Chapter 6

Summarizing discussion

The induction of maturation in the oocyte depends on the completion and integration of a number of essential factors. Among these, first, the oocyte must have completed its cycle of growth and acquired all the molecules before the induction of maturation begins. A second key requirement is that essential endocrine and paracrine signals must be generated at the appropriate time and at sufficient concentrations to induce all the required intracellular changes associated with both nuclear and cytoplasmic maturation and thus developmental competence. However, the concept of developmental competence is not clearly defined since no particular mechanism is associated with it. But, it is believed that the acquisition of developmental competence is associated with different changes like, the synthesis and accumulation of specific RNAs and proteins, relocation of cytoplasmic organelles such as cortical granules, lipids and mitochondria. The consequences of failing in any of these processes results in developmental failure.

This thesis investigated the gene expression in bovine oocytes during meiotic resumption, at 6 h post LH surge, coinciding with germinal vesicle breakdown, which was supposed to give a picture of the major cell cycle regulation changes, cytoskeleton rearrangement and chromosome alignment.
The first study, **(Chapter 2)**, explains the methods used to collect the material used in this thesis and explains the criteria used to select the functional preovulatory follicles, and hence the presumptive competent and non-competent oocytes at different stages of maturation. In the second study **(Chapter 3)**, the collected oocytes were used to construct and hybridize the DNA microarray to unravel the potential transcripts involved in meiotic resumption and possibly developmental competence. The identification of several transcripts in the second study and, its association with spindle assembly and chromosome segregation allowed us to follow up on study three **(Chapter 4)** and investigate the levels of these transcripts in oocyte matured in vivo as normal or deviant based on selection criteria in study one, and oocytes collected from slaughterhouse ovaries and matured in vitro.

Because the action of the products of the different transcripts identified in chapter 4, and also in chapter 3 is ATP-dependent, and because the correlation of oocyte ATP content and developmental competence is well established, energy requirements constitute an important factor to accomplish competence during maturation. In general, lipids form an energy source but there is a lack of information on the role of lipids as energy source in bovine. Therefore, in the fourth study **(Chapter 5)**, based on information in somatic cells, we investigated the involvement of various pathways for lipid transport, β-oxidation and *de novo* fatty acids synthesis during final maturation of bovine oocyte using QPCR. This

discussion summarizes the major findings, and gives suggestions for practical applications and prospects for future research.

Effects of follicle size on steroid hormones levels in the bovine preovulatory follicles after FSH stimulation

Assisted reproductive technologies routinely use controlled ovarian stimulation for oocyte recruitment and ovulation induction. Thus, an increased number of oocytes can be collected, though at the possible risk that not all gametes will show the same developmental competence [1, 2]. This heterogeneity is probably due to intrinsic differences in the oocytes. It is well known that the hormonal milieu of the follicles is altered in cows stimulated with exogenous gonadotropin to a varying degree depending partially on the type of protocol and the hormonal treatment used [3-7]. However, at present little information is available on how follicular steroid production is related to the size and health of the follicles. In **Chapter 2**, we demonstrated that follicular concentrations of steroids are influenced by the size of the preovulatory follicle after oFSH stimulation. In general, one of the main findings is the influence of follicle size on the progesterone levels after LH surge, where the levels increase with follicle size. However, as yet, the exact relationship between oocyte developmental competence and its respective intra-follicular environment is currently unknown. Nevertheless, in sheep, [8] and human [9], the physiological state of the follicles appears to affect subsequent oocyte maturation and competence in vivo. Asynchrony of follicle and oocyte maturation occurs after superovulation and may reduce oocyte developmental competence [1, 2].

In addition, from the present study, we do not have proven reliable criteria to enable us distinguish clearly between follicles with competent and non-competent oocytes. However, in an earlier study in our lab, oocytes collected from cows stimulated with recombinant human FSH have shown decreased concentrations of estradiol at onset of maturation, which were related to impaired distribution of cortical granules to the periphery at finishing of maturation. Normal distribution of these granules is considered a well known sign for developmental competence [10]. Therefore, it was assumed that competent oocytes are primarily enclosed by functional preovulatory-sized follicles showing the changes in steroid concentrations as reported for untreated, normal cyclic cows. Steroid hormones are involved in a wide array of physiological responses, including regulation of glucose [11] and lipids, for instance, in aromatase-deficient (ArKO) mice, exogenous estradiol is necessary to maintain the gene expression and enzyme activity of the genes involved in hepatic lipid metabolism. Steroid hormones have been shown to regulate cell cycle

progression [12, 13], inhibition of apoptosis [14], and modulation of calcium release [15, 16]. In the mammalian ovary, the follicle is the major site of synthesis and secretion of steroid hormones during preovulatory development and maturation of the oocyte. Regulation of steroid production by the ovarian follicular cells varies remarkably at different stages of development. During the preovulatory period, the selected dominant follicle is characterized by cyclical fluctuations in the levels of these hormones [17]. Before the preovulatory LH surge, granulose cells synthesize and secrete estrogen, while after LH, granulose cells luteinize and secrete more progesterone in concert with decreases in mRNA for 17alpha-hydroxylase and P450 aromatase [18]. The specificity of the steroid actions is due to the presence of intracellular receptor proteins. Despite the wealth of information about steroid receptors in different tissues and their importance in reproduction, only the mRNA receptor for estradiol (ERβ) has been identified in bovine oocyte [19]. Progesterone receptor mRNA in granulosa cells of the bovine preovulatory follicles is transiently induced within 5 to 7 h of the LH surge [20-22]. However, nothing is known about the expression of nuclear or membrane progesterone receptors in the oocyte of any mammalian species. In primates, androgen receptors (AR) mRNA activity is essential to early follicular development and oocyte quality [23], and in rats, complete disruption of AR activity is associated with intensive granulosa cell apoptosis in preovulatory follicles and poor quality cumulus oocyte complexes (COCs) [24]. Further, androgen receptors have been reported to translocate from the oocyte cytoplasm to GV, and then to the nucleolus suggesting a role as a ligand-activated, transcriptional factor [25]. In view of these observations, the identification and characterization of the patterns of mRNA changes, and functional analysis of the steroid hormone receptors that are expressed in the oocyte, if any, may provide a fundamental understanding of the critical roles of steroid during oocyte maturation in vivo. In clinical practice, there is a clear need to optimize the ovarian stimulation protocol, and proper design of superovulatory treatment should consider, LH concentration and half life in the FSH preparation, and steroid content and steroidogenic enzyme expression in the preovulatory follicles.

Messenger RNA expression in bovine oocyte during meiotic resumption

The growing mammalian oocyte, although morphologically simple, undergoes a series of discrete differentiation events. A relatively large number of genes are required to program its entire development. A small fraction of these genes are oocyte specific [26] while the large majority are expressed both in the oocyte and in the somatic cells. Transcription and repression of genes is a dynamic process that can be expected to vary in the oocyte with the culture conditions. In order to assess which genes may be regulated by

specific stimuli, it is necessary to have the capability of examining genes under a variety of exposure condition. The triggers for change in gene expression in the oocytes are critical for understanding the molecular mechanism of oocyte maturation. In fact, the information currently available on molecular mechanisms regulating oocyte maturation has been largely obtained from studies using in vitro matured oocytes. Perhaps even more important, in the cow, most in vitro studies have been carried out with oocytes collected from small or medium follicles (3 to 6 mm follicles), which lack the prematuration stage [27, 28]. Prematuration begins at an average follicle diameter of 8.5 mm, that is the beginning of the difference in growth between the two largest follicles [29] and is associated with a differentiation of the concentration of estradiol [30].

Because in vivo oocyte maturation relies on a subtle balance between different follicular regulatory compounds, and also probably between different oocyte receptors, the molecular and biochemical alterations triggered by artificial ligands in vitro may not necessarily reflect the normal in vivo processes. The complexity of meiotic resumption regulatory mechanisms is also well demonstrated in bovine oocytes [31]. The bovine preovulatory follicle appears an attractive experimental model for study of the regulation of oocyte maturation and its ability to develop after fertilization. The preovulatory follicle contains sufficient follicular fluid for the analysis of steroid, proteins and various regulatory compounds. It contains also sufficient amounts of granulosa and cumulus cells, which offers an excellent opportunity to investigate functional interactions between various regulatory factors.

Differentially expressed genes between the oocytes exposed to LH and those collected before can help us understand the molecular basis of meiotic resumption in vivo. The identification and characterization of oocyte genes expressed exclusively or preferentially in the 6 h in vivo matured oocyte will hopefully shed light on the mechanisms of the maturation process and provide useful information for the development of efficient maturation media. The suppression subtractive hybridization (SSH) method allows identifying overexpressed genes (designated forward +SSH) but also underexpressed genes (designated reverse -SSH) by exchanging the driver and tester populations during the procedure (Clontech, Palo Alto, USA) [32-37]. SSH is still widely used technique since it enables the recovery of abundant as well as low copy number mRNA transcripts. However, because it still needs a lot of initiating RNA which will burden using in vivo matured oocytes, the use of the *S*witch *M*echanism *A*t the 5` end of the *R*everse *T*ranscript (SMART) amplification method overcomes this limitation.

The development of microarray technologies permits thousands of genes to be screened in a single experiment to establish differential gene expression in treated versus control cells and population. Consequently, the use of DNA microarray should significantly aid in minimizing the effort required to screen the many variables required to effectively examine gene expression patterns. Microarrays are developed to represent expressed mRNA transcripts (cDNA arrays), or distinguishable portion of an mRNA transcript (oligonucleotide arrays). The popularity in use of this technique is demonstrated by the exponential growth in publications using microarrays since its inception in 1995. Microarrays have been widely used to study issues in pathology, pharmacology, oncology, cell biology and recently, oocytes [26, 38, 39].

Different techniques have been used for gene discovery to design oocyte specific cDNA microarrays for possible use in assessing reproductive technologies performance. Two of these techniques employ a method for selectively segregating cDNA clones or fragments found in one cell or tissue population and absent in another. At present, the complete gene database for bovine is becoming available. Therefore, genomic information must be employed to construct a microarray to use in screening transcripts in the bovine oocyte. The ideal approach to gene expression profiling is to use full genome microarrays to identify genes up or down-regulated in response to certain treatment. However, because microarrays are not likely to become a routine test in the near future, reproductive biotechnology studies will probably require to identify a small subset of genes whose expression can be applied in the development of gene-based quality test. Therefore, in **Chapter 3** we used SSH and microarray technique and oocytes matured in vivo to identify genes involved in regulating the maturation of bovine oocyte, suggesting that LH and maturation in vivo is instrumental in regulating several aspects of oocyte function.

Using SSH and defining a 1.3 fold difference as threshold, 115 regulated genes were identified from initially 945 DNA clones. This relatively small difference in the gene expression pattern due to LH surge may point to the fact that only a small subset of genes needed to regulate the meiotic resumption and developmental competence. The microarray analysis undertaken in this study has uncovered novel mRNAs with potential roles in proper oocyte function, maturation and/or meiotic competence. We identified important changes in genes involved in cell cycle regulation, signal transduction, transcription and mRNA processing, cytoskeleton, cell adhesion, as well as in metabolism. Following the identification of these genes at the mRNA level, the challenge is to utilize efficiently this information to develop a better understanding of meiotic resumption mechanism. The proteomic approach may provide information that could not be obtained at the RNA level,

due either to poor correlation between mRNA and protein levels or due to post-translational modifications that may result in several isoforms generated from one mRNA. For many genes identified, both the putative ligands that activate them and their targets of action remain unknown and represent challenges for future studies to unravel the mechanism of oocyte maturation and developing efficient IVM system.

Messenger RNA expression of genes involved in organelle transport and chromosomal segregation

While the spindle and chromosome morphology of the meiotic events is known for over a century, most of the basic molecular mechanisms regulating the chromosome segregation in mammalian oocytes are widely unknown. Meiosis within the oocyte must be precisely regulated to ensure proper division of the genetic material. Missegregation of chromosomes results in aneuploidy and could lead to inviability. An estimated 10 to 30% of fertilized human eggs have the wrong number of chromosomes, with most of these being either trisomic or monosomic. This has profound clinical consequences: approximately one-third of all miscarriages are aneuploid, which makes it the leading known cause of pregnancy loss and, among conceptions that survive to term, aneuploidy is the main genetic cause of developmental disabilities and mental retardation [40].

The real-time QPCR analysis of six of the genes having a significant role in the spindle formation and maintenance of accurate chromosomal segregation and construction of the cytoskeleton, between oocytes from stimulated cows classified as originating from normal and deviant follicles showed deregulated and aberrant mRNAs levels in the latter category. This may explain the chromosomal abnormality frequently seen in the oocyte and early human preimplantation embryos cultured in vitro, which is commonly associated with impaired cleavage, poor embryo quality and increased fragmentation, all of which may compromise the implantation potential of the embryos [41-43].

While the polarity in mammalian embryos is a well-known phenomenon, existing polarity in mammalian oocyte is still controversial [44-46] and little is known about the genes regulating polarity and related activity in oocytes. In **Chapters 3** and **4**, we identified several transcripts in the oocyte known to play a role in polarity axis formation like par-3, formin, KIF3, β-catenin and CDC42 (unpublished results). Organelles and cortical actin distribute asymmetrically in the oocyte of many species as the dorsal/ ventral axis forms [46]. The identification of genes regulating polarity in the oocyte and the recent finding that

Par-3 protein is associated with meiotic spindles [47], may point to their important function during meiotic resumption and possible role in oocyte polarity.
Moreover, although most of the genes identified in chapter 4 are also common to somatic cells during mitosis, there is a fundamental difference: the chromatids are held together during prometaphase of meiosis II only at the centromere, whereas during prometaphase of mitosis they are joined (at least initially) along their entire length. This raises the interesting possibility that chromosome disjunction during mitosis also requires two different sets of machinery: e.g. one that is normally present during meiosis I that separates the chromatid arms, and another normally found in meiosis II (or during the preceding interphase) that leads to separation in the centromere region. In the future it will be important to determine the differences between the two mechanisms that operate during meiosis to separate the chromosomes. The separation of sister chromatids is a complex process and there are certainly other factors involved in regulating the attachments and separation of sister chromatids.

Regulation of lipid metabolism during final oocyte maturation and early embryonic development

A full understanding of the physiological effect of maturation in vivo on gene expression requires identification of the transcripts having an impact on metabolic pathways, their mode of action, and their consequences for growth, differentiation and survival. At a more practical level, gene identification is essential for formulating a successful maturation medium to support oocyte development after fertilization.

Changes associated with ultrastructure of the growing oocytes related to the accumulation of nutrients like lipids are prerequisite of energy for meiotic resumption and subsequent embryonic development. The origin of lipids reaching the oocyte is not fully understood. Lipids stored in the oocyte have been shown to be accumulated in the oocyte during follicular development [48] and start to decrease during the maturation process [49]. Kim et al. [50] showed that lipid content in bovine oocytes reflects the lipid content in the maturation medium, indicating that lipids accumulating in the oocytes must originate from the medium. It is not known whether these lipids pass via junctions between the oocyte and its surrounding cumulus cells or are taken up directly from the follicular fluid. Since many genes are conserved across human and animal species, function of certain genes can be extrapolated. Therefore, mRNAs representing the major metabolic pathways involved in lipid metabolism were investigated in **Chapter 5** in normal oocytes. From the results, it is possible to suggest a model for long-chain fatty acid (LCFA) transport into the oocyte. The

fatty acids are translocated from the extracellular environment to the cytoplasm by the fatty acids translocase (FAT/CD36) and then solubilized and transported by fatty acid binding proteins (FABPs) to the site where they are metabolized [51, 52]. Once transported across the membrane, LCFA are targeted to specific metabolic fates. These findings together with the higher level of CPT-1 mRNA propose that fatty acid is directly required for meiotic resumption and, indicate that β-oxidation is the major pathway contributing to the energy requirement during oocyte maturation and increased rate of lipogenesis at the blastocyst stage which may be needed to support earlier embryogenesis. Then, the mRNA involved in lipid metabolism were compared to deviant oocytes collected from stimulated cows and those matured in vitro to pinpoint impairment of particular pathways of lipid metabolism. The aberrant levels of several mRNAs may indicate that intracellular fatty acid composition is not proper, decreased β-oxidation and may explain the lower progression of meiosis, lower ATP levels and lower developmental competence of these oocytes. The significant lower mRNA levels of Acetyl CoA carboxylase α (ACCα) the main enzyme controlling *de novo* fatty acid synthesis may explain partially the lower developmental competence of in vitro produced blastocysts. This may indicate a change in the quantity of synthesized lipid, resulting in a lack of certain LCFAs needed for membrane integrity and structure.

Although our study measured mRNA of the main pathway of lipid metabolism, the interpretation of the data on fatty acid metabolism has a number of limitations. For instance, considering that over 30 reactions are required to convert acetyl-CoA to triglycerides, there could be many steps or genes that control the yield of end product. Beside that, in addition to fatty acids (FA), glucose is another main oxidized metabolic substrate, however, its role during oocyte maturation as energy source is controversial [53-55]. Interactions between these substrates are thought to control the extent of their respective oxidation, i.e., to control the reciprocal relation between glucose and FA oxidation. However, which of the two substrates, glucose or FA, is the primary regulator of energy in the oocyte is not clearly known and needs further investigations.

CONCLUSIONS AND FUTURE PROSPECTS

This thesis has revealed many of the new basic properties of the bovine oocyte, and based on this information a plausible model for regulation of meiotic resumption and proper oocyte function is possible. Based on this work we now know from **Chapter 3** that mechanisms of meiotic arrest and resumption in bovine oocyte require different phosphodiesterases (PDE7), the involvement of G-proteins, and regulators of G-protein

signaling (RGS) in meiotic resumption, molecular components involved in chromatids separation, regulation of Ca^{2+} oscillatory activity and cell cycle regulation. Most of these genes were identified for the first time in the mammalian oocyte. However, the challenge is to identify the potential ligands that activate these genes which may provide an answer for how meiotic arrest is released. Future research will certainly provide answers to the open questions regarding these issues. Additionally, in **Chapter 4**, we studied different mRNAs crucial for the correct organelles translocation, and chromosome segregation, which may explain the disturbed function of polar body emission and defects in cytoplasmic maturation usually seen in the deviant oocytes from stimulated animals and those matured in vitro. **Chapter 5** provides new information regarding long chain fatty acid transport into the oocytes and regulation of energy requirement during maturation and blastocyst stage using lipid as a substrate. Furthermore, the aberrant expression of lipid metabolism mRNAs levels in the oocyte matured in vitro, as well as that in deviant oocyte from stimulated cows, in addition to that in the in vitro produced blastocysts suggests a defect in fatty acid metabolism.

Although knowledge on oocyte maturation has grown rapidly during the last few years, the field needs to include completion of the molecular details, determination of key molecular structures, assignment of physiological functions, elucidation of physiological regulatory mechanisms, and exploration of interfaces with other cellular systems. Many missing components will be found with available biochemical assays, and molecular and classical genetics along with genome-wide sequencing, and functional analysis will not only be essential for understanding the mechanisms involved, but also will speed the complete elucidation of the regulatory system of the oocyte. Moreover, the discovery of microRNA genes as one of the more abundant regulatory genes in animals [56] may add more complexity to our understanding of oocyte maturation regulation. The current challenge is how to reap the benefits of these numerous techniques and biological knowledge in the field of reproductive biotechnology.

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