way. In female AMH knock-out mice a decrease of primordial follicles and an increase of growing follicles was observed at 4 months of age. At 13 months of age almost no primordial follicles were present in the ovary (Durlinger, et al., 1999).

Contradicting results have been reported in ovarian tissue culture with AMH added to the culture medium. In mice and human an inhibitory effect on early follicular growth was observed (Carlsson, et al., 2006b; Durlinger, et al., 2001). In mice AMH also attenuated the FSH induced growth of pre-antral follicles indicating a role in the cyclic recruitment (Durlinger, et al., 2001). However, another study using human ovarian tissue showed an increase of primary and secondary follicles with recombinant human AMH stimulation (Schmidt et al 04). Also, in the rat, AMH has a stimulatory effect of the FSH-induced pre-antral growth (McGee, et al., 2001).

1.6.1.4 Bone morphogenetic protein (BMP) 2, 4, 6 and 7

Several other BMPs, BMP 2, 4, 6 and 7 are also localised in the ovary but have not been as extensively studied. Most studies so far, have been carried out in rodents.

BMP 2 expression has been observed in theca cells in antral bovine follicles, it is also occasionally expressed in the bovine oocytes (Fatehi, et al., 2005).

BMP 4 and 7 are expressed in pre theca cells and later in theca cells and promote primordial to primary transition in the rat (Lee, et al., 2001). They also increase the sensitivity of GC's to FSH stimulation and act anti-luteinizing by inhibiting progesterone synthesis (Shimasaki, et al., 1999). BMP 4 also acts as a survival factor by preventing apoptosis in the rat (Nilsson and Skinner, 2003).

Bone morhogenetic protein 6 is expressed in the oocyte and GC's of the rat, BMP 6 decreases FSH action in rat GC's by down regulating adenylate cyclase activity (Otsuka, et al., 2001a). Expression of BMP 6 drop in dominant follicles, this drop may be necessary to allow the continued FSH stimulated follicle development (Erickson and Shimasaki, 2003). The BMP 6 knock-out mice have no effect on fertility (Solloway, et al., 1998).

In sheep BMP 6 are detected in the oocyte of all follicular stages, BMP 2, 4 and 7 are only weakly expressed in GC's. All these factors have an inhibitory effect on

progesterone synthesis however, the proliferation and survival rate of sheep GC's are not affected (Juengel, et al., 2006).

1.6.2 Epidermal Growth Factor (EGF)-family

Epidermal growth factor (EGF) is one member of a large family of closely related proteins including HB-EGF, amphiregulin (AREG), epiregulin (EREG), betacellulin (BTC), epigen and neuregulins (Yarden and Sliwkowski, 2001).

All the EGF-like growth factors bind with different specificity and activate four different receptors. The EGFR subfamily belongs to the receptor tyrosine kinases and includes: EGFR(ErbB1), (ErbB2/neu/HER2), ErbB3(HER3) and ErbB4(HER4) (Holbro and Hynes, 2004; Yarden and Sliwkowski, 2001). EGF-receptors are present in pre-ovulatory follicles in hamster, rat, porcine and human ovaries (Assarsson, et al., 1995; Garnett, et al., 2002; Tamura, et al., 1995). The EGF factors that have been shown to act in the ovary and influence oocyte maturation are AREG, EREG and BTC. In mice, HCG injection causes a rapid increase of mRNA coding for these factors (Park, et al., 2004). HCG/LH induced expression of EGF-like factors have also been reported in human granulosa cell cultures (Freimann, et al., 2004).

EGF induces cumulus expansion in COC cultures as efficiently as FSH. (Tirone, et al., 1997). AREG, EREG and BTC induces oocyte maturation as efficiently as LH, however maturation induced by AREG and EREG was quicker than LH induced maturation, suggesting that they act downstream of LH. (Downs, et al., 1988). Cumulus cells are required for AREG and EREG actions (Park, et al., 2004). AREG stimulates expression of genes involved in cumulus expansion (Ptgs2, Has2 Tnfaip6) and also genes regulating steroidogenesis and steroid hormone action (StAR, Cyp11a1 and Pgr) (Shimada, et al., 2006).

1.6.3 Growth Hormone (GH)

Growth hormone is mainly synthesized in the pituitary but the presence of GH mRNA in GC's and oocytes in the bovine ovary suggests that it can also be synthesized locally and act in a paracrine and autocrine way (Izadyar, et al., 1999). Growth hormone receptors (GHR) have been observed in the rat ovary (Lobie, et al., 1990; Tiong and Herington, 1991), in bovine GC's, cumulus cells and oocytes (Izadyar, et al., 1997) and in the GC's of human antral follicles and corpus luteum (Sharara and Nieman, 1994), indicating a direct GH effect on the ovary.

In the GH-R/GHBP KO mouse, sexual maturation is delayed and the fertility is impaired with reduced litter size and higher mortality of the offspring (Zhou, et al., 1997b). These effects are likely to be a defect of the ovary rather than of the pituitary. Moreover, the IGF-1 expression in the ovaries of the KO mouse was not impaired, indicating that the IGF-1 expression in the ovary is GH-independent (Bachelot, et al., 2002).

GH is likely to stimulate growth and prevent atresia in small follicles. GH administration in vivo increases the number of small follicles in horse (Cochran, et al., 1999), and in GH-treated heifers the number of antral follicles was increased (Gong, et al., 1991; Gong, et al., 1993).

In vitro studies in mice show a stimulatory effect of GH on preantral follicles and follicular cell proliferation that is independent of IGF-1 (Kobayashi, et al., 2000; Liu, et al., 1998). Moreover, over-expression of bovine GH in a transgenic mouse model decreases apoptosis in mouse follicles (Danilovich, et al., 2000). Further, *in vitro* studies show that GH has a direct inhibitory effect on apoptosis in early follicles in the bovine (Sirotkin and Makarevich, 1999) and in the rat (Chun and Hsueh, 1998; Eisenhauer, et al., 1995). The increased follicular survival and increased proliferation caused by GH may be caused by a potentiation of LH, as GH deficiency is associated with low LH responsiveness in rats (Advis, et al., 1981).

GH may also be involved in the selection of the dominant follicle as binding sites for GH are missing in atretic follicles in pigs (Quesnel, 1999) also GHR-deficient cows have a disturbed development of a dominant follicle (Chase, et al., 1998).

The effects of GH on the GC's are partly mediated by alterations of the steroid synthesis. In human GH stimulates differentiation and proliferation in lutinized GC's (Ovesen, et al., 1994), increases estrogen production (Mason, et al., 1990) and modulates the expression of steroid hormones by increased expression of enzymes involved in steroid synthesis (Doldi, et al., 1996). It has also been shown that GH acts in synergy with the gonadotrophins to increase steroid production in the human (Carlsson, et al., 1992; Lanzone, et al., 1992).

1.6.4 Insulin and Insulin-like growth factor I and II

Insulin and Insulin like growth factor I and II are involved in uptake of glucose and amino acids and are also thought to be important regulators of follicular development.
The IGF-I knock-out mice are infertile due to a block in follicular development at the secondary stage and ovulation can not be induced with the addition of exogenous gonadotrophins (Baker, et al., 1996).

In the human ovary the IGF-II is strongly expressed on granulosa and theca cells of small antral follicles and weaker expression was observed for IGF-I, expression the IGF receptors type one and two was observed in all ovarian compartments (Voutilainen, et al., 1996). The insulin receptor is expressed on mural granulosa cells and insulin can bind both to its own receptor and to the IGF-I receptor (Giudice, 1995). In human ovarian cultures insulin, IGF-I and IGF-II increase viability during early folliculogenesis and may also promote granulosa cell mitogenesis in primary and secondary follicles (Louhio, et al., 2000). In later follicular stages IGF-I has a positive effect on FSH induced steroid production, increases LH receptors and stimulates cell growth (Erickson, et al., 1989; Giudice, 1992).

However, the effects differ between species, in bovine insulin has a positive effect on activation and survival on early folliculogenesis, while IGF-I either does nothing or abrogates the insulin effect (Fortune, 2003). Furthermore, in the rat insulin promotes follicle activation, an effect that is enhanced by leukemia inhibitory factor (LIF) and kit ligand (Kezele, et al., 2002).

1.6.5 Kit ligand/ Stem Cell Factor (SCF)

Kit ligand (KL) also named stem cell factor (SCF) or steel factor and its receptor c-kit are expressed in the follicular cells. In most species the receptor is expressed by primordial germ cells (PGCs), theca cells and oocytes while KL is expressed by the GC's (Hutt, et al., 2006).

In the human c-kit is weakly expressed in the oocytes of primordial follicles in fetal ovaries (Hoyer, et al., 2005; Robinson, et al., 2001; Stoop, et al., 2005). Some evidence also implies c-kit expression in newly formed primordial follicles (Hoyer, et al., 2005). Expression of c-kit has also been observed in primary and secondary follicles (Carlsson, et al., 2006a). Increased expression levels are then found in preantral follicles and drop again when the antrum is formed (Hoyer, et al., 2005; Robinson, et al., 2001; Stoop, et al., 2005). KL is expressed in GC's in primary follicles in adult human ovaries (Carlsson, et al., 2006a). In fetal tissue GC's of primordial, preantral and early antral follicles expresses KL (Hoyer, et al., 2005).

Many studies carried out in rodents indicate that these factors are important regulators of primordial to primary follicular growth. Blocking the c-kit receptor in rat causes impaired follicular recruitment (Nilsson and Skinner, 2004; Parrott and Skinner, 1999). Similar studies in mice indicate a role for KL/ c-kit in follicular recruitment, primary follicle growth decreased fluid formation in antral follicles and final follicle maturation (Yoshida, et al., 1997).

Expression of the receptor on theca cells suggests a role for KL and c-kit in recruitment of theca from the stromal cells and regulation of theca cell proliferation (Faddy, 2000; Kang, et al., 2003; Parrott and Skinner, 1999).

Studies using a KL neutralizing antibody propose a role in GC controlled oocyte development in the mouse (Packer, et al., 1994). It has also been shown that KL has an anti apoptotic effect on both PGCs (Sakata, et al., 2003) and oocytes of primordial and primary follicles in the mouse (Jin, et al., 2005) and human (Carlsson, et al., 2006a).

1.6.6 The phosphatidyl inositol 3 kinase (PI3K) pathway

Recent data from Reddy et al suggests that the phosphatidyl inositol 3 kinase (PI3K) pathway plays an important role in activation of primordial follicles. This pathway is suggested to be a part of a network in the oocyte that works downstream of the KL/c-kit interaction (Liu, et al., 2006; Reddy, et al., 2005). The PI3K phosphorylates and thereby activates proteins involved in proliferation survival and metabolism. This pathway is

negatively regulated by a lipidphosphatase named phosphatase and tensin homolog deleted on chromosome 10 (PTEN) (Cantley, 2002). When this negative regulator was conditionally knocked out in mouse ovaries all the resting primordial follicles became activated. This off course resulted in a depletion of follicles at an early age and caused premature ovarian failure. Thus the PI3K pathway seems to be important for the activation of primordial follicles into the growing pool (Reddy, et al., 2008).

1.7 Fertility preservation and Fertility treatments

1.7.1 Premature ovarian failure

Many infertile couples can be helped with the most conventional assisted reproductive techniques such as IVF and ICSI, but there are still challenges to improve and develop new methods so that even more patient groups can be treated. One such group is women suffering from premature ovarian failure (POF). This condition affects approximately 1% of women and is characterized by menopause before the age of 40 years (Coulam, et al., 1986). The majority of the POF cases are idiopathic but there are also a number of known reasons for this condition. First, chromosomal abnormalities like Turners syndrome, which is characterized by partial or total loss of one of the X chromosomes or genetic defects, for example the mutation of the FSH gene (Aittomaki, et al., 1996). Secondly, autoimmune disorders can be involved in the development of POF (Forges, et al., 2004). Third, it can be induced by cancer treatments such as radiotherapy or chemotherapy (Meirow and Nugent, 2001; Wallace, et al., 2003) and finally, environmental factors such as viruses and toxins can induce POF (Goswami and Conway, 2007).

1.7.2 Fertility preservation

For women who are at risk of losing their fertility different treatments are suitable depending on their age and their situation of life at the time point when treatment is needed. If the woman has a partner, one option is to cryopreserve embryos: this method is to date the most efficient, resulting in an acceptable pregnancy rate (Revel and Schenker, 2004). However, the time needed to perform IVF may cause an unacceptable delay of the cancer treatment (Amorim, et al., 2003). Another option is to cryopreseve oocytes however, oocytes are more susceptible to freeze-thawing damage than embryos, though the results are improving also with this technique (Leibo, 2008). To gain time and avoid the hyper stimulation it is also possible to harvest immature oocytes from a natural cycle and mature them *in vitro* (IVM) before freezing.

There is also the possibility to cryopreserve the ovarian tissue. In the 1990s survival of follicles in ovarian tissue after freezing and thawing, using protocols with dimethylsulphoxide (DMSO), propanediol (PrOH) or ethylene glycol (EG) as the cryoprotectant agent was presented (Hovatta, et al., 1996; Newton, et al., 1996). Cryoprotective agents prevent the formation of ice crystals in the cells. The most

commonly used protocol is slow controlled-rate freezing and rapid thawing where 70%-90% of the follicles survive this procedure (Hovatta, et al., 1996; Newton, et al., 1996). Prior to freezing the tissue is cut into small fragments in order to improve the permeation of the cryoprotectant into the tissue. The quiescent primordial follicles are, for several reasons, the most likely to survive. The cryoprotectant equilibrate easier in smaller cells, their metabolic rate is low, they lack a zona pellucida and the oocytes of the primordial follicle is arrested in the prophase of the first meiotic cell division and lack metaphase spindle which makes them less vulnerable to cryodamage (Shaw, et al., 2000). Another advantage of this method is that the tissue, hopefully, contains quite a good number of oocytes: a $4mm^3$ piece from a 30 year old patient contained approximately 120 small follicles (Gosden, et al., 2002).

Vitrification is a more recently developed freezing technique that uses high concentrations of cryoprotections and very short freezing time. This transforms the aqueous parts of the cells to a solid amorphous vitreous stage and prevents formation of ice crystals. This technique is applicable and promising for both oocytes and ovarian tissue. In human good morphology of the follicles after vitrification of ovarian tissue has been presented, however slow freezing gave better results. Further research and evaluation is needed for this technique (Hovatta, 2003), Keros et al. 2008 submitted.

1.7.3 Transplantation

Retransplantation of ovarian tissue to the patient is applicable in cases where there is no risk of re-introducing the disease. This is beneficial for the patient as it can re-establish the hormonal balance and thereby delay menopause and also hopefully restore fertility. Re-transplantation can be done orthotopic (to its original site) or heterotopic (to a different site) the tissue can also be transplanted to another species (xenotransplantation). Hormone production and follicular growth was first observed after subcutaneous re-transplantation (Oktay and Karlikaya, 2000) and later also after orthotopic re-transplantation (Radford, et al., 2001).

A few successful cases have been reported in human with live births after re-transplantation of frozen-thawed ovarian tissue. The first one was a spontaneous pregnancy obtained after transplantation of the tissue under the pelvic peritoneum (Donnez, et al., 2004). Later the same year a woman became pregnant after IVF of oocytes from frozen-thawed tissue re-transplanted under the tunica albuginea of the ovary (Meirow, et al., 2005).

For some hematological and ovarian cancers the risk of re-introducing the disease is too high, which makes re-transplantation impossible (Gosden, et al., 1997; Shaw, et al., 1996). For these patients, the remaining possibility to obtain mature fertilizable oocytes is to mature the follicles *in vitro* in tissue cultures followed by *in vitro* maturation of the oocytes.

1.7.4 Tissue Culture

As mentioned earlier there are patients who can not have their cryopreserved tissue re-transplanted. For this patient group culture of ovarian tissue to mature the follicles *in vitro,* is a promising technique under development. So far, the only live birth from *in vitro* cultured tissue was obtained with the birth of the mouse Eggbert (Eppig and O'Brien, 1996). Later the same group improved their protocol which resulted in a higher success rate (O'Brien, et al., 2003). The protocol included several steps, first ovarian tissue from newborn mice were cultured for 8 days. The next step was 14 day culture of granulosa cell and oocyte complexes that were enzymatically isolated from secondary follicles and finally 15-17 h of *in vitro* maturation of oocytes before IVF and embryo transfer.

There have been many attempts to isolate follicles from human ovarian tissue but the results have been very poor as human ovarian stroma, in which the follicles are embedded is very dense and the follicles easily get damaged during the isolation procedure. Although it is possible to isolate small primordial and primary follicles (Oktay, et al., 1997b) the isolated follicles do not survive more than 24 hours in culture (Abir, et al., 2001; Abir, et al., 1999) therefore, culture of isolated follicles is not applicable for fertility preservation. A more successful approach has been to culture pieces of tissue to keep the structure of the follicles intact and also maintain the support from the different cell types present in the ovarian tissue (Hovatta, et al., 1997; Hovatta, et al., 1999). When the tissue is cut into small pieces and put in culture initiation of growth starts within one week (Hovatta, et al., 1997; Hovatta, et al., 1999; Wright, et al., 1999), this is thought to be due to the lack of inhibitory factors present *in vivo*.

In order to improve the maturation process many different factors have been tested in ovarian tissue culture by our team and others. To comprehend every specific factor's particular effect, only one component at a time can be tested, therefore it takes time to get the complete picture of this complex system. When the different factors that promote follicular growth from primordial to the antral stage have been identified these can be added to the media in combination or possibly to different media which are used sequentially. Ideally oocytes could then be aspirated and matured *in vitro* (IVM), a method that is already in use in several units around the world.

1.7.5 Clinical In Vitro Maturation (IVM)

Already in 1935 Pincus and Enzman showed that immature oocytes spontaneously resume meiosis when they are removed from the ovary (Pincus and Enzmann, 1935). This discovery was confirmed in several animal species, as well as humans in the 1960's, (Edwards, 1965a; Edwards, 1965b). Almost 20 years later pregnancies were achieved using *in vitro*-matured oocytes collected from stimulated cycles (Veeck, et al., 1983). Similar findings were reported by several other investigators (Liu, et al., 1997; Nagy, et al., 1996; Prins, et al., 1987). Ten years later the first IVM pregnancy using immature oocytes from a patient with PCOS was described by Trounson et al. (Trounson, et al., 1994). Since then clinical results showing pregnancy rates of 25 to 35% (Chian, et al., 2004; Hreinsson, et al., 2003; Lin, et al., 2003; Mikkelsen, et al., 1999; Soderstrom-

Anttila, et al., 2005) per cycle have been reported, which is not yet as good as those obtained following conventional ovarian stimulation.
When performing IVM immature GV oocytes are aspirated from antral follicles when at least one follicle has reached a diameter of 10-14 mm. The cumulus-oocyte complexes are then cultured for 28-36 hours in maturation media enriched with recombinant FSH, recombinant LH and serum collected from the patient (Mikkelsen, et al., 1999). IVM treatment can be carried out completely without hormone stimulation, or using low doses of FSH to initiate follicular development. The low dose of FSH is helpful for timing of the oocyte retrieval in PCO patients (Mikkelsen and Lindenberg, 2001). Pregnancy rate of 23% per embryo transfer was reached when either hrechCG or hrecLH was used to induce the maturation of oocytes in a clinical IVM programme (Hreinsson, et al., 2003).

One problem with conventional IVF treatment is the risk of developing ovarian hyper stimulation syndrome (OHSS) which has a prevalence that ranges from 0,5-5% per cycle (Delvigne and Rozenberg, 2002). This syndrome can cause massive enlargement of the ovaries, thromboembolism, renal impairment, pleural effusion and can, in rare cases, be life threatening. IVM treatment is particularly useful for women suffering from PCOS as they have a high risk of developing OHSS. Another group that can benefit from this milder treatment is women in couples where the male factor causes the infertility problems. As the treatment in IVM is faster than in regular IVF, it may also be useful for women who are facing chemotherapy for cancer treatment. Immature oocytes can be collected and matured in vitro and then either oocytes or embryos can be cryopreserved for later use. Furthermore, if /when culture of ovarian tissue comes to clinical practice IVM of the oocytes will be the final step before IVF or ICSI can be performed.

Further advantages with milder fertility treatments are the lower costs that follow with the use of fewer hormones and also less discomfort for the patient. There are also indications that ovarian hyper stimulation is associated with higher incidence of chromosomal abnormalities of the oocytes (Baart, et al., 2007).

1.8 Reflection

This complex area has been rewieved with many references to animal studies although we are very aware of the differences between various species. Performing research with the valuable and scarce human material takes a lot of time and requires ethical thoughtfulness. Comparisons with other species are therefore helpful when designing studies using human material. In this thesis we have studied several aspects of the female reproductive system and tried to provide new tools to investigate this interesting area.

2. AIMS OF THE STUDIES

- Find a molecular tool for evaluation of human embryos and oocytes.
- Study the effects of hormones and growth factors on immature granulosa cells and ovarian tissue culture to investigate their role in follicular development and oocyte maturation.
- Optimize the culture conditions to improve the possibilities of maturing oocytes *in vitro*.
- Set up a system for real-time studies of oocyte maturation *in vitro* to clarify the timeline for the maturation process.

3. MATERIALS AND METHODS

We have ethics committee approval from the Regional Ethics Board in Stockholm for all of the studies included in this thesis. Oral or written consent was given by the patients participating in the different studies.
The methods are described in a general manner in this section, for further details please see the designate articles.

3.1 Oocyte and Embryo Preparations (Article 1)

3.1.1 Mouse
Female (CBAxB6)F1 mice underwent superovulation by hormonal induction according to standard procedures (Hogan, et al., 1994). The fertilized oocytes were cultured further at 37 °C in 5% CO_2 in micro-drops of KSOM medium covered with mineral oil. After culture to the indicated stages, the zona pellucida was removed using acidified Tyrode's solution (Vitrolife AB, Göteborg, Sweden), and zygotes, and 2-cell, 4-cell and 8-cell embryos were transferred to 2 µl ddH_2O with RNAsin (Promega, Madison Wisconsin, USA), snap-frozen in liquid nitrogen and stored at -70 °C.
Single blastomeres were obtained by first removing the zona pellucida from 2-cell to 8-cell embryos (using acidified Tyrode's solution), which were then mechanically separated by pipetting up and down, using a thin glass pipette. Single blastomeres were placed in 2 µl ddH_2O with RNAsin (1 U/µl) (Promega, Madison Wisconsin, USA).

3.1.2 Human
Couples undergoing fertility treatment at the IVF unit at the Karolinska University Hospital Huddinge donated pre-implantation embryos. Oocytes that failed to be fertilized, embryos that were of too poor a quality to be transferred and surplus embryos following transfer and freezing were used in this study. Surplus embryos were cultured further in 20 µl droplets of CCM medium (Vitro Life AB, Göteborg, Sweden) and transferred to fresh medium on day 4 of embryo development. Oocytes and embryos from the indicated pre-implantation stages were treated with acidified Tyrode's solution to remove the zona pellucida and washed in Dulbecco's phosphate-buffered saline (PBS) (Gibco BRL, Grand Island, USA) before being lysed in ddH_2O with RNAsin (Promega, Madison, Wisconsin, USA) at 1 U/µl, snap-frozen in liquid nitrogen and stored at -70 °C until reverse transcription was performed.

3.2 Gene expression analysis (Article I and II)

3.2.1 RNA isolation
RNA from granulosa cells was prepared according to Rneasy Mini-kit (Qiagen, Hilden, Germany) protocol for animal cells.

3.2.2 Reverse Transcription

Synthesis of cDNA was performed directly on the oocyte or embryo lysates and on RNA isolated from GC's using random hexameres, according to the protocol for Superscript First Strand Synthesis System for RT-PCR (Gibco BRL, Grand Island, NY. The samples were then stored at -20 °C or -70°C until real-time PCR was performed.

3.2.3 Primers and Probes

Primers were designed to generate short amplicons of between 50 and 150 bp, which is reported to be optimal for real-time PCR. In article I taqman probes were used to detect the amplified product and pre-developed primers and taqman probes from Applied Biosystems were used for the controls, 18s rRNA and GAPDH. In article II Sybrgreen that binds to all double-stranded DNA was used as the fluorescence detector for both target genes and controls.

3.2.4 Real-Time PCR

Real-time PCRs were performed using an ABI PRISM 7700 or later ABI PRISM 7900 sequence detector (Applied Biosystems, Foster City, CA, USA), in which the thermal cycler, fluorescence detector and analysis software is integrated in one instrument.
The cDNA was heated to 50 °C for 2 min and denatured at 95 °C for 10 min. The template was then amplified over 50 cycles of 15 seconds melting at 95 °C and 1 min at 62 °C for annealing and extension. During the PCR an argon ion laser excites fluorescent dyes in each reaction. Fluorescence data was acquired by measurements taken approximately every 7 seconds and presented as a plot of fluorescence intensity versus cycle number.

Standard curves are constructed using duplicates of six serial dilutions of cDNA prepared from RNA of known concentration. Standard curves were generated and run in parallel with the unknown samples in each experiment. Fluorescence was measured and the threshold cycle (C_t) values at each point in the standard curve were plotted against the log (ng) of the initial concentration (Higuchi, et al., 1993). The relative concentration of unknown samples was determined from the standard curve and calibrated against the internal control of the assay.

3.3 Granulosa Cell cultures (article II)

Mural granulosa cells were collected from follicular aspirates after oocyte retrieval. Cells were centrifuged out of follicular fluid and then treated with hyaluronidase for 30 min in 37˚C. To separate the GC's from red blood cells, the cell suspension was layered on Ficoll (Amersham, Uppsala, Sweden) and centrifuged for 30 min at 300 g. The granulosa cell fraction was then washed with PBS and the cells were seeded 50 000 cells/well in a 24-well plate in 500µl basic media, TCM-199 (Invitrogen-Gibco, Paisley, UK), 2,5%

FCS, 1% penicillin/streptavidin (Invitrogen-Gibco, Paisley, UK) over night to attach to the plastic and to be separated from white blood cells. The following day, one of five different stimulations was given to the cells.
Cell cultures from two groups of patients were studied, non luteinized GC's from IVM patients (n=12) mean age 31.7±3.7 and as a control group luteinized GC's from IVF patients n=6) mean age 33±5.0.As a second control we used luteinized GC's from IVF patients n=6 mean age 34± 2.3 and cultured them for 72 hours before stimulation. This was done in order to see if the IVF GC's can regain a more native reaction pattern after a 72 hours pre-culture period before stimulation.

3.4 Hormone measurements (article II)

3.4.1 Assay of estradiol-17β and progesterone

Concentrations of estradiol-17ß (E2) and progesterone (P) in the culture fluid were determined by clinical routine immunoassays intended for analysis of human serum. The culture fluid was found to possess the same matrix properties as serum in both assays**.** Culture fluid concentrations of E2 and P were determined by competitive chemiluminiscence immunoassay using commercial kits obtained from Roche Diagnostica GmbH, Mannheim, Germany (E2: "Modular E170) and from Diagnostic Products Corp., Los Angeles, CA (P: "Immulite 2000"). Practical detection limits and within and between assay coefficients of variation was for E2 100 pmol / L, 4% and 5% and for P 0.6 nmol/L, 8% and 10% respectively.

3.5 Tissue cultures (article III)

Small ovarian biopsies were donated by eighty-three women aged 24-40 years (mean 33 ± 4.01 years) undergoing cesarian sections (n=82) or gynaecological laparoscopies (n=1) at the Karolinska University Hospital Huddinge, Sweden. The biopsy specimens were collected and placed in pre-equilibrated Flushing Media; MediCult Jyllinge, Denmark. The tissue was then transferred immediately to the culture laboratory. Ovarian tissue samples were cut into designate pieces, using a needle and scalpel and sterile techniques. One piece of tissue, the uncultured control was fixed directly for histological analysis. The others were treated according to the different protocols for the separate experiments in article III.
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) previously coated with 100 µl of extracellular matrix diluted 1:3 with the base media being used minus the supplements (Growth Factor Reduced Matrigel; Becton Dickinson). Culture medium was αMEM supplemented with 10% human serum albumin (HSA; Pharmacia Upjohn, Sweden or Octapharma, Stockholm, Sweden), 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). 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 inserts.

3.6 Histological analysis (article III)

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 embedded in paraffin. The tissue pieces were serially sectioned at a thickness of 4 µm. The sections were then mounted on the slides with eight sections omitted between each mounted section, to prevent double counting of follicles. Following staining with haematoxylin and eosin, the slides were analysed for follicle development and viability. 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 GC's 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.

3.7 GDF-9 agonists and antagonists (article III)

3.7.1 Agonists

To stimulate the GDF-9 pathway first mouse GDF-9 was used. We used conditioned media from 293T cells producing the protein, and in this version the proregion was still attached to the mature region (Kaivo-Oja, et al., 2003). The mGDF-9 was used in the dose-response study and in the first long-term culture.

In the second study testing long-term culture a human recombinant GDF-9 was used. In this protein the proregion had been removed from the active mature region and this product has been characterized *in vitro* (Mottershead, et al., 2008).

3.7.2 Antagonists

To inhibit the effect of endogenous GDF-9 action in tissue culture, we used GDF-9Ab-37 and BMPRII-Fc. GDF-9Ab-37 neutralizes the effect of 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 it has the ecto-domain of the BMP type II receptor (which is utilized by both GDF-9 and GDF-9B/BMP15) in the N-terminal and the Fc domain of human IgG1 as its C-terminal. This protein effectively neutralizes recombinant rat or mouse GDF-9 (Gilchrist, et al., 2006; Vitt, et al., 2002).

3.8 *In vitro* maturation of oocytes (article IV)

3.8.1 Oocytes

The immature oocytes (n=40) used in this system were obtained from women undergoing ICSI treatment. Cumulus-oocyte complexes (COCs) were collected under transvaginal ultrasonographic guidance, using HEPES-buffered flushing media (MediCult a/s, Jyllinge, Denmark). The oocytes were then transferred to Universal IVF Medium (MediCult a/s, Jyllinge, Denmark) until denudation. They were denuded from their granulosa cells (GC's) by pipetting in SynVitro Hyadase (MediCult a/s, Jyllinge, Denmark), and were evaluated by the embryologist for stage of maturity. If they were found to be immature (GV or MI stage) they were donated to research. The immature oocytes were then transferred to Universal IVF Medium (MediCult a/s, Jyllinge, Denmark) while the maturation medium was prepared and equilibrated. Finally, the oocytes were transferred to pre-equilibrated maturation medium with the GC's, and the plate(s) were placed in the Cell-IQ® equipment.

3.8.2 Granulosa cells

Granulosa cells from the same patient were co-cultured with the oocytes. They were washed in Universal IVF Medium (MediCult a/s, Jyllinge, Denmark) to remove hyaluronidase and then transferred to a 48-well plate with 600µL of maturation medium that had been pre-equilibrated in a traditional incubator for 1 hour. The GC's were spread over the plate and not too concentrated near the oocyte, as they would otherwise grow over the oocyte and block it from view.

3.8.3 Culture medium

The maturation medium for the oocyte-cumulus cell co-cultures was TCM-199, Invitrogen, Paisley, UK, containing human serum (10%), 0.3 mM pyruvate, 1% penicillin Invitrogen, Paisley, UK, 1.5% streptavidin Invitrogen, Paisley, UK, hCG (Ovitrelle®; Merck-Serono, Stockholm, Sweden) at 0.5 IU/mL and 0.075 IU rhFSH(Gonal F®; Merck-Serono, Stockholm, Sweden).

3.8.4 Cell-IQ®

The Cell-IQ® is a research instrument that combines the culturing environment with an imaging system in order to allow continuous morphological studies without disturbing the culture conditions. We used this system to study *in vitro* maturation of oocytes. As oocytes are large round cells, floating in culture medium some adjustments were needed as only programs for adherent cells were available before the present study.

The incubator has a temperature-controlled space (normally 36.5 ±0.2 ºC) and a special well plate lid for control of gas both in and out. The incubation gases are piped directly into the well plate through the lid, and each well has the same gas volume. The lid has inlet and outlet connectors and appropriate valves and filters, so that the desired gas environment can be maintained throughout the culture period. The gas is humidified using sterile water placed between the wells of the plate and/or in wells not in use for cultures.

The light source used in this system is a green 530 nm LED that minimizes harmful exposure to light but still enables high quality phase contrast images. The imaging set-up comprises a phase contrast microscope (Nikon light source, Nikon ×10 objective, Infinity Optics Tube), a digital camera (Jai cv-A10CL) giving image resolution of 0.9 µm/pixel and a motorized z-stage for focusing (1.25 µm resolution).

The x,y position of the oocyte is localized by means of the motorized x,y stages (2.5 µm accuracy) that set the well plate in place for imaging. As the oocyte is much larger than, for example, adherent cells, several positions in the z direction have to be set to be able to observe changes that occur at different levels of the oocyte.

The Cell-IQ® instrument uses an all-in-focus imaging method to produce information-rich images (IRIs) that are sharp in the entire field of view, regardless of the dimensions of the target. The depth of focus in the Nikon ×10 objective is about 3 µm, which is often smaller than the maximum depth of the target object. Therefore, it is not sufficient to take only one focused image to obtain a picture that is well focused overall; instead a stack of images at intervals of 1.25 µm is taken at each z position and the system composes one focused picture from the stack. The composite picture created by this imaging principle is called an all-in-focus image. The system then allows you to choose how often a picture should be taken. (Chip-Man Technologies Ltd. Website: www.chipmantech.com).

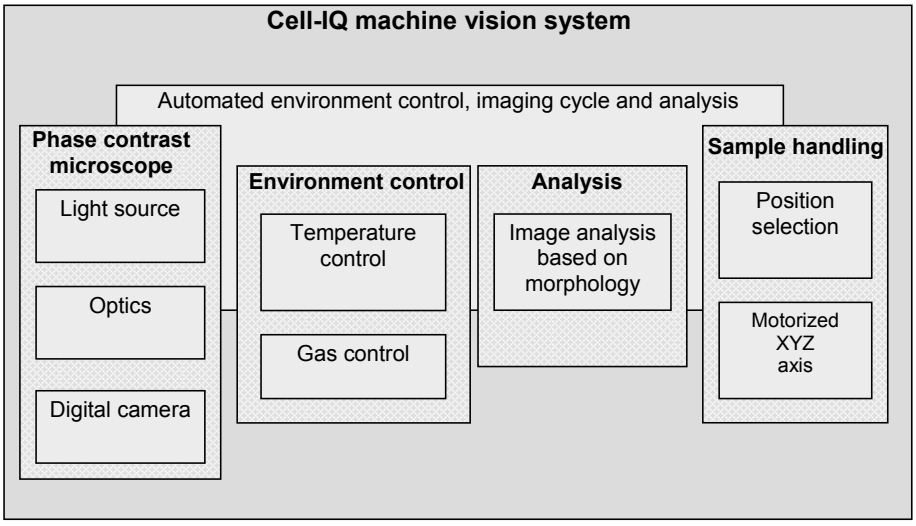

Figure 3. Schematic of the Cell-IQ® system

3.9 Statistical analysis

Article I and II
Differences in gene expression were analyzed using 2-tailed t-tests, significance is reported at the 0.05 level.

Article III
Proportions of follicles at different developmental stages, proportions of viable follicles, density of follicles and follicle and oocyte diameters within each patient sample were compared for calculation of overall means and standard error of the means. Statistical comparisons between groups for all data were determined using a one-way analysis of variance non-parametric, one-way ANOVA. Significance is reported at the 0.05 level. Data are presented as the mean ± SEM.

4. RESULTS AND DISCUSSION

4.1 Article I

During the process of oocyte maturation the oocyte accumulates a large number of proteins and mRNAs to prepare for the initial stages of embryo development. These maternal factors control the first cell divisions until the embryo itself becomes transcriptionally active and takes over further development.

When selecting embryos for embryo transfer during IVF treatment morphology is the leading, if not the only, criteria used in clinical practice. Our intention with this study was to explore the possibilities to quantify gene expression in single embryos and more important single blastomeres. The optimal goal for such measurements would be to provide a way to measure the expression level of a marker gene known to indicate good quality of an embryo. The level of gene expression in biopsied blastomeres could then serve as additional criteria in the selection of embryos for transfer. Hopefully that could increase the chances of a successful treatment.

We used real-time PCR to study the expression of eIF-1A in fractions of mouse and human preimplantation embryos and in single blastomeres from mouse embryos. The expression of this gene was already known to increase at the time of embryonic gene activation (EGA) in mouse and bovine embryos (De Sousa, et al., 1998). In our study we confirmed this fact in mouse embryos and showed that this was also the case in human embryos, as the expression level per blastomere significantly increased at the 8-cell stage which corresponds to the EGA in human. We also showed that this method was sensitive enough to measure gene expression in single mouse blastomeres as the measurements performed on separated cells corresponded to the values on whole embryos.

We conclude that this is a suitable method to study gene expression in single cells.
Since we performed this study the technique has been further improved and the accuracy and sensitivity of the methodology today makes it even more suitable. However, the technique needs to be optimized and the identification of suitable markers is a crucial step before this method can approach a clinical setting.

4.2 Article II

In our second study we wanted to study follicular cells which participate in the complex network of communication that leads to maturation of the oocyte. Here we used the unique model of non-luteinized (GC's) from patients who undergo *in vitro* maturation (IVM) treatment.

This is a recently established method not yet commonly used. Hence, it offers a model for studying the expression and actions of several growth factors, hormones and their receptors on the GC's at a stage where they can still influence the oocyte maturation.

Using these cells we can obtain completely new information regarding human follicular development. All information until now has been obtained using luteinized granulosa cells, which have already been under the influence of FSH and hCG during controlled ovarian hyper stimulation.

We have chosen to study the effect of gonadotrophins (FSH and hCG) on the non-luteinized GC's by measuring secretion of estrogen and progesterone before and after treatment. We also measured expression levels of $p450_{aromatase}$ and $p450_{scc}$ (side chain cleavage enzyme), two enzymes involved in the synthesis of these hormones. We have also measured how the receptors for LH and FSH react to the different treatments.
To relate our findings to earlier studies we also investigated how luteinized GC's from conventional IVF-treated patients reacted to the same treatments. Further, we also studied how the luteinized GC's reacted to these stimulations after a "resting period" in culture.
When we stimulated the non-luteinized GC's with gonadotrophins we observed a different hormone response pattern compared to that in luteinized cells. ELISA measurements demonstrated that hCG and FSH alone and in combination induce a significant increase in progesterone production, and FSH alone and in combination with hCG increases estrogen production. In luteinized GC's estrogen and progesterone levels were not influenced by the different treatments. The "rested" luteinized GC's reacted similarly to the luteinized GC's that were treated directly.

We also used real-time RT-PCR to study the gene expression of enzymes involved in estrogen and progesterone synthesis and the receptors for FSH and LH. We found that in non-luteinized GC's the expression levels of $p450_{aromatase}$ increases with all treatments and $p450_{scc}$ expression increases with the combined FSH and hCG treatment. In the luteinized GC's hCG treatment induced a decreased $p450_{aromatase}$ expression. Expression of 450_{scc} also decreased in cells treated with FSH and hCG separately but not with the combined treatment. In IVM GC's the LH receptor expression increased with FSH treatment but the expression of FSH receptor did not change with different treatments.

We conclude that by studying non-luteinized GC's we are likely to observe a different reaction pattern compared to the luteinized cells. As the cells are harvested at an earlier time point they are still in a stage where they can influence the oocyte. The results from non-luteinized cells may be more likely to reflect the reaction pattern of GC's during late follicular development in vivo.

4.3 Article III

The Growth Differentiation Factor 9 (GDF-9) is known to be an important regulator of folliculogenesis in many species. An earlier study from our group has shown that addition of crude mammalian HEK 293 cell expressed rat recombinant GDF-9 stimulates the progression and survival follicles in human ovarian tissue culture (Hreinsson, et al., 2002). The development of new compounds acting as stimulators or inhibitors of this important growth factor encouraged us to perform further studies in our human ovarian tissue culture system.

In this study we tested how two exogenous agonists to GDF-9 influence the follicular development in human ovarian tissue. We also tested two different ways to antagonize the effect of endogenous GDF-9. (For details about the different compounds see section 3.7).

The first compound tested in this study was a mouse GDF-9 protein produced in a similar way as the rat protein. This protein was produced as a whole complex with its proregion still attached and had shown good bioactivity in both human and rodent GC assays (Gilchrist, et al., 2004; Kaivo-Oja, et al., 2003; McNatty, et al., 2005b). However in our system the effect was moderate.
In later studies we used recombinant human GDF-9, where the mature region is purified to obtain higher bioactivity: this compound turned out to be more efficient in our system. We investigated the effect of this protein in long term cultures for up to 6 weeks.
In all tissue treated with rhGDF-9 the follicular development progressed at all time points when compared to untreated tissue. The viability decreased in all treatment groups when compared to the uncultured control. Although after 14 days of culture with rhGDF-9 treatment the viability was higher than after both 14 and 28 days of culture without rhGDF-9 treatment. We also saw a positive effect of rhGDF-9 on follicle size at all time points studied. Also, the oocyte became larger after 28 and 42 days of culture with rhGDF-9.

The results from the inhibition studies varied with the two different compounds. Using the GDF-9 neutralizing antibody only minor effects were observed. However with a high dose of 3000 ng /ml a suppressed initiation from primordial to primary follicular stage was observed. The other compound, BMPRII-Fc that works as a soluble receptor has been shown to neutralize the effect of both, GDF-9 (McNatty, et al., 2005a; Mottershead, et al., 2008) and GDF-9B/BMP15 (Kaivo-Oja, et al., 2003). At a concentration of 1000 ng/ml BMPRII-Fc the proportion of primary follicles was higher and consequently the proportion of secondary follicles was lower compared to the uncultured control, indicating an inhibition of the transition from primary to secondary stage. Treatment with BMPRII-Fc at a concentration of 100 and 1000 ng/ml also resulted in a decrease in follicular size when compared to the cultured control tissue. The oocyte diameter was not affected by BMPRII-Fc treatment.

This study highlights the significance of using a compound that is purified when working with ovarian tissue culture. The modest effect of the mouse protein may be caused by the fact that the proregion needed during synthesis of the protein is still present in a complex with the mature region. This proregion may cause difficulties for the mature region to access the receptors. It may also bind to the extra cellular matrix present in the tissue and thereby inhibit the activity of the mature protein.
However, the results with the purified rhGDF-9 protein were very promising.

This study also shows the importance of endogenous GDF-9 in human follicular development. Earlier studies have shown that mitogenesis in GC's induced by recombinant mice GDF-9 can be inhibited by the GDF-9 antibody used in this study (Gilchrist, et al., 2004). However they used fairly high doses to obtain this effect.

Blocking the endogenous GDF-9 with this compound was not very efficient in our system.
By using the BMPRII-Fc both the effect of GDF-9 and GDF-9B/BMP15 are blocked. This resulted in more obvious effects; however specific inhibitors would be needed to distinguish the different contributions of the two proteins.

In conclusion, these results show the importance of endogenous GDF-9 in follicular development. We also demonstrated that the progression of follicles is improved by adding GDF-9 to the tissue cultures; this can hopefully bring us closer to successful *in vitro* maturation of human follicles.

4.4 Article IV

In vitro maturation is becoming an attractive alternative to conventional IVF treatments especially for certain patient groups such as PCOS sufferers, as they exhibit a high risk of developing OHSS. However, IVM does not yet have the same efficiency as IVF treatments and the miscarriage rate is higher in IVM pregnancies. To be able to improve the maturation of oocytes *in vitro* we need to learn more about the maturation process. In our fourth study we have used a recently developed research instrument called the Cell-IQ® and adapted it for the studies of oocytes in culture. The Cell-IQ® is an instrument where the incubator is integrated with the microscope and a camera and this setup allows continuous observations without disturbing the culture conditions. This instrument was originally developed for the study of adherent cell cultures. As the oocytes are much larger three dimensioned floating structures, several adjustments to the system were necessary to adapt it to our requirements.

For developing the method, we used denuded immature oocytes from women scheduled for intra-cytoplasmic sperm injection (ICSI) in co-culture together with the cumulus cells denuded from the oocytes of the same subject. The GC's not only served as a source of growth factors and hormones required for the maturation of the oocyte, they were also helpful in keeping the oocyte in place to prevent disappearance from the field of view.
By using this system we could time the different phases of oocyte maturation very accurately, as well as study other parameters such as the shape and structure of the oocyte.

We were able to time the different maturation steps such as germinal vesicle breakdown (18min ± 7), polar body extrusion (16 min ± 5) and the interval between these two events (20 hours ± 5) with very high accuracy. We also measured the time interval from oocyte pick-up until maturation to the MII stage of these immature ICSI oocytes to 36.62 hours ± 11.12. Parthenogenic division of an oocyte was also monitored. The shape of the oocyte altered dramatically during development, and major movements occurred, particularly before PBE. Furthermore, during the maturation process the structure of the cytoplasm changed from granular to smooth. The degree of fragmentation also varied as fragments were extruded and reabsorbed. This information told us that the maturation

process was dynamic and as its features change over time an oocyte could easily be misjudged as being poor if it is infrequently observed.

We think that this system offers an excellent opportunity to study how different factors influence these different maturation steps. The knowledge gained from such studies can be used to optimise culture conditions for maturation of oocytes *in vitro*, leading to the improvement of clinical IVM.

5. CONCLUSIONS AND FUTURE PERSPECTIVES

We have explored the possibility to analyze gene expression in single cells and found that this is possible using real-time RT-PCR. With the fast development and refinements of this technique it is likely that today even more reliable and stable data can be obtained. Furthermore, we have utilized the fact that IVM treatments provide a unique and seldom investigated source of non-luteinized GC's. These cells which are at an earlier stage of development, proved to react very different from the more frequently studied luteinized GC's. We are presently performing more studies with non-luteinized cells to learn more about their response to different stimulations. The increased knowledge about what factors are secreted from these cells gives us useful information on how we can improve *in vitro* maturation of oocytes. In our combined imaging and culture system developed in article IV the effect of different factors can be tested and observed continuously and provide new insights to oocyte maturation.

In article III we have studied the effect of the oocyte secreted factor GDF-9 on follicular development in ovarian tissue cultures. We found that the different protein preparations and also different inhibiting approaches can provide varying results. We also concluded that the human recombinant protein was most suitable to use when studying human material. Our experiences with the different compounds used in this study will be very useful for planned future studies on GC's and oocytes.

Working with different techniques and looking at various phases of the development is instructive and gives an insight to the complexity of the system. The development of the different techniques presented in this thesis will hopefully be helpful for further research trying to increase the understanding of the female reproductive system. Such knowledge can help in reaching the aim to obtain mature and fertilizable oocytes.

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