Expression analysis of genes implicated in meiotic resumption in vivo and developmental competence of bovine oocytes

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Expression analysis of genes implicated in meiotic resumption in vivo and developmental competence of bovine oocytes

Analyse van de expressie van genen betrokken bij de hervatting van de meiose en de ontwikkelingscompetentie van rundereicellen in vivo

(met een samenvatting in het Nederlands)

Proefschrift

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بسم الله الرحمن الرحيم

وفي أنفسكم أفلا تبصرون

صدق الله العظيم

To my family

To my mentor Mart Bevers

CONTENTS

Chapter 1

General introduction

In 1672, Reinier de Graaf first described the transformation of ovarian follicles into corpora lutea. He reported that both age and coitus cause very great changes in eggs (follicles). In young animals, the follicles are very small, and in more developed ones they are larger. After coitus, the follicles alter to resemble the globules (the corpora lutea), with the number corresponding to the number of fetuses the animal will produce. Ovarian follicles have since been shown to be the basic functional unit of the ovary. When follicle growth is initiated, the oocyte enlarges and the somatic cells expand clonally to produce mural and cumulus granulosa cells within the Graafian follicle [1]. Initiation of primordial follicle growth and transition from a primordial to a primary follicle depends on changes in the ovarian microenvironment. However, the temporal regulation of this process is still poorly understood [2].

The maturation of preovulatory follicles has been studied extensively and involves combined actions of various hormones and locally produced growth factors that prepare the follicle to respond to luteinizing hormone (LH); follicle maturation is gonadotropin dependent and is associated with increasing production of estrogens, mainly estradiol-17β (E2) [3]. This is a result of the action of gonadotropins on the theca and granulosa cells of the developing follicle. Maximal estrogen production occurs at the level of the preovulatory follicle, when the combined actions of FSH and LH are exerted on granulosa cells. The LH surge acts to terminate follicular growth and activate genes that are necessary for follicle and oocyte maturation and ovulation.

There are many similarities in the function of different somatic cells and oocytes. However, oocytes show some remarkable phenomena. The process of meiosis in an oocyte is marked by specific periods of arrest, which are not seen in other germ cells. Moreover, the oocyte has the capacity to remain in a dormant state for years without comprising its ability to resume meiosis and grow. Another important feature of the oocyte is its ability to store large amounts of RNA in a very stable state for many years [4]. Furthermore, the zygote inherits almost whole of its cytoplasm from the oocyte, and the early pre-implantation embryo is therefore dependent on maternally transcribed RNA until activation of its own genome. The successes obtained in cloning certain mammals have further highlighted the enormous potential of the oocyte to remodel, transform and reprogram an older donor cell nucleus [5-7]. These characteristics make the oocyte the most important cell in the body. It is, therefore, essential to understand the mechanisms regulating the resumption of meiosis.

The correct regulation of gene expression is a demanding and vitally important process. As most eukaryotic cells carry an entire organism's worth of genetic information, controlling which genes are turned on, and when, is essential for normal growth and development. A complex interplay between transcriptional regulators (transcription factors) and chromatin structure establishes a barrier to gene activation, which ensures that genes are transcribed only when appropriate signals make their way to the nucleus. Moreover, the transcription proteins themselves have to be present at the right place, at the right time and in the correct amounts, and their activity has to be fine-tuned to produce levels of transcription that are appropriate for each gene. Using several approaches, genes have been identified that are expressed in the oocyte. Using IVM techniques, a number of studies have made important strides in elucidating genes involved in developmental competence and to explore different cellular pathways and mechanisms involved in oocyte maturation [8], cellular response to DNA damage, growth [9], apoptosis induction [10] and transcription regulation [11]. However, although approximately 90% of oocytes collected from 3- to 6-mm antral follicles and matured using IVM techniques can progress through meiosis normally, only 30 to 40% will reach the blastocyst stage, and few will yield viable offspring after transfer. Therefore, the relevance of the information obtained from in vitro culture systems to the physiological situation remains unclear.

Publication of the complete DNA sequence for the human genome has provided the starting point for understanding the genetic complexity of man and the role of genetic variation in diverse processes. It is also clear that primate and rodent models have played an invaluable role in understanding this information. Nevertheless, model animals other than primates and rodents have also played an important role in developing reproductive technology for man. Indeed, the bovine model has been the fundamental research platform for developing human assisted reproductive techniques; treatments and techniques used for superovulation, oocyte maturation, in vitro fertilization and embryo culture and transfer, were all based upon many years of research with bovine oocytes and embryos [12-14]. Comparative genetic maps have also indicated that the bovine and human genomes are more similar than either is with the mouse genome. The mean length of conserved segments between man and cow is approximately twice as long as that between man and mouse [15]. Furthermore, orthologous exonic alignment of non-coding DNA sequences from cattle, pigs, cats and dogs consistently grouped them closer to man than rodent (mouse and rat) sequences [16]. In addition, distinct mechanisms for embryonic genome activation during early preimplantation development exist in man and mouse; during this period the embryo is unable to respond to external stimuli by altering its pattern of gene expression. In human embryos, embryonic genome activation does not occur until the 4 to 8 cell stage [17, 18],

whereas in the mouse embryonic genome activation takes place very early in development, at the late 1-cell stage [19, 20]. In the cow, induction of transcription occurs at the late 4-cell stage [21] making the cow is a better model for man in this regard. Moreover, a recent study showed significant differences among different mice strains induced to ovulate with regard to cytoplasmic microtubule organizing center (MTOC) number [22]; the relatively outbred nature of cattle avoids the limitations inherent to strain-to strain variations. In addition, because of the wealth of information on reproductive techniques in cattle and physiological similarities to man such as being a monovular species, the cow appears an appropriate choice for studying the molecular mechanisms of oocyte maturation and early embryonic development to ultimately help improve human assisted reproductive techniques.

Farm animal biotechnologies that are based on ovarian stimulation, in vitro maturation of oocytes, in vitro fertilization, and embryo culture, and also specialized techniques such as pronuclear injection and somatic cell nuclear transfer have been shown to suffer a number of side effects. These include a high frequency of pre- and post-implantation developmental arrest, abnormal fetal development, increased perinatal loss and elevated birth weight [23-25]; problems that are all unusual under natural reproductive conditions. Moreover, oocytes matured in vitro are less competent of producing offspring than those matured in vivo [26-28], while oocytes recovered from gonadotropin stimulated cows are not all of equal quality [29, 30]. It is clear that the developmental fate of an embryo is largely dictated by the quality of the oocyte from which it is derived, and that the oocyte's microenvironment during maturation but also additional factors can explain or indicate the health, maturity and competence of that oocyte (for review see [31].

Oocyte maturation

The term "meiotic maturation" generally refers to the resumption of meiosis in an oocyte that was arrested in the dictyate stage of meiotic prophase I. Structurally, these events are represented by the breakdown of the germinal vesicle (GVBD) and dissolution of its inner lining, a fibrillar network of laminae. Subsequently, chromosomes move from the center of the nucleus towards the undulating membranes, where condensation takes place. Chiasmata move to the ends of the chromosomes and the chromatin becomes heterochromatic. After completion of condensation, the chromosomal bivalents appear V-shaped and telocentric. Once highly condensed, the chromosomes aggregate in the center of the oocyte, waiting to line up on the metaphase spindle. The spindle apparatus increases in size and moves to the periphery of the oocyte. Metaphase I (Fig. 1, 8) lasts for only a few hours and gives way to anaphase I, when the chromosomal bivalents move towards

opposite ends of the spindle. During telophase I, preparations for the extrusion of the first polar body are made. One set of the recently separated homologous chromosomes is extruded with a small amount of cytoplasmic material into the perivitelline space (Fig 1, 11). This takes place in late telophase I. Once the oocyte reaches metaphase II [32], it arrests until sperm penetration, fertilization and the subsequent events that mark the initiation of embryonic development. The events related to the nuclear changes of meiosis II are unusual in that the chromosome and the DNA content of the oocyte are halved. Moreover, the transition from meiosis I to II is peculiar because progression of the cell cycle occurs without DNA replication between these two stages. Finally, a truly haploid ootid never exists in mammals because the final reduction in chromosome number does not occur until fertilization. All of these nuclear events are encompassed within the term: **nuclear maturation**. Proper and coordinated reductional division must occur to ensure the success of meiotic events. As in the mitotic cell cycle, checkpoint machinery operates in meiosis to ensure that one event does not occur until the preceding event has been completed. To date, two different checkpoints have been shown to operate in meiosis. The recombination checkpoint ensures that cells do not exit the pachytene stage until recombination intermediates have been resolved. The metaphase checkpoint prevents cells from exiting metaphase I until all chromosome pairs have been properly oriented on the spindle apparatus [33]. Meiotic chromosomes frequently do not segregate properly leading in man to a variety of well described birth defects and a very high frequency of miscarriages [34, 35].

The nuclear maturation events of meiosis are accompanied by changes in the **cytoplasm** that prepare the oocyte to undergo the events related to fertilization and activation of embryonic development. Currently, a number of processes are considered to be components of cytoplasmic maturation: these include events that ensure the occurrence of normal fertilization, such as acquisition and redistribution of the smooth endoplasmic reticulum (SER) and calcium stores [36]; an increase in the number and redistribution of the receptor operated calcium channels [37-39], migration of the cortical granules to a position close to the oolemma and acquisition of the ability to control the remodeling of sperm chromatin into a paternal genome that, in concert with the maternal genome, directs embryonic development [40]. The coordination of oocyte cytoskeletal (microtubule and microfilament) dynamics which is critical to the normal progression of nuclear maturation is another important event closely associated with proper cytoplasmic maturation [41].

Transitions between different phases of the cell cycle are driven by cytoplasmic maturation-promoting factor (MPF), which consists of cyclin-dependent kinase (Cdk1 or p34cdc2) and cyclin B [42-44]. Inactive Cdc2-cyclin B is present in the cytoplasm during

interphase but, after it is activated, it accumulates in the nucleus where it phosphorylates multiple targets and initiates nuclear envelope breakdown [45]. Cdk1 activity plays an important role in driving the cell cycle, at least up to the metaphase stage. Completion of the cell cycle is then accomplished by proteolytic machinery anaphase promoting complex (APC) which destroys the Cdk activity by degrading cyclin [46]. An inability to destroy cyclin leads to cells becoming arrested in anaphase and failing to undergo cytokinesis [47]. Although the major events of the cell cycle in somatic cells have been studied in detail, there are still many aspects that are not fully understood. In addition, oocytes and embryos show some differences to somatic cells; oocytes grow very rapidly without dividing, and fertilized oocytes divide rapidly in the absence of growth. For these reasons, it is possible that the pathway leading to activation and cell cycle arrest is different. To date, the molecular and biochemical mechanisms mediating meiotic resumption in vivo are not clear, even though many studies have been conducted in this field [48, 49].

Successful coordination of nuclear and cytoplasmic maturation is of critical significance in ensuring normal fertilization and embryogenesis [50]. In this respect, maintaining the correct nuclear position within the cytoplasm, segregation of genetic material and movement of the male and female pronuclei towards each other, are all processes dependent on microtubule and actin cytoskeletal elements [51-55]. It is widely accepted that oocyte quality as reflected by the ability to give rise to healthy offspring is affected by exogenous hormone administration and by in vitro culture conditions, where the latter are known to affect structural integrity [56], normality of chromosomes [57, 58] and metabolism [59, 60].

In vivo versus in vitro oocyte maturation

In the absence of an LH surge, fully-grown oocytes arrest in prophase I of the first meiotic division. The preovulatory LH peak initiates resumption of meiosis. Subsequently, oocytes undergo GVBD, and then progress through metaphase I, anaphase I, telophase I, before arresting again at metaphase II (MII) until sperm-induced oocyte activation occurs [62]. Alternatively, mechanical removal of oocytes from their normal environment and subsequent culture in vitro induces spontaneous meiotic resumption, without the requirement for gonadotropic hormone stimulation [63, 64].
These observations led to the development of a model for the regulation of meiotic maturation and arrest in mammalian oocytes based on the assumptions that meiotic arrest and resumption is modulated by putative factors in the follicular fluid [65] and cyclic adenosine mono-phosphate (cAMP) levels within both the follicle and the oocyte [66, 67]. However, while a rise in follicular cAMP mediates LH-induced meiotic maturation,

maintaining high levels of cAMP within an oocyte by exposure (in vitro) to dibutyryl-cAMP (dbcAMP) [68] or a phosphodiesterase (PDE) inhibitor [69] prevents that oocyte from resuming meiosis spontaneously. Such opposing actions of cAMP in the two follicular compartments, i.e., the somatic cells and the oocyte, have been explained by the differential localization of subtype-specific PDEs in the granulosa cells and oocyte [70]. Cyclic AMP in turn activates protein kinase A (PKA), resulting in phosphorylation of specific substrates within the oocyte [71, 72].

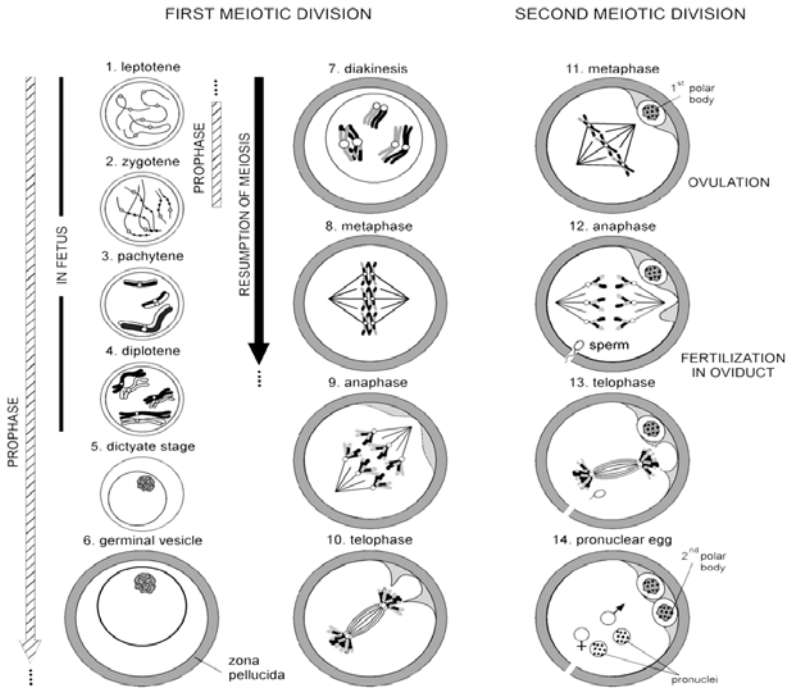

Fig. 1: Schematic representation of meiosis in the mammalian oocyte. Adapted from [61]. Prophase I: 1-5 in fetal ovary, 6-7 germinal vesicle formation. After the LH surge, oocyte meiosis resumes, Metaphase I: 8, Anaphase I: 9, Telophase I: 10, and arrest at Metaphase II: 11untill fertilization. After fertilization, the oocyte enters Anaphase II: 12, Telophase II: 13 which completes the second meiotic division and results in second polar body extrusion 14.

In vitro oocyte maturation (IVM) has been widely used for studying regulation of oocyte maturation, and for the production of embryos, in various species, including cattle [73, 74], sheep [75, 76], pig [77] and man [78]. In recent years, it has become increasingly clear that

oocyte maturation media have a profound effect on the oocyte's ability to resume meiosis and develop into a blastocyst [79, 80]. In cattle, steroid hormones, growth factors and meiotic inhibitors have been shown to modulate the developmental potential of oocytes [9, 81-84]. Nevertheless, the events occurring during spontaneous maturation cannot strictly be compared to the hormone-induced meiotic resumption that occurs in vivo because of the lack of interactions between the oocyte and follicle components in vitro. Moreover, despite the vast amount of bovine oocytes reaching the Metaphase II stage (90%), only 30 to 40% reach the blastocyst stage after IVF and IVC, and only 5 to 10% result in the birth of a live offspring after embryo transfer [31, 85]. It has been postulated that in vitro maturation systems adequately support nuclear maturation in mammalian oocytes, but fail to produce oocytes with a fully mature cytoplasm [86]. Cytoplasmic maturation involves numerous metabolic and structural modifications in preparation for subsequent fertilization [87-90]. Deficient cytoplasmic maturation may be reflected by specific cytoplasmic abnormalities including cytoplasmic inclusions, vacuoles, abnormal lipid accumulation and deficient organelle migration [91, 92].

In vivo, in cow and woman, usually only one follicle becomes dominant and grows beyond 8 mm in diameter; the other, subordinate follicles regress. The dominant follicle continues to develop into a preovulatory follicle, which will eventually release a mature and competent oocyte [93, 94]. There is a number of steps associated with selection and establishment of follicle dominance once a follicle reaches 8 mm in diameter. These include the appearance of LH receptors on the granulosa cells [95], increased production of estradiol [3, 96, 97], leading to an decreased amplitude and increased pulse frequency of pituitary LH release [98].
For experimental studies, cattle oocytes are generally collected from the ovaries of slaughtered cows and from small follicles of 2 to 6 mm in diameter. The acquisition and expression of meiotic competence in IVM oocytes has been related to changes in oocyte growth, germinal vesicle break down, meiotic cell cycle status and transcriptional activity [99-102]. Moreover, oocytes harvested from small follicles may not have undergone an essential pre-maturation step, such that there are various possible reasons for the limited developmental competence of oocytes originating from small follicles following in vitro maturation.

Exogenous hormone stimulation

Follicular development in cattle occurs in a wave like pattern. The emergence of each follicle wave is stimulated by an FSH surge. After emergence, a group of follicles enters a

common growth phase and the FSH surge begins to decline, reaching nadir levels at around the time of follicle size deviation. A transient increase in circulating LH, and enhanced LH receptor expression in granulosa cells has also been reported around the time of selection of the future dominant follicle. At this point, LH stimulates an increase in E2 secretion by healthy follicles which is involved in suppressing FSH secretions to levels lower than required by smaller growing follicles, thereby facilitating the establishment of dominance [103-105].

Ovarian stimulation with exogenous gonadotropic hormones is usually initiated at the onset of the second follicular wave (between days 8 and 12 of the estrous cycle) [106], when FSH and LH are effective in inducing multiple follicle development and ovulation in cattle (Figure 2).

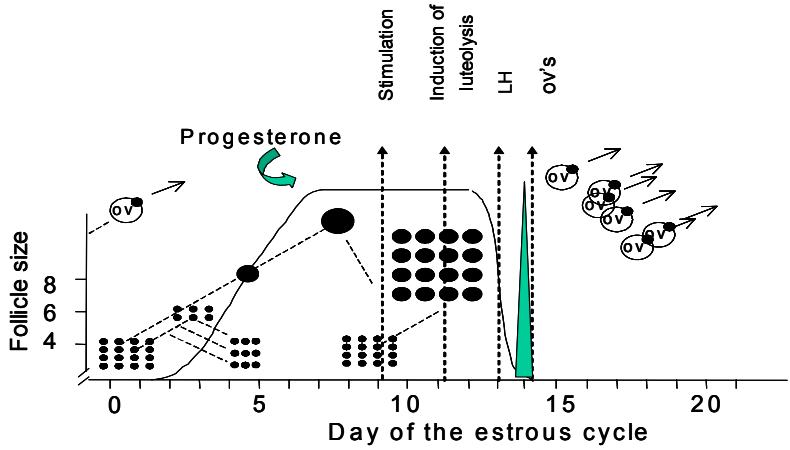

Figure 2. Schematic model of follicular dynamics on the ovary and changes in progesterone and LH concentrations in the peripheral blood during the estrous cycle in the cow. A commonly used scheme for superovulation is depicted at the top of the figure. (Adapted from van de Leemput, E. E, Thesis, 1998)

Despite drawbacks of current superovulation techniques, this basic approach is still the only method available under practical conditions. The only way in which hormone stimulation increases the likelihood of achieving a pregnancy is by increasing the number of oocytes or embryos retrieved, and which can be used for various assisted reproductive techniques. It is well known that the hormonal milieu of the follicles is altered in cows stimulated with exogenous gonadotropin, to a degree depending partly on the type of protocol and the exact hormone regime used [29, 107-110]. Studies in vivo and in vitro

have demonstrated that different gonadotropin preparations have different effects on the expression of steroidogenic enzymes and subsequently on steroid hormone production by ovarian follicle cells [111-114]. Ovarian stimulation in mice has been shown to result in a significant increase in the frequency of oocyte spindle defects, resulting in chromosomal errors [115-117]. Reduced fertility and increased pre- and post-implantation mortality have also been reported as consequences of using standard doses of gonadotropins [118-127]. Cytogenetic analysis of pronuclear stage mouse embryos after ovarian stimulation demonstrated a higher incidence of chromosomal aberrations in the female pronucleus compared to the male pronucleus, indicating a developmental abnormality of oocyte origin [125]. Although findings from various species have contributed to our understanding of ovarian stimulation effects, few studies have examined the effect of ovarian stimulation on gene expression. Proper spindle positioning and accurate chromosome segregation have common molecular requirements in diverse organisms: microtubules, actins, dynein, myosin, kinesins and formins. Bovine preovulatory follicles are much larger than those in rodents, which makes it possible to study steroid levels as a marker for follicle quality and examine the latter's relevance to gene expression.

Molecular motors and chromosomal segregation genes

The active transport of organelles, proteins, RNAs, and chromosomes along the microtubules and microfilaments of the cytoskeleton to specific destinations within a cell is essential for normal development. Microtubules are polarized in such a way that the minus ends are always located at the microtubule organizing center, near the nucleus while the plus ends extend to the periphery. Dynamics and interactions of molecular motor proteins with the cytoskeleton provide the machinery for most membrane trafficking within the cytoplasm of higher eukaryotes. In most mammalian cells, two classes of motor proteins with opposite directionality, kinesins and cytoplasmic dyneins achieve oriented transport using microtubules.

Cytoplasmic dynein is a minus end directed motor complex that consists of heavy chains, intermediate chains and light chains [128]. The heavy chain contains the motor domain [129] and the other chains appear to target the motor to various cargos [130, 131]. Although the full complement of microtubule motors has been identified in several model organisms, their functions and hence the mechanisms of many active transport processes remain poorly defined. Dynein has been shown to participate in a variety of processes, including nuclear migration, centrosome separation, spindle formation [132], spindle alignment, chromosome segregation [133], nuclear envelope breakdown [134], mRNA localization [135, 136], and

organelle transport [137]. Kinesin was first found in neural tissue, where it functions to generate plus end directed movements [138] and subsequent studies have suggested an essential role in meiosis [139, 140]. Since microtubules undergo profound changes in organization during meiotic maturation in mammalian oocytes [141] and these changes are implicated in the regulation of organelle movement, distinct expression patterns may occur during meiotic maturation and early embryonic development.

Actin-based motor cytoplasmic myosin has been demonstrated to participate in many organelles and cellular movements during oogenesis and embryonic development [41, 142-146]. A recent study showed that in fertilized mouse oocytes, inhibition of myosin II or myosin light chain kinase (MLCK), which belongs to the family of Ca^{2+} /calmodulin-dependent protein kinases and specifically phosphorylates myosin regulatory light chains, inhibited second polar body formation and reduced cortical granule exocytosis [147].

Mammalian cells frequently exhibit an asymmetric distribution of organelles, proteins or cytoskeletal components along a particular axis. This internal organization is referred to as 'cell polarity' [148]. Meiotic division requires the correct positioning of the spindle within the cell, in addition to assembly of the spindle apparatus and segregation of the chromosomes. Formins are proteins that mediate interactions between microtubules and the cell cortex to establish spindle position, they also function as nucleators of actin filaments and are involved in motile processes such as the formation of actin cables in yeast, assembly of actin filaments in the cytokinetic ring [149] and mitotic spindle position in yeast [150]. Partition defective protein (par) genes have been identified as essential regulators of egg polarity in *C.elegans* and with a specific protein kinase C (PKC-3) form a complex in the anterior half of the *C. elegans* zygote [151, 152]. Recent studies in mice showed that, during oocyte maturation, spindle associated par-3 was translocated to the cortex, suggesting a role for par-3 in establishing asymmetry in the oocyte and defining the future site of polar body emission [153].

Meiotic and developmental competence is acquired progressively during follicle and oocyte maturation and is associated with a series of nuclear and cytoplasmic changes [154]. It is well known that oocytes undergo various structural changes during maturation including mitochondrial redistribution, cortical granule translocation and mRNA localization. Since oocytes matured in vitro or resulting after ovarian stimulation, show inferior cytoplasmic competence, in terms of defects in cortical granule translocation and chromosomal mis-segregation [30, 116, 117, 155, 156], it seems likely that the motor proteins regulating chromosome segregation play a role in several other aspects of oocyte

maturation and developmental competence. However, the function and expression of these proteins within the oocyte is currently rudimentary.

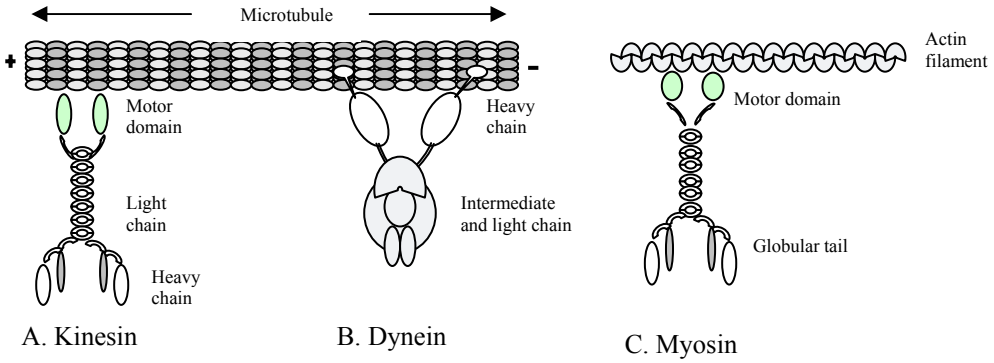

Fig. 3. A basic overview of the structure of the molecular motors; kinesin, dynein and myosin. All of the molecular motors are large multiprotein complexes with a dimeric structure. They each consist of a globular head domain, which contacts the cytoskeleton and provides the force for motility by hydrolysing ATP, and tail domains, which are more divergent and likely to be involved in cargo recognition. **(A)** Kinesin is in general a plus-end-directed MT motor. However, there are many kinds of kinesins, including minus-end-directed motors. **(B)** Cytoplasmic dynein, the major MT minus-end-directed motor in the cell, is responsible for the transport of many cargos. It is the largest of the motors and has an accessory multiprotein complex, **(C)** Non-muscle myosins are a large and diverse family of actin-based motors. Modified from [157]

Fatty acid transport and metabolism

The mammalian oocyte and embryo are able to use a variety of substrates for the production of ATP. Lactate and glucose, and to lesser extent amino acids, can all be metabolized to produce ATP [60, 158]. The extent to which these exogenous sources contribute to ATP production varies, and depends on the stage of maturation or on the stage of development after fertilization [159, 160].

Lipids comprise one of the most important classes of complex molecules present in animal cells. Within any mammalian cell, lipid diversity and concentrations are determined by the process of lipid metabolism, including lipid transport, utilization and *de novo* synthesis. Lipids are used as a means of communication, for regulating of a variety of physiological processes (as hormones), and as cellular constituents for the protection against a desiccating environment (phospholipids). Moreover, the ability to synthesize and

store fat and then utilize it as an energy source is central to the development and survival of many mammalian cells. An imbalance in lipid metabolism can lead to developmental failure. Therefore, understanding the regulatory pathways that govern energy intake and storage (i.e., fat accumulation) versus energy expenditure (i.e., fat oxidation) is a key to understanding energy requirement of the oocyte during maturation and of early embryonic development during culture.

Long-chain fatty acids (LCFA) are thought to be the class of lipids serving the most diverse functions. Fatty acids are the primary substrate for energy in liver and muscle cells, and are needed for the formation of complex lipids, amongst which the phospholipids are of central importance as constituents of cell membranes [161, 162], and as signaling compounds [163] to modulate the expression of specific genes [164, 165]. These various dynamic functions of fatty acids require that their availability, both outside and inside cells, be tightly controlled, not only to make sufficient amounts of fatty acids available when needed, but also to prevent them from local accumulation since this may dramatically impair cell function [166, 167].

There are different mechanisms by which lipids can move intracellularly. In general, medium chain fatty acids move to a desired site within a cell by diffusion while long and very long fatty acids use specific lipid-transfer protein-mediated transport [166]. Several membrane proteins that increase the uptake of LCFAs when over-expressed in cultured mammalian cells have been identified. The best characterized of these are FAT/CD36 [168] and fatty acid transport proteins (FATPs; solute carrier family 27). CD36, also known as fatty acid translocase, is a receptor for several ligands, including oxidized LDL and long-chain FFAs [169, 170]. Expression of CD36 in fibroblasts which do not endogenously express CD36, is associated with an increase in FA uptake and incorporation into phospholipids [171]. The distribution of CD36 favors tissues with a high metabolic capacity for FAs such as adipose tissue, heart and skeletal muscle [169], where it is involved in high-affinity uptake of fatty acids [173, 174]. In addition, mice null for CD36 have been shown to exhibit increased serum FA, triglyceride and cholesterol levels [172]. Although lipid transfer proteins have been described extensively in various tissues [175, 176], evidence for their active presence in mammalian oocytes and early embryos is lacking. Six FATP genes have been described in the human and mouse genomes (FATP1-6). FATP1 was the first family member identified [177] and is thus far the best studied. Expression of FATP1 in mammalian cells increases the import of LCFAs and very long chain fatty acids (VLCFAs), but not of medium chain substrates [178]. However, the mechanism of LCFA import and whether its regulation is relevant to oocyte maturation and early embryonic development is

unknown. Understanding these expression patterns may contribute to improvements in IVM culture techniques to produce competent oocytes.

Once transported across the cell membrane, LCFAs are targeted to specific metabolic pathways. A candidate factor capable of coordinating responses to different lipid constituents is AMP-activated protein kinase (AMPK), it is a heterotrimeric enzyme that is conserved from yeast to man and functions as a gauge to monitor cellular energy stores [179-181]. AMPK regulates the expression of genes involved in lipid synthesis by modulating the activities of different transcription factors and coactivators [182]. The nuclear receptors, transcription factors and peroxisome proliferator-activated receptors (PPARα and PPARγ) that are activated by AMPK, and the fatty acid derivatives released as a result regulate gene networks that promote lipid synthesis and storage in adipocytes (PPARγ), or activate oxidation pathways (PPARα) [183]. There are numerous reports linking the expression of PPARs in granulosa cells to ovarian function [184-187], but their expression in the oocytes itself has not been documented in detail. Identifying the pattern of changes in expression of these transcription factors will not only provide information about energy requirements during oocyte maturation and early embryonic development but may clarify whether fatty acids and changes in culture conditions can modify oocyte maturation through direct interactions with transcription factors within the oocyte.

AMPK also phosphorylates acetyl-CoA carboxylase (ACC) leading to inhibition of ACC activity and decreased malonyl-CoA content. Two major mammalian ACC isoforms have been identified. ACCα and ACCβ are products of distinct genes with different tissue expression patterns, physical and enzymatic properties, the expression of which responds to hormones or changes in dietary composition during development [188-194]. Both ACC isozymes produce malonyl-CoA which is required for fatty acid synthesis and, together with fatty acid synthase [195] for fatty acid chain elongation systems, and also inhibits mitochondrial carnityl palmitoyltransferase (CPT), the rate-limiting enzyme in the import and oxidation of fatty acids in mitochondria [196]. Moreover, ACC catalyzes a pivotal step in fuel metabolism because it links fatty acid and carbohydrate metabolism through the shared intermediate acetyl-CoA. To date, however, there has been no evidence to support the existence of this system in mammalian oocytes. Moreover, lipid transfer activities have not been described in either oocytes or in developing embryos.

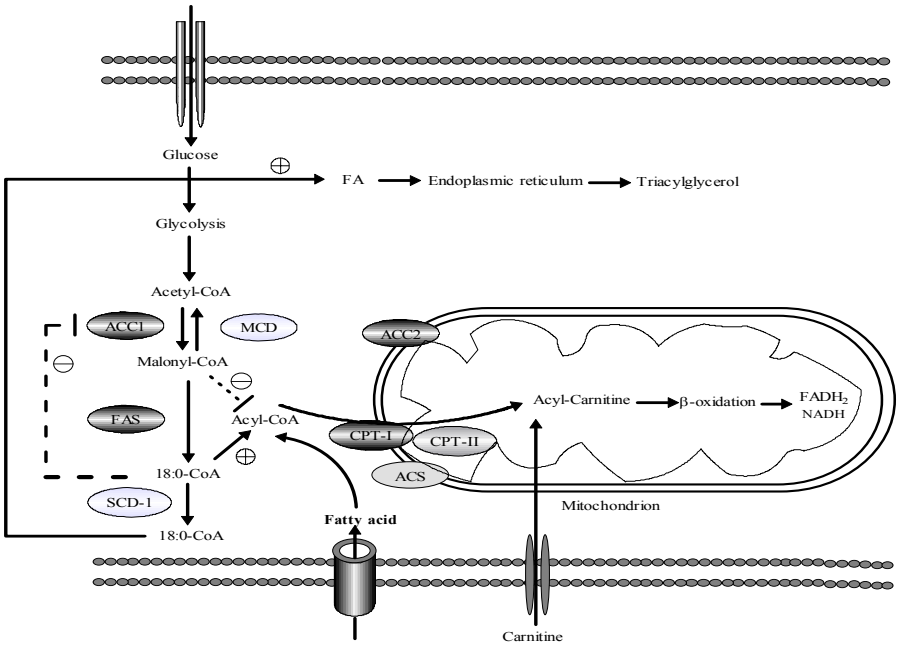

Figure 4. Metabolic pathways involved in lipid metabolism of liver and skeletal muscle. Among the lipid metabolic enzymes, acetyl-CoA carboxylase (ACC), fatty-acid synthase (FAS) and carnitine palmitoyl transferase (CPT) are the three that primarily regulate the synthesis of malonyl-CoA, which is the principal inhibitor of fatty-acid entry into mitochondria for β-oxidation. Stearoyl-CoA desaturase-1 (SCD1) regulates lipid oxidation by converting stearic acid (18:0) to oleic acid (18:1). The saturated fatty acyl-CoAs are known to allosterically inhibit ACCα, FAS (FAS) and acyl-carrier protein (ACP) and are involved in fatty-acid synthesis; however, their role in lipid metabolism remains unclear. ACS, acyl-CoA synthase; MCD, malonyl-CoA decarboxylase. Modified from [197].

Methods for studying differential gene expression

Different techniques have been developed for the screening of genetic alterations at the mRNA level. The most widely used being subtractive hybridization, differential display, serial analysis of gene expression and cDNA microarray hybridization.

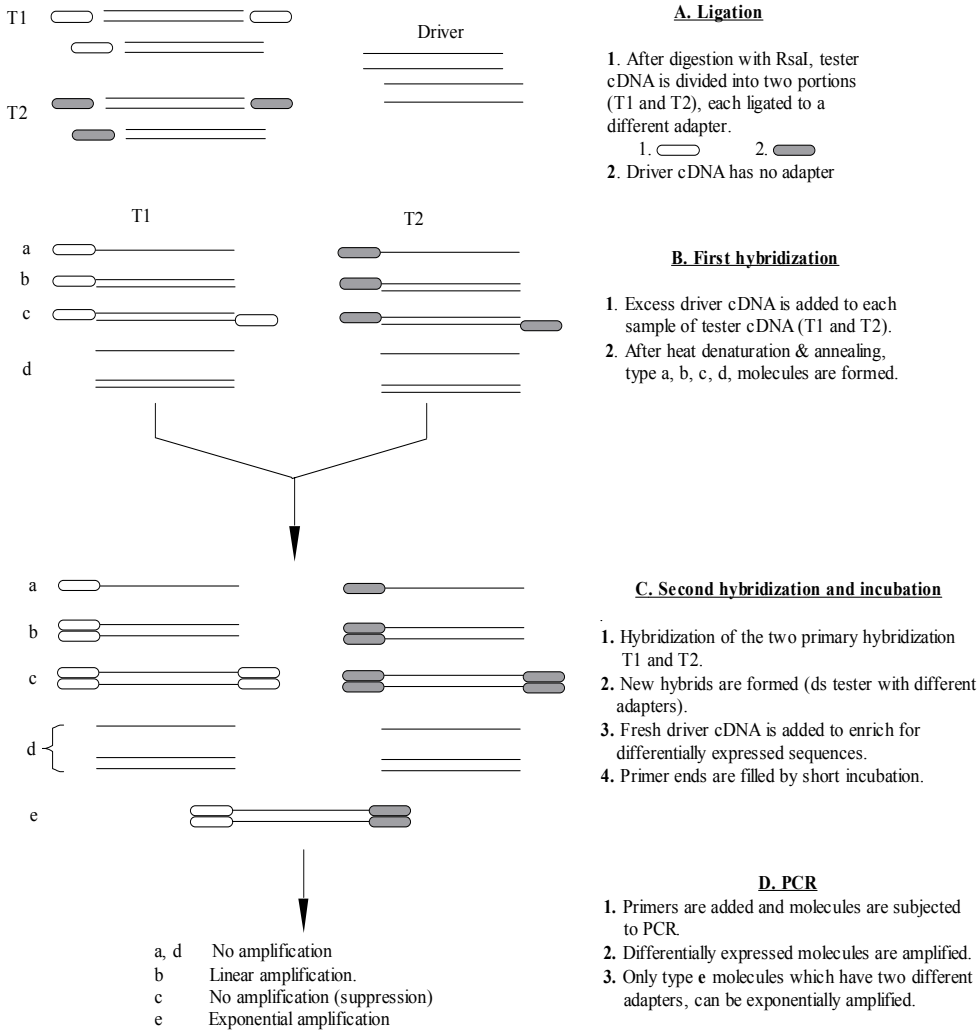

Fig. 5. Schematic diagram of the cDNA library construction procedure using suppression subtractive hybridization. Modified from [200]

Suppression Subtractive hybridization (SSH)

SSH is a technique used to isolate nucleic acids present in one sample but not in another. The two samples compared are the "tester" and "driver" (Fig. 5). The tester is the sample from which differentially expressed sequences are to be isolated, and the driver is a reference sample. Subtraction is based on hybridizing the sequences present in both the tester and the driver samples, and subsequent separation of driver and tester-driver hybrids from unhybridized tester DNA. To achieve as complete a subtraction as possible, an excess of driver DNA is used during hybridization [198]. The most developed version of subtraction hybridization SSH, combines normalization with subtraction in a single step [199]. As a result, concentration of the high and low abundance cDNA species is equalized, subtraction is effective with all sequences, and the probability of detecting rare, differentially expressed sequences is increased. In selection, a suppression PCR is used to selectively suppress amplification of non-target DNA molecules, while the target molecules are exponentially amplified in the same reaction. SSH is a powerful technique for enriching differentially expressed genes. The subtracted cDNA population can be used to construct a cDNA library followed by screening with another method like microarray. SSH is also suitable for detecting novel sequences.

DNA microarray

A DNA microarray consists of thousands of individual gene sequences printed in a high-density array on a glass microscope slide. It provides a practical and economical tool for studying gene expression on a very large scale [201, 202]. The introduction of array technology to the study of mammalian oocyte transcription has been held back by the low number of oocytes available for array analysis. Indeed, the ideal approach to gene expression profiling would be to use full genome microarrays to identify genes up- or down-regulated at specific stages of maturation. This requires a fully sequenced genome something which currently exists only for a few organisms including man, *Drosophila, Caenorhabditis elegans,* yeast, mouse and rat and not the cow [203]. In organisms lacking a fully sequenced genome, screening and identifying genes requires alternative techniques such as differential display polymerase chain reaction (DD PCR) and SSH.

Nucleic acid microarrays primarily use short oligonucleotides (15– to 25 nt), long oligonucleotides (50 to –120 nt) and PCR-amplified cDNAs (100 to –3,000 base pairs) as array elements. Both types of elements have shortcomings, for example short oligonucleotides sometimes lack the specificity required to ensure single-gene specificity in

complex hybridizations; for this reason, a 'tiling approach' with multiple short oligos per gene is often used. Because of their extended length, PCR amplified cDNAs produce strong signals and high specificity. The cDNA inserts are readily obtained from cDNA libraries, and are typically used for organisms for which only limited genomic sequence is available [204-206].

During a DNA microarray assay, two RNA samples are labeled separately with different fluorescent tags (for example, cyanine 3 and cyanine 5 (Cy3, Cy5)), hybridized to a single microarray and scanned to generate fluorescent images from the two channels. A two-color graphical overlay can then be used to visualize genes that are activated or repressed (Fig. 6). All microarray strategies allow comparisons of tissue types such as heart versus brain, normal versus diseased tissue samples, or time-course samplings of cell cultures subjected to different treatments or conditions, and both schemes yield high-quality gene expression data.

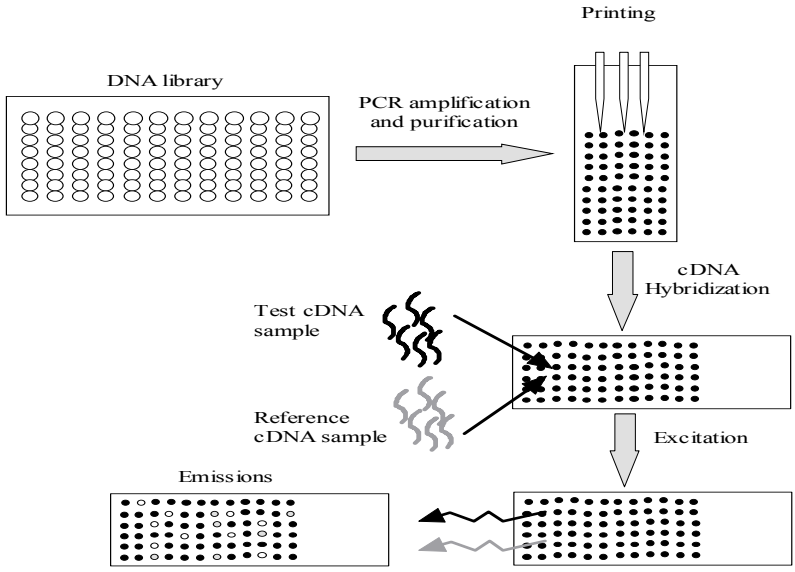

Fig. 6. Schematic diagram of a microarray procedure. Modified from [212]

Several groups have used microarray techniques to capture the total molecular information by making SSH cDNA libraries [203, 207-209]. However, because of the

difficulty in obtaining large numbers of mammalian oocytes, molecular studies have been limited to examining transcripts in oocytes from slaughterhouse material matured in vitro. Currently available approaches to overcoming limitations inherent to gene expression profiling of cell and tissues types of limited abundance are to amplify starting RNA or to amplify cDNA using PCR based strategies. The *S*witch *M*echanism *A*t the 5` end of *R*everse *T*ranscript (SMART) amplification method, available as a kit from Clontech overcomes this problem [210]. This method utilizes a modified oligo dT primer (CDS II/3PCR primer) to prime the reverse transcription, and the SMART II oligonucleotide in the same reaction to facilitate template switching by reverse transcriptase [210, 211]. The template switching generates single-stranded full-length cDNA libraries that can then be used for different purposes.

Quantitative polymerase chain reaction (QPCR)

In general, regulation of protein abundance in a cell is accomplished solely by regulation of mRNA; virtually all differences in cell type or state are correlated with changes in the mRNA levels for specific genes. Therefore, knowing when a gene is expressed often provides a strong clue as to its biological role. In a conventional PCR, the amplified product is detected by an end-point analysis, by running DNA on an agarose gel after the reaction has finished. In contrast, real-time PCR allows the accumulation of amplified product to be detected and measured as the reaction progresses, that is, in "real time". This is made possible by including a DNA-binding fluorescent dye in the reaction; specialized thermal cyclers equipped with fluorescence detection systems are then used to monitor fluorescence during the exponential phase of the reaction [213, 214].

QPCR is one of the most sensitive and reliable quantitative methods for measuring gene expression. It allows the amplification of mRNA in low abundance and permits the analysis comparison of gene expression in low numbers of samples. It has been broadly applied to quantification of transcripts in oocytes and early embryos [11, 207, 215-217]. Accurate quantitative PCR analysis depends on the integrity of the purified RNA. Real-time RT-PCR needs high quality, DNA-free, and un-degraded RNA [218]. Similarly, the reverse transcriptase step (RT) is a source of variability. In addition to reaction conditions and the enzyme used; priming method used to initiate cDNA synthesis can affect cDNA synthesis efficiency. Target gene nonspecific primers using random primers is widely used to circumvent high inter-assay variation in the RT reaction [219], using nonspecific primers can also maximize the number of genes that can be assayed from a single cDNA pool.

Another important marker for kinetic PCR is the fluorescent probe used to quantify detection. Two methods have become established for the detection of amplicons, Gene-specific fluorescent probes like Taqman and FRET, and non-sequence specific fluorescent dsDNA binding dyes like SYBR Green I and ethidium bromide (for review [213, 214]). The level of expression can be measured by absolute or relative quantitative real-time PCR. Absolute quantification relates the PCR signal to input copy number using a calibration curve, while relative quantification measures the relative change in mRNA expression level (for review see [220]).

Scope of the thesis

The objectives of this study were to characterize and clarify the regulation of meiotic resumption in in vivo matured oocytes at the transcriptional level, including the possible role of expressed genes in developmental competence. Following a brief introduction on general aspects of oocyte biology, oocyte maturation, different reproductive biotechnologies and the methods used to study gene expression in **Chapter 1**, we investigated the temporal changes and relationships between follicular fluid steroid concentration and follicle size at various stages of follicle and oocyte maturation; the methods used to distinguish between follicles based on steroid profile are discussed in **Chapter 2.** The objective of **Chapter 3** was to describe the transcriptome of the bovine oocytes at the onset of meiotic resumption, and to elucidate the functional importance of these genes in regulating meiotic resumption. In **Chapter 4,** the transcription levels of selected genes known to be involved in spindle formation and chromosome segregation were quantified using QPCR at different stages of maturation in vivo in oocytes collected from cows stimulated with oFSH, and classified as normal or deviant based on follicular steroid profiles; the same transcripts were quantified in oocytes at corresponding stages of maturation in vitro. Because of the lack of information about lipid metabolism in the oocyte, in **Chapter 5** the expression patterns of eight mRNAs that play key roles in lipid metabolism were analyzed at various stages of oocyte maturation and early embryonic development in vivo; the same transcripts were quantified in oocytes matured and blastocysts produced in vitro to examine the impact of in vitro culture conditions. Finally, the overall implications of the results obtained during this PhD study are discussed in **Chapter 6.**

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Chapter 2

Effects of oFSH stimulation on steroid concentrations in the fluid of preovulatory follicles during final maturation in relation to the LH surge in the cow

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Abstract

FSH-stimulation is used to collect in-vivo oocytes to study maturation and embryonic development. However, not all oocytes are competent. Therefore, selection of functional follicles is a prerequisite. Steroid concentrations in the follicular fluid known to characterize normal preovulatory follicles are not yet defined for FSH-stimulated follicles during the 24 h prior to ovulation. We evaluated the steroid concentrations in all follicles ≥ 5 mm at 2 h before, 6 and 22 h after LH in cows (n=26) treated with oFSH plus GnRH-controlled LH peak. In small follicles (n=101) the concentrations were significantly different from those in large follicles > 8 mm (n=352). In large follicles estradiol 17β was high before LH and decreased thereafter, and progesterone increased at 6 h and further at 22 h after LH, which resembled the pattern in normal preovulatory follicles. Estradiol and androstenedione were distinctly lower before and at 6 h after LH compared to normal follicles or after eCG-stimulation, which could be due to low LH-activity of the oFSH.

Comparison of steroid concentrations in large vs. small follicles indicated that selection criteria for follicles > 9 mm can be limited to: estradiol before LH (> 0.9 µmol/L) and at 6 h after LH (> 0.5 µmol/L), and to progesterone at 22 h after LH (> 0.5 µmol/L). Disqualifying also the few follicles with proper steroid profile but deviating cumulus-oocyte-complex, 51% of the follicles > 9 mm could be considered to enclose a competent oocyte. The stimulation protocol with oFSH and controlled LH surge facilitates the collection of 6 to 7 competent oocytes per cow at specific stages of final maturation.

Keywords: FSH stimulation, final maturation, steroid concentration, oocyte competence, follicle quality

Introduction

Ovarian stimulation protocols are widely used to unravel the molecular and biological mechanisms defining the competence of cow oocytes to develop into viable embryos [1-3]. But not all such oocytes are of equivalent quality. In contrast, in normal cyclic cows, usually only one growing dominant follicle acquires specific structural and functional characteristics to differentiate to the preovulatory stage. During this stage, when a certain level of estradiol 17β is reached in the circulation produced by the growing follicles, the feedback of estradiol 17β on gonadotropins is changed from negative to positive resulting in the preovulatory LH surge that elicits the resumption of meiotic maturation of the oocyte. Six hours after the maximum of the LH surge, concomitant with germinal vesicle break down (GVBD) the estradiol 17β concentration decreases rapidly in the microenvironment of the maturing oocytes and the follicle turns to a progesterone producing structure [4,5]. Only at the completion of a varied set of intracellular changes, including a decrease in the size of Golgi complexes, undulation of the nuclear membrane and a more superficial location of the clusters of cortical granules, the oocyte can finally acquire its full capacity to support fertilization and embryogenesis [6].

Exogenous gonadotropins are used to stimulate development of multiple preovulatory follicles in the mammalian ovary [7] for the collection of increased numbers of oocytes. However, not all oocytes will show the same developmental competence due to deviations in preovulatory follicular development [8, 9]. This heterogeneity in quality is probably due to intrinsic differences between oocytes originating from different follicular microenvironment as can be inferred from the considerable evidence for endocrine regulation changes after stimulation compared to normal cyclic cows. Firstly, a reduction occurs of endogenous basal secretion, pulse frequency and amplitude of FSH and of pulse frequency of LH by more than 50% [10,11], as well as a shortening of the period of preovulatory follicular development from 61 to 41 h in comparison to unstimulated cows [12]. Secondly, superovulation treatment has been shown to induce abnormal amounts of steroids in serum compared to the physiological levels seen during natural cycles [12-16]. Thirdly, different studies have also shown that follicular cells derived from stimulated cows have altered gonadotropin receptor mRNAs [17] and altered abundance of several transcripts of steroidogenic enzymes genes [18].

To explain and to improve the variability in oocyte competence to develop into viable embryos, the amount of required LH bioactivity in the follicle stimulating gonadotropin has been studied extensively. Equine chorionic gonadotropin (eCG) and gonadotropins with high LH bioactivity have been shown effectively to induce multiple follicle development, final oocyte maturation, ovulation and corpus luteum formation [19-21]. However, eCG has an extended half life and can be still detected in serum days after the end of the treatment [12]. Continued support of the follicular cells by eCG increases steroid levels in follicular fluid [22] and therefore prolongs the exposure of the oocyte to estradiol 17β which has been shown to be detrimental to spindle formation in vitro [23,24]. Currently, in clinical applications with purified pituitary FSH is used either with added LH to a bioactivity ratio of 1:1 or with only a low remaining LH bioactivity, both products showing similar yields of viable embryos. Stimulation with FSH with low LH is now more common and has been proven to be an effective alternative to eCG protocols in terms of embryo quality [25-27]. However, in contrast to eCG, this type of FSH results in lower concentrations of estradiol 17β in serum and follicular fluid, and of progesterone in serum [16, 28]. When LH bioactivity is completely absent upon stimulation as with human recombinant FSH, development of preovulatory follicles still takes place but these follicles have a markedly reduced estradiol 17β concentration and contain oocytes that lack cytoplasmic maturation shortly before ovulation [29]. Therefore, balanced amounts of both FSH and LH are required for proper stimulation of follicles in the cow. The resulting steroid levels in the follicular fluid appear to be important determinants in the production and complex interaction of growth factors ensuring follicle growth and differentiation, and ultimately oocyte nuclear and cytoplasmic maturation [30, 31].

The developing ovarian follicle is one of the most rapidly proliferating normal tissues known in vivo, and granulosa cells account for the majority of this follicle expansion. During the preovulatory period a large change in the steroid content occurs concomitant with this proliferation [32]. Since superovulation leads to varying degrees of preovulatory follicle size, changes in steroid concentration may also arise in these follicles that most likely concur with varying degrees of oocyte developmental competence. Recently, a significant relationship has been reported during GnRH induced ovulation between follicle size, serum progesterone and pregnancy rates [33]. However, in cows stimulated with exogenous gonadotropins, serum steroid levels reflect a sum of steroid produced by many follicles. As a result, predicting oocyte quality based on serum steroid concentration is not possible. In striking contrast to the wealth of information available regarding superovulatory response and embryo quality

after treatment with FSH, little is known concerning steroid concentrations in preovulatory follicles after FSH treatment in relation to size of the follicles. Therefore, follicular fluid concentrations of estradiol 17β, progesterone, testosterone and androstenedione were studied in cows stimulated with FSH with low remaining LH activity at onset, after initiation and at completion of final maturation of the oocyte.

2. Materials and methods

2.1. Experimental design

Normally cyclic Holstein-Friesian cows were treated for superovulation using the protocol as described before [34] with oFSH and a Crestar/GnRH-controlled LH surge (Fig. 1). The concentration of LH in plasma was monitored to determine the time of ovariectomy for the collection of follicles relative to the maximum of the LH surge, and to exclude the few, eventual cows with deviating LH profiles [35]. Cows were allocated at random to three experimental groups: 1) at onset (2 h before LH), 2) after initiation (6 h after LH), and 3) at completion (22 h after LH) of final maturation. The experiment was carried out as approved by the Ethical Committee of the Veterinary Faculty of Utrecht University. For comparison of steroid levels, data was used that had been collected previously for preovulatory follicles from eCG-stimulated cows [36] and from untreated, cyclic cows [37].

2.2. Animals and treatments

For the FSH group, thirty-three lactating Holstein-Friesian cows were selected after clinical examination, that were on average 4.4 y of age and 185 d post partum. While on pasture, progesterone concentrations in peripheral blood samples were measured three times a week. After at least four weeks, cows (n=30) with normal cycles were pre-synchronized preceding the experiments in groups of 4 animals using an ear implant for 9 days and prostaglandin as described before [34]. Following the synchronized estrus, the cows were housed inside, and were fed silage and concentrate with water supplied ad libitum. On Day 9 of the estrous cycle (Day 0 is estrus), the cows received another ear implant (3 mg norgestomet, Crestar; Intervet International B.V., Boxmeer, The Netherlands) for 5 d but not further combined with norgestomet and estradiol valerate im. From Day 10 onwards, the cows were treated with oFSH im (Ovagen ICP, Auckland, New Zealand) twice a day in decreasing doses during 4 d (3.5, 2.5, 1.5 and 1 mL; in total 17 mL equivalent to 299 IU NIH-FSH-S1). Prostaglandin (PG; 22.5 mg

Prosolvin im; Intervet International B.V.) was administered together with the fifth dose of FSH. Ear implants were removed at 49 or 50 h after PG, and GnRH (0.021 mg Receptal im; Intervet International B.V.) was administered concomitantly with the 50 h after PG implant removal to induce the LH surge. Follicular fluids were collected following ovariectomy (OVX) at 50, 58 and 74 h after PG corresponding with 2 h before, 6 and 22 h after the maximum of the LH surge, respectively.

For the eCG group, pre-synchronized cows in a previous experiment had been treated with 3,000 iu eCG (Folligon im; Intervet International B.V.) on Day 10 and 22.5 mg PG 48 h later on Day 12 [36]. Follicular fluids had been collected by transvaginal ultrasound-guided puncture from preovulatory-sized follicles at 12 h before, 4, 12 and 22 h after the maximum of the endogenous LH surge.

For the untreated, cyclic group, pre-synchronized cows in a previous experiment had been monitored for the onset of luteolysis by frequently determining the concentration of progesterone in peripheral blood from Day 14 onwards [37]. Preovulatory follicular fluids had been collected following OVX at 48 to 62 h after luteolysis while, in cows, the interval between onset of luteolysis and LH surge is 61 h [38].

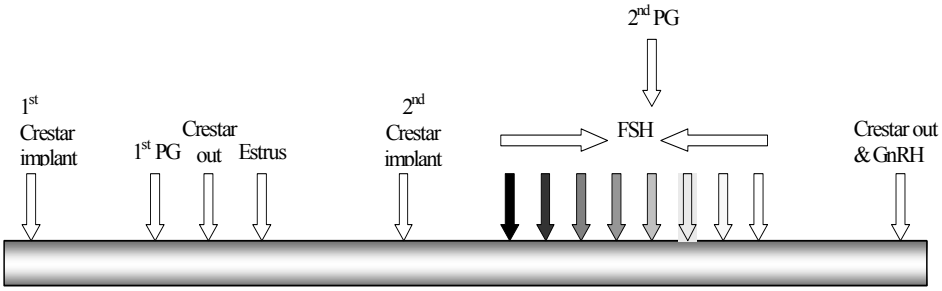

Figure 1. **Schedule of treatment for pre-synchronization and superovulation with a Crestar/GnRH-controlled LH surge to obtain oocytes at specific times of development;** PG=prostaglandin. The precise timing of the administration of PG during the FSH treatment was determined at intervals of 1 h to allow for periods of 1 h between each cow at ovariectomy. Similarly, removal of the 2nd ear implant (Crestar out) and administration of GnRH were carried out at 1 h intervals; a maximum of 4 cows was used every treatment run.

2.3. Blood sampling

Heparinized blood samples were collected from the jugular vein every day during the experimental cycle, every 3 h starting 12 h before removal of the second implant and every hour thereafter for 6 h. After immediate centrifugation at 4°C, plasma was stored at -25°C.

2.4. Collection of follicles

For every treatment run with a group of 4 cows OVX was performed at 1 h intervals (See text of Fig. 1) by laparotomy through flank incision under local infiltration anesthesia [5]. Ovaries were collected in 0.9% NaCl at 37°C and immediately transported to the laboratory. The numbers of follicles were recorded per size class 2 to 5, 5 to 8 and > 8 mm. The content of each follicle > 8 mm was aspirated using an 18-ga winged infusion set needle attached to 15 ml polystyrene conical tube under low pressure by means of a suction pump, and were then immediately stored on ice at 4°C. The size of the follicles was calculated from the volume of follicular fluid after collection which resulted in a slight underestimation due to some eventual loss of fluid and not accounting for the thickness of the follicular wall. As can be derived from the formula for volume (= 4/3*pi*r3 with 2r=diameter) underestimation is more manifest with small follicles. Follicles with a calculated diameter of > 9 mm can be assumed to have been > 10 mm. After retrieval of the cumulus oocyte complexes (COC) under a stereo microscope, the follicular fluids were centrifuged 3,000 *g* for 10 min at 4°C and stored at -25°C until analysis for steroids. The morphological appearance of COCs at the successive stages of in vivo maturation was rather different from that of slaughterhouse oocytes. Therefore, the standard qualification as used for in vitro oocytes was not applied to distinguish between grades. The COCs were further processed and stored for other studies into the transcriptome of oocyte and cumulus. The fluid of follicles of 5 to 8 mm as established by ruler was aspirated by a 2 ml syringe to estimate the volume, and was stored at -25°C until analysis for steroids.

2.5 Assays for hormones in plasma and follicular fluid

Concentrations of LH were determined in duplicate aliquots of 100 μL plasma by a homologous double-antibody RIA as described previously [4]. Bovine LH (bLH-7981) was used for iodination and standards, and rabbit anti-bLH (8101) as antiserum. Specificity of the RIA was high as indicated by low cross-reactivity for other pituitary hormones and by the observed parallelism with NIH-LH-B4 and NIH-LH-B9 for the range of 0.2 to 50 μg/L. The limit of quantitation was 0.2 μg/L LH.

Concentrations of progesterone in plasma were determined in duplicate aliquots of 100 μL by solid-phase ^{125}I RIA method (Coat-A-Count TKPG; Diagnostic Products Corporation, Los Angeles, CA, U.S.A.) as validated previously [39]. The limit of quantitation was 0.8 nmol/L progesterone.

Concentrations of steroid hormones in follicular fluid were determined in aliquots of 1 to 25 µL fluid dependent of the hormone and the size of the follicle by solid-phase ^{125}I RIA methods (Coat-A-Count, Diagnostic Products Corporation; progesterone (P): TKPG; estradiol 17β (E): TKE2; testosterone (T): TKTT ; androstenedione (A): TKAN) as validated for blood plasma of cows [39] with slight modifications. Briefly, the follicular fluid samples were extracted with 2 mL freshly opened diethyl ether (BDH Laboratory Supplies, Poole, England). To determine and correct for the efficiency of extraction approx. 10,000 dpm ^{3}H-steroid were added before extraction to a separate parallel series of similar aliquots of fluid. After evaporation of the organic solvent the samples and efficiency series were dissolved in 250 µL borate buffer (for E) or in 250 µL zero plasma of the manufacturer. Duplicate volumes of the samples were then incubated in the antibody coated tubes (Coat-A-Count). Specificity of the RIAs was high as indicated by low cross-reactivity for other steroid hormones of physiological importance, for details see the manufacturer's manual. (Coat-A-Count). The limits of quantitation were 8, 5 to 92, 4, and 5 to 13 nmol/L dependent of the sample volume used and the standard series applied for P, E, T and A, respectively.
Calculation of all hormone results was done applying the spline approximation for the standard series from RIASmart (Packard Instruments Company, Meriden, CT, U.S.A.). The calculated doses were < 4 % different from the defined doses over the entire range. In general, the intra- and inter-assay coefficients of variation were < 10 and < 15% for all assays, respectively.

2.6. Statistics

Results are presented as means ± SEM, and data were analyzed by using SPSS statistical package, version 12.0 for Windows (SPSS Inc., Chicago, IL). Results that were not normally distributed were log-transformed before analysis. Concentrations of follicular fluid steroid between different follicle sizes were analyzed by the linear mixed model procedure including follicle size as fixed effect and the individual cow as a random effect. Bonferroni correction was used when significance tests were performed. Bivariate correlation analysis (calculation of Spearman's coefficient) was used to assess the correlation between follicle size and follicle steroid content on raw data. A *P* value less than 0.05 was considered significant.

3. Results

Data of four cows were not included, two had no proper LH suppression during Crestar treatment, and two showed < 3 preovulatory-sized follicles (POF) with a diameter 10 mm. The average number of POF was 8.6, 13.0 and 10.4 per cow of the 2 h pre LH, 6 h and 22 h after LH group, respectively. The follicles were subdivided into the size categories small: 5 to 8 mm, and large: > 8 to 10, > 10 to 12 and > 12 to 16 mm as calculated from the volume of follicular fluid. No significant differences were observed between the 3 groups regarding the number of follicles per size category (Table 1). A total of 429 follicles (≥ 5 and ≤ 16 mm) were analyzed, 150 follicles 2 h pre LH, 157 follicles 6 h and 122 follicles 22 h after LH, respectively. Variable numbers of 2 to 5 mm follicles were recorded (mean: 21 per cow).

Table 1. Average number of follicles per size category per cow at different times preceding ovulation in cows treated with an oFSH protocol for superovulation with a controlled LH surge.

Time (h) relative to LH peak (n cows)	Size category (mm)[1]				
	> 5 to 8	> 8 to 10	> 10 to 12	> 12 to 16	> 16
-2 (10)	3.9	4.7	3.3	3.4	0.6
6 (8)	5.0	4.3	5.9	4.6	0.6
22 (8)	2.8	3.6	5.3	3.6	0.8

[1] size calculated from collected follicular fluid volume.

3.1. Estradiol 17β

The concentration of estradiol 17β was highest in the fluid of follicles > 8 mm at onset of final maturation that is 2 h pre LH, compared to the level at later stages after the LH peak (Fig. 2). The concentrations in the three larger size categories were not significantly different but all higher ($P < 0.05$) than in 5 to 8 mm follicles. The average level (1.41 ± 0.10 μmol/L, n=113) in follicles sized > 8 to 16 mm of the pre LH group treated with oFSH was significantly ($P < 0.05$) lower than that found for pre LH follicles following eCG treatment (4.78 ± 0.23 μmol/L, n=86) and also than the level in the preovulatory follicle of untreated cyclic cows just before the LH peak (4.25 ± 0.30 μmol/L, n=11); only the average (4.46 ± 0.43 μmol/L, n=5) of the highest five of the

oFSH group was similar to that of the other treatment groups. At the later stages during final maturation, at 6 and 22 h after LH, the differences between the concentrations of estradiol 17β in the fluid of the different size categories remained, with the level in the follicles sized 5 to 8 mm being significantly lower ($P < 0.05$) than that in the other categories. A significant correlation between size and concentration of estradiol 17β was observed 6 h after LH.

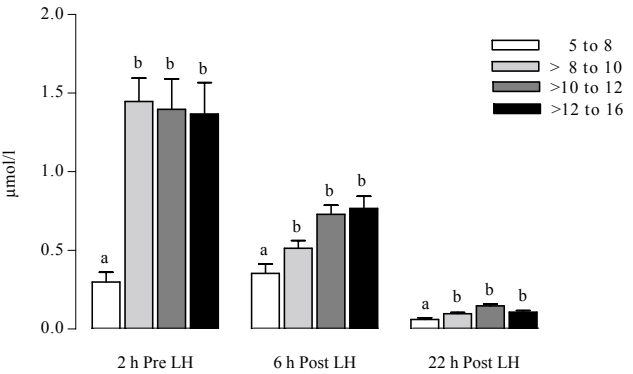

Figure 2. Estradiol 17β concentrations in the fluid of follicles collected during final maturation relative to the maximum of the controlled LH surge in oFSH treated cows; concentrations are mean ± SEM, indices a,b indicate significant differences within a time group.

3.2. Progesterone

The concentration of progesterone in the fluid of the different size categories varied considerably during final maturation (Fig. 3). In the pre LH group, the progesterone concentration was at a similar and low level in all follicles which was significantly different from that found in preovulatory follicles of untreated cyclic cows (0.26 ± 0.07 µmol/L, n=11) but the level in both groups was lower ($P < 0.05$) than that in pre LH follicles following eCG treatment (0.34 ± 0.08 µmol/L, n=86). At 6 h after LH the progesterone concentration increased in particular in follicles > 10 mm which was similar to the increase found after eCG treatment (0.46 ± 0.05 µmol/L, n=59; at 4 h after LH). Shortly before ovulation the highest level was observed in the largest category of follicles (> 12 to 16 mm; 0.89 ± 0.17 µmol/L, n=29) which was higher than that found after eCG treatment at 22 h after LH (0.54 ± 0.04 µmol/L, n=108), but the level in the follicles > 8 to 12 mm was similar to that after eCG treatment. A significant correlation

between size and concentration of progesterone was observed 6 and 22 h after LH that is in the period of initiation and at completion of maturation.

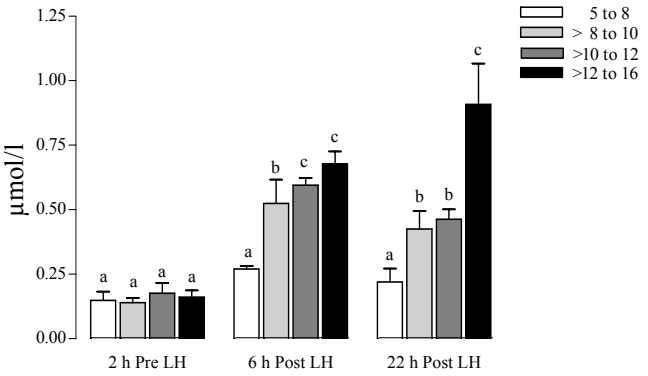

Figure 3. Progesterone concentrations in the fluid of follicles collected during final maturation relative to the maximum of the controlled LH surge in oFSH treated cows; concentrations are mean ± SEM, indices a,b,c indicate significant differences within a time group.

3.3. Testosterone

The most marked changes in the concentration of testosterone occurred in the small follicles of 5 to 8 mm (Fig. 4). At all stages of final maturation these levels were significantly ($P < 0.05$) higher than that of the other larger size categories in which a significantly higher ($P < 0.05$) level was observed in > 8 to 10 mm follicles at pre LH and 6 h after LH. At all respective stages during final maturation a significant correlation was observed between size and concentration of testosterone. The molar ratio of estradiol 17β over testosterone concentrations showed a significant correlation with size of the follicles in the pre LH group being 20.8 ± 4.3 (n=32), 36.7 ± 4.6 (n=45) and 57.9 ± 8.2 (n=32) (average ± SEM) for > 8 to 10, > 10 to 12 and > 12 to 16 mm diameter, respectively. A similar increase with size but at lower values was observed for the 6 h post LH group.

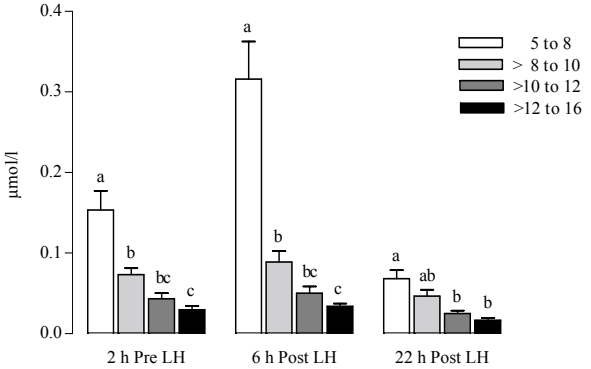

Figure 4. Testosterone concentrations in the fluid of follicles collected during final maturation relative to the maximum of the controlled LH surge in oFSH treated cows; concentrations are mean ± SEM, indices a,b,c indicate significant differences within a time group.

3.4. Androstenedione

The concentration of androstenedione varied between small vs. large follicles but no significant differences were observed between the large size categories of pre LH or 6 h after LH follicles (Fig. 5). The levels of androstenedione at 22 h after LH were not measured since this steroid can be considered to play no role at the stage of completion of maturation. The average level in large follicles > 8 to 16 mm before LH (0.11 ± 0.01 µmol/L, n=104) was markedly lower ($P < 0.05$) than that in follicles of eCG treated and of untreated normal cyclic cows at the corresponding stage (0.83 ± 0.09 µmol/L, n=86 and 1.33 ± 0.20 µmol/L, n=11, respectively). At 6 h after LH the average level in large follicles was the same as before LH (0.11 ± 0.01 µmol/L, n=115) which was still lower ($P < 0.05$) than that following eCG despite the obvious decrease in the latter group (0.58 ± 0.06 µmol/L, n=62). A significant correlation was observed between size and concentration of androstenedione for follicles at 6 h after LH.

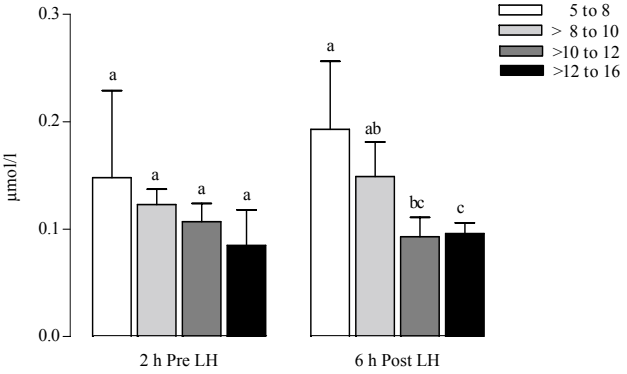

Figure 5. Androstenedione concentrations in the fluid of follicles collected during the first two stages of final maturation relative to the maximum of the controlled LH surge in oFSH treated cows; concentrations are mean ± SEM, indices a,b,c indicate significant differences within a time group.

3.5. Threshold steroid levels for large follicles

To discriminate between follicles being functional *vs.* non-functional it was assumed that the normal pattern of changes of steroid concentrations occurred only in healthy preovulatory sized oFSH stimulated follicles. Therefore, the concentrations of steroids in small 5 to 8 mm follicles were used as reference. The threshold value for size of preovulatory follicles was set at > 9 mm as calculated from the volume, since at about this size follicles either develop further or undergo atresia.

In the pre LH group 18/122 (: 15%) of the large follicles were > 8 to 9 mm. Taking then the estradiol 17β (E) concentration + 2SD of small follicles as threshold value (Fig. 6) 38/104 (: 36.5%) of the follicles > 9 mm showed a lower E concentration. All of the remaining large follicles (n=66) met the criteria derived from the small follicles for progesterone (P) and testosterone (T). In the few follicles with a too high P concentration this deviation always coincided with an E concentration below the threshold value.

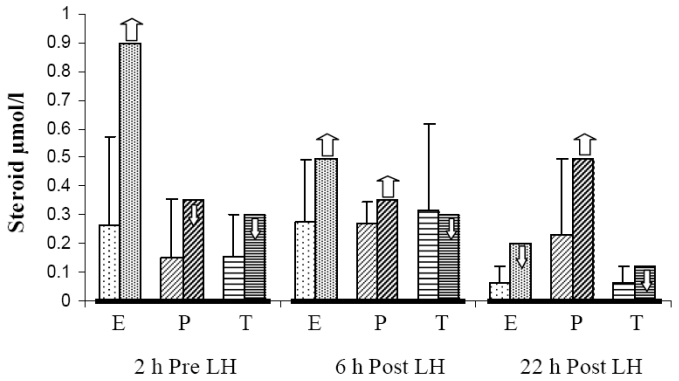

Figure 6. Threshold levels of steroid concentrations in the fluid of follicles collected during final maturation relative to the maximum of the controlled LH surge in oFSH treated cows; E: estradiol, P: progesterone, T: testosterone. Of each pair of bars, the left bars represent the mean ± SD steroid concentration for follicles 5 to 8 mm, and the right bars are the threshold value for large preovulatory follicles; arrows pointing up- or downwards indicate whether the threshold values are lower or upper limits for the qualification functional, respectively.

In the 6 h post LH group 11/123 (: 9%) of the large follicles were > 8 to 9 mm. Taking again the E concentration of the small follicles but now + 1SD as criterion in view of the rapidly decreasing E level at this stage of maturation, 47/112 (: 42%) of the follicles > 9 mm were below the threshold value (Fig. 6). Similar to the findings in the pre LH group all remaining large follicles (n=65) met the criteria for P and T concentrations. In the few follicles with a too low P concentration and in the 9/112 follicles with an exceptionally high P (1.95 µmol/L) this deviation always coincided with an E concentration below the threshold value.

In the 22 h post LH group 15/107 (: 14%) of the large follicles were > 8 to 9 mm. Taking now the P concentration of the small follicles + 1SD as criterion in view of the stage of completion of final maturation, 36/92 (:39%) of the follicles > 9 mm were below the threshold value (Fig. 6). All remaining large follicles (n=56) met the criteria for E and T concentrations.

4. Discussion

In the preovulatory follicle of the normal cyclic cow the successive stages of final follicular and oocyte maturation are characterized by the concentrations of steroids in the follicular fluid [5,40]. Before the LH surge before onset of maturation estradiol 17β is predominant while the oocyte is still at the germinal vesicle (GV) stage. Coinciding with the LH peak the level of progesterone in the normal preovulatory follicle shows a temporary increase lasting about 6 h [5], and at 6 h the level of estradiol although decreasing is still high while the oocyte is undergoing GVBD. At completion of maturation at 22 h after the LH peak progesterone is predominant and the oocyte is at metaphase II showing also cytoplasmic maturation [6]. After oFSH stimulation the relative pattern of changes with regard to estradiol and progesterone concentrations in large follicles is in agreement with that in the normal preovulatory follicle. However, before the LH surge the concentration of estradiol is distinctly lower also in comparison to follicles stimulated with eCG. Even when the follicles with an estradiol level below the arbitrary threshold are excluded the average concentration (2.08 μmol/L) is only about half of that in the normal untreated preovulatory follicle. Before the LH surge, estradiol levels in the preovulatory follicle depend on the capacity of theca cells that have acquired LH binding sites to respond to LH with increased androstenedione production, and also to the ability of granulosa cells to convert this androgen to estradiol [41]. The significantly lower androstenedione concentration in the oFSH stimulated large follicles in comparison to that of the normal cyclic preovulatory follicle indicate that the low estradiol levels could be due to the low LH bio-activity of the oFSH preparation used. Treatment with eCG having a high LH bio-activity resulted in higher androstenedione and consequent estradiol concentrations.

Synthesis of estradiol and progesterone is regulated by the relative levels of steroidogenic enzymes of the delta 4 (for P) and 5 (for E) pathways expressed in theca and granulosa cells. The precursor for both pathways pregnenolone, is produced in the mitochondria from cholesterol that is mobilized from the peripheral blood [42]. Since the progesterone levels in the fluid of the oFSH stimulated large follicles were comparable to those in the normal cyclic preovulatory follicle in particular at 6 h after LH [5] contrary to the levels of estradiol, it is unlikely that the reduced estradiol concentration in oFSH stimulated follicles at onset of final maturation is due to a defect in cholesterol mobilization or synthesis of pregnenolone. Further in the delta 5 pathway, P450 17α-hydroxylase ($P450_{17\alpha}$) which is expressed exclusively in theca cells [43] and 3β-hydroxysteroid dehydrogenase (3βHSD) are responsible for converting

pregnenolone into androstenedione. Soumano et al. [18] have shown that eCG and FSH treatments have different effects upon follicular $P450_{17\alpha}$, but not upon 3βHSD and P450 aromatase mRNA abundance. The finding that 5 to 8 mm follicles contain higher concentrations of testosterone and androstenedione, and the lack of this accumulation in oFSH stimulated large follicles strongly indicate an active aromatase enzyme complex. In summary, it can be concluded that the low estradiol level is due to a reduced activation of $P450_{17\alpha}$ as a result of low LH activity in the oFSH.

Despite the significantly lower estradiol level in the large follicles at 2 h before the LH surge a substantial proportion of the oocytes will be developmentally competent. Previous studies applying the same stimulation protocol with oFSH and a controlled LH surge have demonstrated that a proportion of 43% of such oocytes develop in vitro to morula and blastocyst stages [1] and in vivo result in embryos with a normal incidence of apoptotic cells [44] and a low proportion of cells with chromosomal aberrations [45].

Although it is known that in the periovulatory period, ovulation and luteinization are dependent on local progesterone actions [46], little information is available regarding the role of progesterone in mammalian oocyte maturation and developmental competence. Recently, inhibition of LH induced progesterone production by granulosa and cumulus cells in Rhesus monkeys in vivo [47] or during IVM of porcine cumulus oocyte complexes [48] resulted in suppression of GVBD suggesting an important role for progesterone in meiotic resumption. Interestingly, in the present study, when final maturation was initiated at 6 h after LH, the concentration of progesterone in follicles > 10 to 16 mm was significantly higher than in those < 10 mm while with completion of maturation at 22 h after LH the follicles > 12 mm showed a sharp increase of the progesterone level. This latter increase likely reflects increased numbers or more differentiated granulosa cells. A significant effect of follicle size during GnRH induced ovulation on pregnancy status has recently been reported [33]. Follicles ovulated at size < 12.8 mm showed a decrease in the ability to support the pregnancy until day 60. Whether this is related only to a reduced function of the corpus luteum originating from such follicles or also to oocyte competence is not clear. It can be speculated that larger follicles with higher progesterone levels have an increased chance to contain competent oocytes. The higher level of progesterone is probably due to a rise of LH receptors in the follicle. A substantial increase of LHr mRNA has been reported when follicle size increases from > 10 to 15 mm [49].

4.1. Selection of oFSH stimulated follicles

It can be assumed that competent oocytes are primarily enclosed by functional preovulatory-sized follicles showing the normal changes in steroid concentrations. To distinguish such follicles after oFSH stimulation, criteria were developed on the basis of the steroid concentrations in the follicular fluid dependent of the stage of final maturation. In view of the reduced estradiol level at 2 h before LH in comparison to that in the normal preovulatory follicle the criteria were derived from the steroid levels in the group of non-responding (or still growing) 5 to 8 mm follicles. During the un-stimulated cycle deviation that is the beginning of the difference in growth rate between the two largest follicles, begins at an average diameter of 8.5 mm [50] and is associated with a differentiation of the concentration of estradiol [51]. This suggests that the functionality of the follicles sized > 8 to 9 mm following the oFSH treatment may be critical. Indeed, this category showed a 65% chance of not meeting the criteria. When the follicles > 8 to 9 mm were excluded for selection, a 52 to 54% of the follicles > 9 mm showed a proper steroid profile at the respective stages of maturation. Few oocytes (16/352) were observed with a (huge) expansion of the cumulus at 2 h before and at 6 h after LH, and without expansion at 22 h after LH. Half of these deviating oocytes were found in follicles not meeting the steroid criteria and the other half in follicles considered to be functional (8/187). Disqualifying also these follicles resulted in an average 51% (179/352) of functional follicles after oFSH stimulation with a controlled LH surge. This proportion was not correlated with size for follicles >9 to 16 mm (data not shown). The similarity of the proportion of functional follicles with that of viable embryos in embryo transfer programmes using the same oFSH (60%; [52]) strongly suggests that these follicles enclose developmentally competent oocytes. Although it has been suggested for human oocytes that developmental competence is related more closely to the ratio estrogen to androgen [53] and a positive correlation of this ratio with follicular size was observed in the groups before and at 6 h after LH, the set of criteria to select follicles can be limited to the concentration of estradiol before and at 6 h after LH, and of progesterone at 22 h after LH.

In conclusion, the stimulation protocol with oFSH and controlled LH surge facilitates the collection of 6 to 7 competent oocytes per cow at specific stages of final maturation. In view of the similarity between early embryonic development in the cow and the human regarding the need for maternally derived molecules during the first cell cycles after fertilization, the data presented here will provide important reference points

for collecting oocytes to study the transcriptome in relation to developmental competence.

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Chapter 3

Isolation and identification of differentially expressed genes involved in meiotic resumption of bovine oocytes in vivo

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Abstract

Meiotic progression is a tightly controlled cascade of interdependent events controlled by sequential expression of a variety of genes. Here we report the construction of a bovine oocyte DNA microarray using Suppression Subtraction Hybridization (SSH). This microarray was used to analyze the transcriptional activity of bovine oocytes during meiotic resumption. A subtractive library consisting of 945 clones was developed from bovine, presumptive competent oocytes matured in vivo, using 30 pooled oocytes collected before LH surge (-2 h) as driver and 30 oocytes exposed to LH (6 h) as a tester. One hundred fifteen transcripts with known homology and a large number of unknown transcripts were found to be up-regulated during meiotic resumption. Thirty-five genes were clustered into six groups according to their function, and included genes related to signal transduction, cell cycle regulation, transcription and mRNA processing, cytoskeleton and cell adhesion, metabolism and genes related to antioxidant and defense mechanisms. The mRNA expression of 10 genes was further studied using quantitative real-time PCR. Among the mRNAs identified were G protein γ12 subunit which plays an important role in signal transduction, regulators of G-protein signaling 10 (RGS10) which act as GTPs-activating proteins by accelerating GTP hydrolysis, cAMP phosphodiesterase 7A1 mRNA (PDE7A1) encoding for an enzyme that has a high affinity and specificity for cAMP hydrolysis, metabotropic glutamate receptor 5 (mGluR5) that has been shown to induce prolonged Ca^{2+} oscillatory activity upon activation and cytochrome c oxidase subunit VIII (COX8) a nuclear gene, playing a vital role in the cellular energy generation. The expression of most of these genes was not previously associated with oocyte maturation. The use of the SSH and microarray approach combined with in vivo matured oocytes have uncovered novel important genes associated with oocyte maturation in the bovine. The roles of these genes during maturation have not been entirely characterized, but the results presented here will support the establishment of new hypothesis regarding the resumption of meiotic arrest in bovine oocytes.

***Key words*:** meiosis, oocyte maturation, SSH, PDE7A, mGluR5

Introduction

During growth, the mammalian oocyte is characterized by extensive decondensation of chromatin and a high level of transcriptional activity [1]. In bovine, this activity gradually decreases as the oocyte reaches a diameter ≥110 μm and comes to be silent through the completion of meiosis, fertilization, until the 8- to 16- cell stage [2, 3]. Clear evidence in several species supports the concept that with timed activation of the fully grown oocyte stores of presynthesized transcripts are needed for meiotic resumption and early embryogenesis [4, 5].

Inside the ovary, mammalian oocytes are arrested at the G2 prophase of the first meiotic cell cycle. G2 arrest is terminated by specific signals, induced through LH binding to LH receptors on granulosa cells, followed by a decrease in oocyte cAMP which is then translated to a decrease in cAMP dependent protein kinase A (PKA) activity leading to complex signaling transduction pathways and release from cell cycle arrest and progression into the meiotic cell cycles [6, 7].

Resumption of meiosis, represents transition from G2 to M phase and involves condensation of the chromosomes, dissolution of the nuclear membrane referred to as 'germinal vesicle breakdown' (GVBD) and formation of the first metaphase spindle (MI), which is completed by the emission of the first polar body and is immediately followed by the second metaphase (MII), reviewed by Dekel [8]. These nuclear changes are paralleled and supported by certain modification occurring in the cytoplasm, involving, for instance, sister chromatid separation [9], protein degradation [10], and adaptive responses to oxidative stress [11], metabolic processes [12], organelle translocation [13], and membrane fusion. Although many of these main steps in oocyte maturation have been described, our understanding of their mechanistic bases has remained rudimentary.

Precise regulation of nuclear and cytoplasmic events during maturation is crucial to produce developmentally competent oocytes of all mammalian species, and requires cooperative functional interactions between different follicular cells components [14]. Using in vitro culture models to inhibit or to accomplish spontaneous maturation, the mechanisms of oocyte meiotic maturation and developmental competence have been extensively examined by adding gonadotropins, steroid hormones, growth factors, and meiotic inhibitors to the medium. Nevertheless, the insights obtained from spontaneous maturation cannot be extended to the ligand-induced meiotic resumption in vivo. This process demands a strict regulation of oocyte and granulosa cells metabolic activity via the

modulatory action of variety of hormones and growth factors [15, 16]. In vitro models lack the interactions between the oocyte and its follicular components, therefore, the molecular regulatory signals by which the oocyte undergoes intrafollicular activation remain largely unknown [17].

The genome-wide transcriptional activity during oocyte maturation has been investigated. Since no complete microarray is available for bovine, commercially spotted human arrays have been used to analyze the pattern of bovine oocytes gene during maturation. The differential expression of a number of genes demonstrated the correlation of a specific maturation stage with the expression of a different genes subset [18]. Moreover, another study using bovine oocyte microarray and oocytes matured in vitro collected from slaughterhouse ovaries identified several mRNAs involved in developmental competence [19]. Although the use of cell culture systems has contributed to further oocyte mRNAs characterization, and provided insight into changes in the gene expression patterns during maturation and developmental competence, the extent of relevance of these results to the processes in vivo remains unclear. Vice versa, identification of genes that are differentially expressed due to in vivo LH surge may lead to a greater understanding of which processes and signaling pathways are pertinent in meiotic maturation leading to successful fertilization and embryo development.

The aim of this study was to identify and characterize genes that are differentially expressed in bovine oocytes in vivo due to LH surge and therefore are likely to be involved in the initiation and regulation of meiosis and play a role in developmental competence. In this study we examined the differential gene expression in oocytes of bovine 6 h after LH peak by SSH. cDNAs from the forward subtracted library were screened using DNA microarray for differential expression and the up-regulation of 10 transcripts was studied by QPCR. A further 25 genes were clustered based on their function into 6 groups. This study demonstrated that the use of in vivo derived oocytes in powerful molecular techniques can be utilized to identify genes with a potential, thus far unknown, role in developmental competence.

Materials and Methods

Experimental design

Follicle development was stimulated in Holstein-Friesian cows using our standard protocol [20] with oFSH and a Crestar/GnRH-controlled LH surge. Cows were allocated at

random to two experimental groups for ovariectomy (OVX): 1) at onset (2 h before LH), and 2) after initiation (6 h after LH) of maturation to determine changes in mRNA expression related to resumption of meiosis in vivo. Presumptive competent oocytes were selected on the basis of the steroid profile in the enclosing follicle (Table 1), and were assigned to replicates for Suppression Subtractive Hybridization (SSH) and validation by QPCR analysis in such a way that within a group replicates were equivalent with regards to steroid profile and represented maximum numbers of cows. The experiment was carried out as approved by the Ethical Committee of the Veterinary Faculty of Utrecht University.

Animals and treatments

Normally cyclic Holstein-Friesian cows (n=30) were selected and treated for superovulation using the protocol as described before [20] with oFSH (Ovagen ICP, Auckland, New Zealand), prostaglandin (PG; Prosolvin; Intervet International B.V., The Netherlands) and a timed LH surge controlled by norgestomet/GnRH (Crestar ear implant/Receptal; Intervet International B.V.). Cyclicity was verified by measuring the concentration of progesterone in peripheral plasma during at least 6 weeks before FSH-stimulation [21] and the LH surge was monitored in plasma from 38 h after PG until OVX using a validated RIA with bovine LH (bLH-7981) for iodination and standards, and rabbit anti-bLH (8101) as antiserum [22]. Oocytes and follicular fluids were collected following OVX at 50 and 58 h after PG corresponding with 2 h before and 6 h after the maximum of the LH surge, respectively.

Collection and selection of in-vivo preovulatory oocytes

For every treatment run with a group of 4 cows OVX was performed at 1 h intervals, the time needed to collect all oocytes and follicles from one cow, by laparotomy through flank incision under local infiltration anesthesia [23]. Ovaries were collected in 0.9% (w/v) NaCl at 37^{o}C and immediately transported to the laboratory. The contents of each follicle >9 mm were aspirated using an 18-ga winged infusion set needle attached to 15 ml polystyrene conical tube under pressure by means of a suction pump, and were then immediately stored on ice at 4^{o}C. The size of the follicles was calculated from the volume of follicular fluid after collection. After retrieval of the cumulus oocyte complexes (COCs) under a stereo microscope, the follicular fluids were centrifuged 3,000 *g* for 10 min at 4^{o}C and stored at -25^{o}C until analysis for steroids. Collected COCs were rinsed twice in 700 µl PBS (Sigma-Aldrich, St. Louis, MO, U.S.A.) supplemented with 5% (w/v) polyvinyl alcohol (PVA; Sigma). Cumulus cells were removed by continuous pipetting after

incubation for one minute with 100 µl 5% (w/v) hyaluronidase (Sigma-Aldrich) and denuded oocytes were checked for remaining cumulus cells and washed three times with PBS-PVA and stored individually at -80°°C until RNA extraction.

For each collection group relative to the LH surge, oocytes were selected on the basis of the concentration of steroids in the fluid of the enclosing follicle according to criteria as described previously [24]. Briefly, oocytes from follicles with estradiol 17β > 0.9 µmol/L before LH and > 0.5 µmol/L 6 h after LH were considered to be competent (Table 1). The few oocytes that had heavy atretic features (expanded cumulus scattered in dark clumps in a jelly-like matrix) were excluded.

Table 1. Numbers of follicles and presumptive competent oocytes recovered during maturation in FSH-stimulated cows with a controlled LH surge.

	notes	2 h before LH (Pre)	6 h after LH (Post)
Follicles > 9 mm			
- # total per cow (n cows)		11.2 (n=13)	15.3 (n=12)
- # Normal/total (%)	[1]	104/145 (72)	124/183 (68)
Oocytes collected			
- # total/# follicles > 9 mm (%)		119/145 (82)	135/183 (74)
- # competent/total collected (%)	[2]	81/119 (68)	88/135 (65)
Oocytes used			
- # for SSH	[3]	30	30
- # for QPCR		47	50
- estradiol 17β ± SEM		1.77 ± 0.11	0.84 ± 0.03
- progesterone ± SEM	[4]	0.14 ± 0.01	0.53 ± 0.01
- follicle diameter ± SEM	[5]	13 ± 1	13 ± 0.2

[1] Normal: functional follicles on the basis of steroid profile
[2] Competent: oocytes from functional follicles
[3] SSH: Suppression Subtractive Hybridization; QPCR: quantitative PCR
[4] Mean steroid concentration in µmol/L fluid of follicles from which oocytes were used
[5] Mean diameter in mm as calculated from volume of fluid of follicles from which oocytes were used

RIA of steroids in follicular fluid

Concentrations of the steroid hormones estradiol 17β and progesterone in follicular fluid were determined in aliquots of 1 to 25 µL fluid dependent of the hormone and the size

of the follicle by solid-phase ^{125}I RIA methods (Coat-A-Count, Diagnostic Products Corporation, Los Angeles, CA, U.S.A; estradiol 17β: TKE2; progesterone: TKPG) as validated for blood plasma of cows [21] with slight modifications such as extraction with diethyl ether (BDH Laboratory Supplies, Poole, England).

Total RNA extraction and precipitation

Total RNA was isolated using microspin column and DNA was digested with Dnase1 to eliminate possible genomic DNA contamination according to manufacturer's instruction (Absolutely RNA Microprep Kit; Stratagen, San Diego, CA, U.S.A.), the RNA was recovered by two subsequent 50-µl elutions with warm (60°C) elution buffer provided in the kit. RNA was then precipitated with 250 µl of 100% ethanol and 10 µl of 3 M sodium acetate pH 5.2, using 1 µl of 1 mg/ml linear acrylamide (Ambion, Austin, TX, U.S.A.) as co-precipitant. The mixture was chilled at -80^{o}C for 30 min, centrifuged for 20 min at 4^{o}C at 16000g. The pellet was then washed with 75% ethanol and resuspended in 3 µl of nuclease-free water for the RNA used for the SSH experiment and in 15 µl of water for the RNA used in real-time PCR analysis.

Generation of a Subtracted Library by Suppression Subtractive Hybridization (SSH)

The SMART^{TM} PCR cDNA Synthesis Kit (Clontech, Palo Alto, CA, U.S.A.) was used to maximize cDNA yields prior to the subtraction. The PCR-Select cDNA Subtraction Kit (Clontech) was used for SSH as described in the manufacturer's recommendations to isolate and enrich for gene sequences differentially-expressed between the two pools of oocytes: 1) collected before LH surge (-2 h, n=30) as driver and 2) exposed to LH (6 h, n=30) as tester.

The subtracted material was then cloned into the pGEM-T and pGEM-T easy vector system Kit (Promega, Madison, WI, U.S.A.) and transformed into DH5α-T1 max efficiency *Escherichia coli* cells (Invitrogen, Burlington, ON, Canada). Selected white colonies were inoculated individually in LB/ampicillin medium in 96 well-plates at 37^{o}C for 6 h with agitation, and screened for inserts by PCR using 1 µl of bacterial stock culture and SSH adaptor-specific nested PCR primer 1 (5'-TCGAGCGGCCGCCCGGGCAGGT-3') and 2R (5'-AGCGTGGTCGCGGCCGAGGT-3') (BD Biosciences,Mississauga, ON, Canada), and 1 U of HotMaster^{TM} Taq DNA polymerase (Eppendorf, Westbury, NY, U.S.A.). Amplified products were visualized on a 2% agarose gel with ethidiumbromide (EtBr).

Microarray preparation

PCR products originating from the subtracted library were purified using the Unifilter 384-well purification plates (Whatman, Clifton, NJ, U.S.A.), speedvac dried (SPD SpeedVac ThermoSavant, Milford, MA, U.S.A.), and resuspended in 5 µl of 3X standard saline citrate (SSC)/dimethylsulfoxide (DMSO; 1:1). Clones (n=945) were spotted twice on GAPS II glass slides (Corning, Acton, MA, U.S.A.) using a VersArray ChipWriter Pro (Bio-Rad, Mississauga, ON, Canada). Various controls were printed on the slide in two replicates: three cDNA products of the SpotReport Alien cDNA Array Validation System (Stratagen) were used as negative controls, a fragment of the Green Fluorescent Protein (*GFP*) as an exogenous positive control, and various housekeeping genes (*actin*, *tubulin*, *H2A*, *and GAPDH*) as internal positive controls. Slides were cross-linked with ultraviolet (UV) rays according to the manufacturer's instructions and quality of the printing was controlled with Terminal Transferase (TdT) (Roche Diagnostics, Laval, QC, Canada) and and Cy3 dye (GE Healthcare Bio-Sciences, Piscataway, NJ, U.S.A.)

Microarray hybridization

Subtracted PCR products were used as probes to hybridize the glass slide in order to select the positive clones for sequencing. Probes were labeled with Alexa Fluor 555 and 647 reactive dye packs (Molecular Probes, Burlington, ON, Canada) using Amino Allyle dUTP (Ambion) according to the manufacturer's instructions. Two dye swap hybridizations were performed with two different protocols. The first protocol is described in detail by Sirard and collaborators [25] and the second using the Solid*Hyb*^{TM} Array Kit (Viridis Biotech, QC, Canada) follows the manufacturers instructions. Loess normalization transformations and background were calculated as described in the *microarray analysis* section below for the second and the third hybridizations.

Microarray analysis

The hybridized slides were scanned using a VersArray ChipReader System (Bio-Rad), the signals were analyzed and normalized with ArrayPro Analyser software (Media Cybernetics, San Diego, CA, U.S.A.). For each hybridized spot, data ratios were calculated and background intensity was subtracted and normalized ($t = m + 2$ x s.d., where t is the calculated threshold, m is the mean of the negative controls raw data and s.d. is the standard deviation of those same negative control raw data). Data points representing the

differentially expressed genes above 1.3 fold were selected as positives. Those clones were sequenced and identified as described previously [25].

Real-time Polymerase Chain Reaction

Reverse Transcription and primers design

Total RNA was reverse-transcribed in a total volume of 20 µl using the iScript cDNA Synthesis Kit (Bio-Rad Laboratories, Hercules, CA, U.S.A.) containing oligo-dT and random hexamer primers. Reactions were incubated for 5 min at 25°C, 30 min at 42°°C, and 5 min at 85°C. Primers (Table 2) used for the real-time PCR analysis of reverse-transcribed mRNAs were designed with the aid of the Beacon Designer 4.0 software (PREMIER Biosoft International, Palo Alto, CA, U.S.A.). The specificity of the primers was confirmed by sequencing the amplified product and visualization on a standard 2% agarose gel with EtBr. The PCR products were sequenced after purification with the QIaquick PCR Purification Kit (Qiagen, Valencia, CA, U.S.A.) and quantified with a spectrophotometer (Nanodrop ND 1000, Isogen, IJsselstein, The Netherlands). PCR products were then diluted from 100 to 0.01 fg as standards to construct the standard curve.

Real-time Polymerase Chain Reaction

Real-time PCR was performed on a Bio-Rad MyiQ system using the 2X iQ SYBR Green Supermix reagents, following the manufacturer's protocols. Reactions were performed using 25 µl duplicate reactions with the quantity of diluted cDNA corresponding to 0.1 to 0.3 of an oocyte, depending on the mRNA abundance as estimated using oocytes from slaughterhouse ovaries matured in vitro, and 0.5 µM of each primer. Each transcript was amplified from four different replicates. Samples and standard curves were amplified on the same run with the same PCR master mix. The thermal cycling program starts with an initial denaturation step at 95°C for 3 min, and is followed by 45 to 50 PCR cycles (dissociation for 5 sec at 95°C, annealing for 5 sec at temperature showed in Table 2, and elongation for 20 sec at 72°C), one melting cycle consisting of 5 sec at 95°C, 30 sec at 72°C, and a step cycle up to 95°C (0.3°C/sec transition rate), and finally a cooling down cycle at 40°C. Amplification of the GAPDH mRNA [26] was performed for each reverse transcribed sample as an endogenous quantification standard. These raw CT values were then analyzed with a modified delta-Ct method with efficiency correction using a PCR data analysis program, qBase (version 1.3.2) (http://medgen.ugent.be/qbase/) to obtain relative quantification values. PCR product was analyzed on a 1.5% agarose gel with EtBr to

confirm amplification. Product sizes and annealing temperatures for each gene are presented in Table 2.

Table 2. Primers used for real-time PCR experiment

Genes	GeneBank accession number	Oligos sequences	Product size (bp)	Annealing temperature (°C)
G-protein γ12	NM_174785	F 5'-agccagtaccaacaacattg-3' R 5'-tccacttcactacttctctataag-3'	222	55
PDE7A1	NM_002604	F 5'-gcttgccaattctgtaactccttg-3' R 5'-attctggcgactgatgtctgtg-3'	286	53
mGluR5	NM_000842	F 5'-gatggctcctcctcttcc-3' F 5'-gcacggctgatacataggtc-3'	262	58
MC1R	NM_174108	F 5'-catcacctactacaaccacaag-3' R 5'-agaggttgaagttcttgaag-3'	311	55
VCP	NM_007126	F 5'-atccgtgaatccatcgagag-3' R 5'-gactctgctgaagggtctgg-3'	202	57
GSTA1	BTU49179	F 5'-cgggaaagacatgaaggaga-3' R 5'-cttgttgcccaccaggtagt-3'	211	58
COX8	NM_174024	F 5'-gctgactccactgctgttg-3' R 5'-catcactcccgcttcttgtag-3'	204	57
β-cat	NM_174637	F 5'-gccgacaagaagattgaag-3' R 5'-atctggtgaaccctctgg-3'	191	56
PPARBP	BC006517	F 5'-atagaccctggagtgaaacc-3' R 5'-agatgttactttgagagccttc-3'	131	56
ARF4	NM_001660	F 5'-ccctcttctcccgactatttgg-3' R 5'-atggtaggaatggtggtgactatc-3'	127	56
GAPDH	NM_001034034	F 5'-ccacgagaagtataacaacacc-3' R 5'-gccagtagaagcagggatg-3'	229	56

G-protein γ12, *Homo sapiens* G protein gamma 12 subunit; PDE7A1, *Homo sapiens* cAMP phosphodiesterase PDE7A1; mGluR5, *Homo sapiens* metabotropic glutamate receptor 5; MC1R, *Bos taurus* melanocortin 1 receptor; VCP, *Homo sapiens* valosin-containing protein; GSTA1, *Bos taurus* glutathione S-transferase subunit A isoform I; COX8, *Bos taurus* cytochrome c oxidase subunit VIII; *Bos taurus* β-cat, beta catenin like 1; PPARBP, *Homo sapiens* PPAR binding protein; ARF4, *Homo sapiens* ADP-ribosylation factor 4; GAPDH, *Bos taurus* glyceraldehyde-3-phosphate dehydrogenase.

Statistical analysis

Data are presented as the mean ± SEM. Statistical differences between the two groups were determined by means of the unpaired Student's t-test. Differences were considered statistically significant at the 95% confidence level ($P < 0.05$).

Results

Collection and selection of oocytes

Two cows of the thirty FSH-stimulated cows were excluded: in one the concentration of LH was elevated during the norgestomet treatment and another cow showed < 3 preovulatory-sized follicles. The 52 oocytes collected from 96 follicles > 9 mm of a further 3 cows were not used for SSH and QPCR since all follicles showed an estradiol 17β concentration that was 5 times lower than the threshold value for selecting competent oocytes. Of the remaining 25 cows on average 6.2 and 7.3 oocytes were collected per cow at 2 h before and 6 h after the maximum of the LH surge, respectively (Table 1). The proportion of functional follicles varied noticeably between cows. Six of the 28 cows (21.4%) had a population with > 90 to 100% of the follicles being functional, another 6/28 cows (21.4%) had < 20 to 0%, and the remaining 16 cows (57.2%) showed a population with 73% (median; range 42 to 89%) of the follicles being functional.

Suppressive Subtractive Hybridization

We used SSH to isolate genes that are differentially expressed in oocytes that were exposed to LH in vivo. The subtraction efficiency was evaluated by the GAPDH housekeeping genes expression, which was dramatically reduced in the subtracted library. Among the 1092 clones screened, 945 showed single insert-containing clones from the subtracted library enriched for LH up-regulated sequences. The insert size range was between 250 and 800 bp. The cDNA fragments were amplified, and then blotted onto glass slides together with control cDNAs and probed with the subtracted cDNA pools.

Table 3. Genes identified in the SSH library with known identity and function

GeneBank name	Accession No.
Genes related to signal transduction and calcium	
1. Guanine nucleotide binding protein (G-protein) gamma 12	NM_174785
2. Heat shock protein 90 alpha (HSP90 alpha)	AB072368
3. Regulator of G-protein signaling 10 (RGS10)	AF493934
4. metabotropic Glutamate receptor 5 (GRM5)	NM_000842
5. Calcium binding protein P22 (CHP)	NM_007236
6. cAMP phosphodiesterase PDE7 (PDE7A1)	NM_002604
7. Melanocortin 1 receptor (MC1R)	NM_174108
8. Rod outer segment guanylate cyclase precursor (ROS-GC1)	AF027201
Genes related to transcription and mRNA processing	
1. SWI/SNF chromatin remodeling complex subunit OSA1 (OSA1)	AF521670
2. PRP8 pre-mRNA processing factor 8 homolog	NM_006445
3. Homeobox-containing protein PKNOX1	AY196965
4. polymerase (DNA-directed), delta 3, accessory subunit	NM_006591
Genes related to cytoskeleton and cell adhesion	
1. Thymosin β4	AY192438
2. β-Actin	AY141970
3. Catenin alpha like 1	AY523969
4. Catenin Beta like	NM_174637
5. Myosin regulatory light chain (MRLC)	AF513721
6. Putative rab5 GDP/GTP exchange factor homologue (RABEX5)	NM_174591
7. Syntaxin 8	BT007319
8. ADP-ribosylation factor 4 (ARF4)	NM_001660
9. Dishevelled associated activator of morphogenesis 1 (Daam1)	NM_014992
Genes related to metabolism	
1. AMP-activated protein kinase subunit gamma 1 (AMPK)	NM_174586

2. Cytochrome c oxidase subunit VIII (COX8)	NM_174024
3. PPAR binding protein (PPARBP)	BC006517
4. ornithine decarboxylase (ODC)	U36394
Genes related to antioxidant	
1. Peroxiredoxin 1 (PRDX1)	NM_174431
2. Glutathione S-transferase subunit isoform I (bGSTA1)	U49179

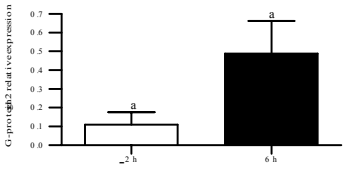
Microarray hybridization

Determination of the background hybridization signal threshold was performed by considering all of the *GFP* negative controls and SpotReport Alien cDNA. All of the poor quality spots were removed from further analysis. Fluorescence signal was calculated using each replicate for each clone. If one of the replicates was lower than the corrected background, then the corresponding clone was completely eliminated from the analysis.

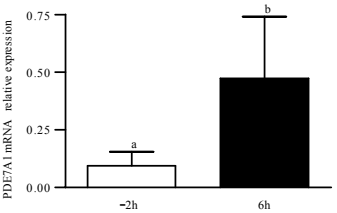
Following the hybridizations, three hundred and thirty transcripts showed more than 1.3 fold increases and were selected for sequencing analysis. The output sequences were analyzed using nucleotide-nucleotide BLAST (blastn) program on the NCBI database in order to find homologies with already known genes. When clones were examined, 115/350 showed identity to known sequences, while 36/350 matched uncharacterized sequences (clone, BAC, RIKEN, or hypothetical protein), 167/330 had no significant match to any known sequence. In the known sequences, 11 were represented twice, 2 represented 3 times, 1 represented 4 times and 1 represented 6 times, and 1 represented 7 times in the sequenced clones. Of the 115 genes whose expression showed similarity to known genes, 79 had homology to human, 27 to bovine and 9 to other species.
Thirty-five genes presented in Table 3 represent a proportion of the genes that have well-known identifiable molecular functions.

Quantitative RT-PCR Analysis of Target Genes

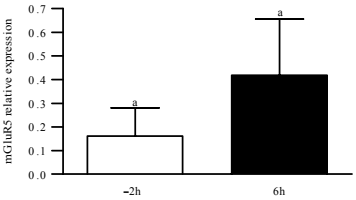
Targets of particular interest found with the arrays were selected based on their reported function in somatic cells, (Table 2) and screened to validate the SSH finding by real-time RT-PCR using RNA extracted from 4 different oocytes pools. The primer sequences used are summarized in Table 2.



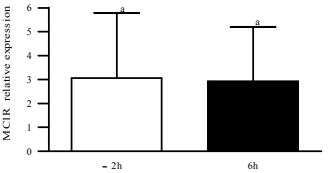
A. G-protein γ 12



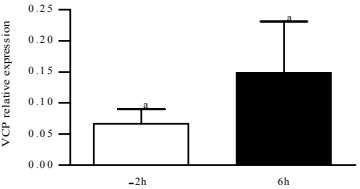
B. cAMP phosphodiesterase PDE7A1



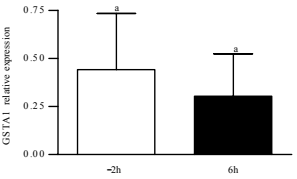
C. metabotropic Glutamate receptor 5



D. Melanocortin 1 receptor



E. Valosin containing protein



F. Glutathione S-transferase subunit A isoform I

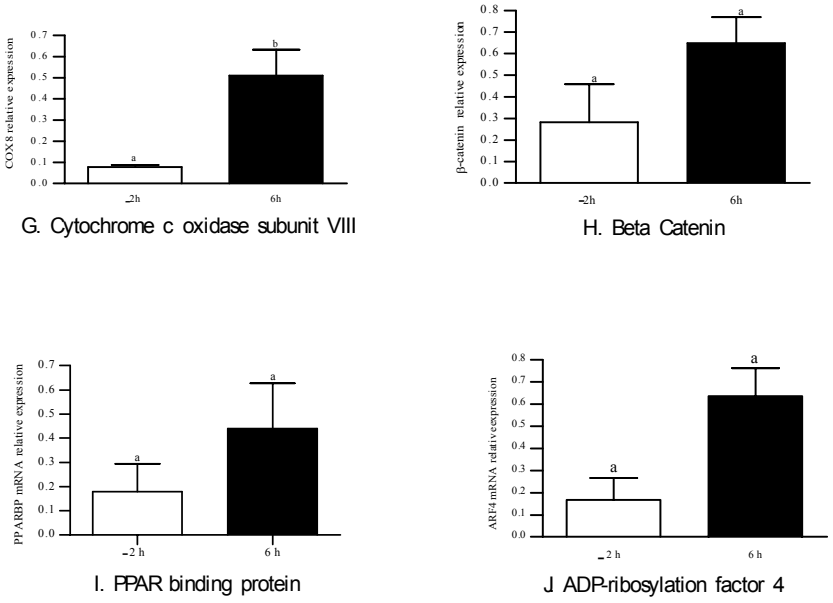


Figure 1. Real-time PCR analysis of mRNA expression of the ten genes (A to J) selected from microarray results; data are shown as mean ±± SEM of the mRNA level of the oocytes collected 2 h pre LH surge and the oocytes collected 6 h following the LH surge. Different letters indicate statistical significance between the two groups as determined by unpaired Student's *t*-test. For all mRNAs, $P < 0.05$ was considered statistically significant.

Real-time PCR experiments revealed the presence of all the genes tested. Two candidates (COX8 and PDE7A1) showed significant differential mRNA levels. We did not detect the statistically significant differences in the amount of other genes by QPCR although 6 of these genes tended ($P < 0.1$) to show increase at 6 h post LH (ARF4, g-protein γ12, PPARBP, ß-catenin, VCP and mGluR5). Figure 1 (A to J) presents the quantification of mRNA levels in oocytes collected 2 h before or 6 h after LH surge.

Discussion

The current study demonstrates the value of using in vivo matured oocytes with SSH and microarray technique to unravel the transcriptional changes occurring during the onset of meiotic resumption within the follicle. The most significant aspects of this study were to examine the effect of in vivo LH surge on gene expression in the bovine oocytes. These data further extend our understanding of the mechanisms of meiotic resumption in the mammalian oocytes. Compared to the first application of microarray analysis with bovine slaughterhouse ovaries oocytes matured in vitro and hybridized to a human cDNA array [18], very few genes are represented in our library, indicating potentially different mechanism regulating meiotic resumption in vitro compared to in vivo. Moreover, comparison of the present results to our recently published results [27] using the same tester cDNA (6 h post LH surge oocytes) and 6 h in vitro matured oocyte as driver led to large proportion of different genes. However, the functional distinctions between the two culture systems do not seem to exist only because of alterations of the culture medium, but we need to consider also the role of cumulus and granulose cells to fully understand the difference between the two systems.

Several of the transcripts identified in our study represent genes well known to participate in oocyte maturation pathways, modulating cAMP level (such as cAMP phosphodiesterase), cell cycle regulation (such as CDC28) and chromatid separation (PTTG1) which provides validation of the SSH approach as an effective high-throughput technology to derive a profile of factors involved in meiotic resumption. Additionally, much of the expression data is consistent, or at least compatible, with current notions of how meiotic resumption might occur, and it is not difficult to construct a tentative hypothesis about how several identified genes might be involved is some aspects of oocyte maturation. However, quantitative real time PCR analysis revealed a significant difference for only tow genes amongst the genes tested. Many of the tested genes show a different level but did not reach the significant level with the number of replicates used in this study. Since these factors are believed to be polyadenylated early during the maturation process [28], the QPCR may detect only a portion of the RNA while the micro array might have shown all possible transcripts since PCR was used to generate the library and the polyadenylation status does not limit the messenger abundance.

Half of the sequenced clones identified have no known matches in the NCBI database (23-3-2004). Bovine genome has now been completely sequenced and it is possible that many of the unidentified sequences will find matches as the database growing. It is also

possible that these orphan genes may be the results of rapid evolution such that the sequence similarity is lost even within relatively short evolutionary time [29].

One of the interesting findings in the current study is the expression of G protein γ12 subunit. During meiotic arrest, the conversion of intracellular ATP to cyclic AMP (cAMP), is catalyzed principally by the precise regulation of adenylyl cyclase activity [30]. Adenylyl cyclases are either activated or inhibited by interactions with Gαα or βγ subunits liberated from heterotrimeric G proteins in response to ligand-activated G protein- coupled receptors like hormones, neurotransmitters and growth factors. Different Gα subunits have different sets of intracellular targets and functions. For example, Gααs is known as adenylate cyclase activator while Gααi as inhibitor. Similarly, there are multiple forms of β and γ subunits, the amino acids sequences of different β subunits are 53-90% identical to one another, for review see [31, 32], whereas different kinds of mammalian γ subunits are present as different isomers, the biological properties of the βγ subunit have been attributed to the γ rather than β subunit. The preferential Gβ/Gγ interaction determines which G αβγ heterotrimers will be available and thus determine the action and direct certain routes of signaling [33]. It has been suggested that meiotic arrest in mouse follicle-enclose oocytes needs functional Gαs. Targeting of Gαs using anti-Gαs inhibitory antibody induced oocyte maturation in follicle-enclosed oocytes, while injection of anti-Gαi inhibitory antibody did not have any effect [34]. However, the finding that γ12 has a function through specific interaction with Gαo and Gαi, and that βγ12 by itself was able to inhibit adenylyl cyclase in rat retinal membranes and stimulate the activity of phospholipase C in the cytosol of HL60 cells [35] may suggest an important role during oocyte meiotic resumption. Moreover, in *C. elegans* and *Drosophila,* G proteins play a direct role in position and orientation of mitotic spindle, but are not required for spindle assembly indicating a direct role in meiosis [36]. Furthermore, to exert their role, G-proteins attach to a family of regulators of G-protein signaling (RGS) which act as GTPs-activating proteins by accelerating GTP hydrolysis because Gα-bound nucleotide has a very slow dissociation rate [37], our results revealed the presence of RGS10 mRNA which has been identified to interact specifically with several activated forms of Gααi [38]. In mitosis, it has been shown that GTP hydrolysis is important for the mitotic function of G proteins. GTP hydrolysis terminates the signal and in RGS mutants, signaling is enhanced and prolonged. Therefore, spindle forces are reduced in RGS-7 mutants of the *C. elegans* embryo. The mitotic role of RGS proteins is conserved in mammals [39], In early embryos of RGS14 knockout mice microtubules had completely disappeared, and in RNAi experiments of RGS14 with HeLa cells severe spindle defects occurred upon complete depletion [39]. Although these findings might help to explain the role of G protein and RGS in mammalian oocytes meiotic resumption, the

mechanism by which these proteins regulate the adenylyl cyclases, cAMP and chromosomal segregation in mammalian oocytes remain unknown. The identification of G protein subunits and effectors that couple GPCR will provide additional clues to the role of G proteins pathways in oocyte maturation.

The cAMP phosphodiesterase (PDE) superfamily is large, showing at least 12 families, These enzymes catalyze the hydrolysis of cAMP and cGMP or both [40]. PDE7 enzymes have a high affinity and specificity for cAMP hydrolysis and are encoded by two genes designated PDE7A and PDE7B [41]. PDE7A occurs as three alternative splice variants, PDE7A1, PDE7A2, and PDE7A3 [42]. The results of the present study demonstrate that bovine oocytes show PDE7 isozymes expression, namely PDE7A1, thereby providing the first characterization of different PDE isozymes in mammalian oocytes. Within the mouse ovarian follicle, PDE3 has been shown to localize mainly in the oocyte while PDE4D localizes to granulosa cells. Inhibitors of PDE3 have been shown to block meiotic resumption of 90 to 100% of rodent oocytes [43, 44], while only 20% of bovine COCs remain indefinitely arrested using the same inhibitor [45, 46]. Interestingly, the expression of PDE7 in the bovine oocyte may explain this variation; the pattern of PDE7 inhibition is quite different from inhibition of PDE3. This enzyme is resistant to all standard PDE inhibitors, including nonselective compounds such as isobutylmethylxanthine (IBMX) [47]. Because no PDE7 selective inhibitors are available yet [48], the role PDE7 may play in cAMP metabolism remains to be determined. Expression of PDE7A in the bovine oocyte may indicate that the mechanism of meiotic maturation differs according to species. Understanding the mechanisms of meiotic arrest and resumption require the characterization the multiple phosphodiesterases activities in various follicular cells compartments which will provide crucial insights into the control of meiotic resumption mechanism, and ultimately, the improvement of the oocyte in vitro culture conditions.

The melanocortin 1 receptor (MC1R) is a G protein coupled receptor (GPCR) expressed preferentially in epidermal melanocytes coupled positively to adenylyl cyclase via the Gs protein and is a key regulator of mammalian pigmentation. MC1R is activated by α-melanocyte stimulating hormone (α-MSH) and its action has been proposed to be mediated by cAMP-dependent activation of the kinases PKA, PKC and MAPKs has been proposed, for review see [49]. However, recent findings showed that specific serine/threonine kinases (GRK2 and GRK6) expressed in melanoma cells of human and mice can recognize and occupy MC1R receptors, transiently inhibiting its responsiveness to its agonist (desensitization) and significantly impairing agonist-dependent cAMP production [50], these results may indicate the existence of other unidentified kinases able

to target MC1R. Moreover, it is possible that MC1R may initiate the meiotic resumption through a distinct receptor(s) on a different signaling route. It has been proposed that the phosphorylated receptors interact with proteins of the arrestin family, which uncouple the complex from the G protein effectors often leading to activation of new signaling events [51]. Collectively, this finding suggests that the process of meiotic resumption via cAMP degradation may be more complex, than originally proposed.

In the present study as in other reports [18, 52], we identified the yeast Cdc2 homolog CDC28 a well known cyclin dependent kinase (CDK) recognized as the central component controlling the timing of events in the *Saccharomyces Cerevisiae* cell cycle [53, 54]. Cdc28 mutants arrest cell cycle progression at the pachytene stage of meiotic G2 [55] and show a defect in mitotic chromosome stability [56], when in complex with cyclins, Cdc28 promote entry into mitosis and can trigger cytoskeletal reorganization [57]. Beside that, it is known that formation of at least some CDC28-cyclin complexes require one or more assembly factors. Interestingly, the molecular chaperone of the heat shock protein 90 (HSP 90), which is required for correct folding of proteins limited to cellular signal transduction, such as kinases and transcription factors [58], has been suggested to participate in CDC28-cyclin complex formation [59, 60]. These data may indicate that CDC28 helps to constitute the active bovine MPF and is essential for progression through meiosis. Co-expression of HSP90 with CDC28 and other genes indicates that it is important for the correct folding of newly synthesized proteins into their final conformation in order to perform their assumed function. Moreover, the expression of pre-mRNA processing factor 8 homolog (*PRP8*) is another interesting finding, fission-yeast pre-mRNA splicing *prp8* and *cdc28* are identical loci. Thus, a mutation in a single gene can cause a specific arrest in progression through the cell cycle and a block in pre-mRNA splicing [61]. This point to a link between RNA metabolism and meiotic progression and, similar action may occur in the oocyte, but further functional experiments will be required to confirm this hypothesis.

We have demonstrated several subunits of anaphase-promoting complex (APC), known to play an essential meiotic role in other organisms by destroying cyclins, in order to produce an alternation between interphase and mitosis [62], like Ubiquitin-specific protease, Polyubiquitin, UBXD2 and valosin containing protein (VCP). VCP is a mammalian homolog of the yeast cell cycle division protein Cdc48 in budding yeast and of p97 in *Xenopus laevis*, acts as a molecular chaperone in many diverse cellular activities, such as membrane fusion, cell cycle regulation, vesicle-mediated transport, stress response, programmed cell death. Almost all these activities are directly or indirectly regulated by the ubiquitin–proteasome system, (for review see ref. [63]). Loss of VCP function has been

shown to inhibit the degradation of many Ub-Pr substrates including cyclin B1 molecules [64]. Moreover, in budding yeast, Cdc48 is essential for cell cycle progression through the degradation of the CDK inhibitor Far1[65]. Our study revealed also Ubx cofactors subunit 2 that has previously been shown to interact and direct the proteolytic activity of Cdc48. In budding yeast, Ubx2 mutant cells have been shown to accumulate CDK inhibitors due to compromised the ubiquitin-proteosome system [66].

Sister chromatid separation which takes place at the metaphase-to-anaphase transition is triggered by the ubiquitin-dependent degradation of an anaphase inhibitor known as securin, which associates with and inhibits an anaphase activator known as separase, a protease that cleaves a subunit of the cohesion complex that mediates the association between the two sister chromatids. The budding yeasts lacking securin (Pds1) are unable to arrest mitotic progression in response to DNA or spindle damage [67]. In *Drosophila*, cells defective in securin function fail to separate sister chromatids [68], and the absence of the human securin, known as pituitary tumor transforming gene (PTTG), results in reduced levels of separase [69]. Protein sequence and immunoprecipitation studies have shown that securin-separase interaction is dependent on Cdc28-mediated phosporylation. Recently, Mourot et al. [27] showed increased accumulation of oocyte PTTG1 mRNA in oocytes from large follicles compared to the small ones, suggesting a prominent role to permit normal and efficient chromosome separations. Taken together, these data demonstrate that despite the cell type differences many of the molecular components involved in chromatids separation are conserved between meiosis and mitosis, which may suggests that some of the mechanisms may also be conserved.

Many types of stimuli function to release Ca^{2+} from an internal stores through generation of Ins (1, 4, 5)P3 (IP3) [70]. The dynamics of IP3 production can be very different depending on the receptor type being activated. Metabotropic glutamate receptor 5 (mGluR5) is a GPCR widely expressed in neurons and is activated by its natural ligand glutamate. Activation of mGluR5 has been shown to induce prolonged Ca^{2+} oscillatory activity when activated by endogenously released glutamate [71], whereas stimulation of other receptors give rapid and short Ca^{2+} transients. Oscillations in cytoplasmic Ca^{2+} levels have been implicated in the control of many different processes in different mammalian cells such as increasing the efficiency and specificity of gene expression [72], exocytosis [73], mitochondrial redox state [74], and moreover, oocyte maturation [75-77]. These receptors stimulate IP3 synthesis and the mobilization of Ca^{2+} from IP3-sensitive intracellular Ca^{2+} stores and also more efficiently through ryanodine-sensitive Ca^{2+} stores [78]. Phospholipase C activation is coupled to the activation of mGluR5 and can induce Ca^{2+} release from intracellular stores through the IP3 pathway [79]. It has also been shown

that phosphorylation and dephosphorylation of mGluR5 by protein kinase C are responsible for Ca^{2+} oscillations [80]. Although Ca^{2+} oscillations have been reported to occur during maturation of bovine oocytes in vitro, [81] and both receptors have been identified in the bovine oocyte [82], the molecular mechanisms responsible for the generation of these oscillations have not been thoroughly investigated. The stimulation of PKC by the agonist PMA can substitute for FSH induced competence in immature bovine oocytes supporting the activation of this pathway in vivo [83]. The critical role of mGluR5 in the generation of Ca^{2+} oscillations using Ca^{2+} influx from extracellular media and from intracellular stores is well documented in several mammalian species, and the same physiological role in mammalian oocyte is possible. Moreover, the presence of multiple messengers for Ca^{2+} mobilization has added further complexity to our understanding of Ca^{2+} signaling, and it is clear that different patterns of Ca^{2+} signals are generated by complex interactions between multiple Ca^{2+} release mechanisms. The identification of calcium binding protein mRNA in the present study indicates that Ca^{2+} can also act indirectly through binding to these proteins.

Our study identified a member of guanylate cyclases involved in photo transduction known as rod outer segment guanylate cyclase (ROS-GC1). Guanylate cyclase regulates cellular physiology by activating protein kinases, directly gating specific ion channels, or altering intracellular cyclic nucleotide concentrations through regulation of phosphodiesterases (PDEs) via the synthesis of cyclic GMP [84]. Synthesis of cyclic GMP in rod outer segments is catalyzed by two isoforms of this enzyme, ROS-GC1 and ROS-GC2 [85]. However, in contrast to the surface receptors guanylate cyclase family, they are not activated by any of the peptide hormones [86]. Alternatively, ROS-GC1 is modulated by the intracellular Ca^{2+} signals via Ca^{2+} binding proteins. Lower Ca^{2+} concentrations stimulated ROS-GC1 and hence guanylate cyclase while the higher Ca^{2+} concentrations inhibited the cyclase [87], consistent with a presumptive role in oocyte maturation.

The translocation of organelles in association with elements of the cytoskeleton is essential for a variety of cellular processes including cortical granules exocytosis and secretion. In the present study we identified several transcripts involved in a late stage of exocytosis. Cortical granules are targeted to designated areas of the plasma membrane via actin cables using myosin motor proteins, in the present study we demonstrated myosin regulatory light chain mRNA. Inhibition of myosin light chain kinase (MYLK2) the enzyme that specifically phosphorylates and inhibits myosin regulatory light chain [88], reduces cortical granules exocytosis in mouse oocytes [89]. The interaction between vesicle and plasma membrane integral proteins (v-SNAREs and t-SNAREs, respectively) (SNARE,

soluble *N*-ethylmaleimide-sensitive fusion attachment protein receptors), lead to the fusion of the bilayers of the secretory vesicles and the plasma membrane [90], which allow the secretion of vesicle contents and the incorporation of membrane proteins at specific plasma membrane domains. We identified Syntaxin 8 which belongs to a subfamily of the t-SNAREs that has been shown specifically required for the fusion of early endosome [91]. Our study also identifies Rab5 a member of rab family of small GTPases function as a master regulator of exocytosis by interacting with vesicle cargo, organelle fusion, organelle transport along microtubules and signal transduction (for review, see [92]). The known functions of most of these genes in other mammalian cells, and their presence in the oocyte strongly suggest roles for these proteins in the control of cortical granules exocytosis, membrane fusion and protein secretion and consequently oocyte developmental competence. However, a better understanding of this overall process will require more detailed characterization of the proteins that mediate this specific exocytosis and fusion events.

The regulation of cell-cell adhesion is a well understood function of beta and alpha catenin, nevertheless, during maturation the contact between oocyte and cumulus cells decreases and it is unlikely that the oocyte may employ the catenin complex to regulate adherent junction between the oocyte and cumulus cells. A potential role for catenin in the Wnt/wingless signaling pathway has been emerged, this pathway plays a key role in control of differentiation and development [93]. The elements of this signaling pathway are associated with cytoskeletal components and activates the cytoskeletal regulator Rho through activation of Dishevelled, and a direct interaction of Dishevelled with dishevelled-associated activator of morphogenesis (Daam1) [94]. The expression of α-catenin, β-catenin and Daam1 in the present study indicates that bovine oocytes may indeed use this complex as one of the functional units to regulate signal transduction.

During maturation, chromosomal DNA is packaged into a highly condensed chromatin structure, which inhibits transcription presumably by preventing the loading of transcription factors onto the target DNA sites. Therefore, the chromatin structure around DNA should be converted into an open configuration before the initiation of transcription processes [95]. Among the various chromatin modifying enzymes, there are two structurally distinct categories, histone-modifying enzymes which covalently acetylate, phosphorylate, ubiquitinate, or methylate histones, and chromatin remodeling complexes that use the energy of ATP hydrolysis to alter nucleosome conformation and/or position [96]. In the present study we identified a member of the ATP-dependent chromatin remodeling complexes (SWI/SNF chromatin remodeling complex subunit OSA1) which are highly

related to their *Drosophila* counterparts and display similar biochemical activities and share several conserved domains (for review, [97]). The SWI/SNF complex is required for the transcription of a subset of yeast genes and also for the function of several heterologous transcription activators in yeast [98, 99]. Different studies have revealed a cross talk between ATP-dependent chromatin remodeling complexes, histone-acetyltransferase (HATs) and the Transcription Machinery in the regulation of gene expression [96, 100]. Direct interactions between the ATP-dependent remodelers and chromatin modifiers could increase their affinity for the chromatin template, which could also affect the activities of each complex. Moreover, alteration of the chromatin template by one complex could make it a better substrate for the other complex. It has been shown that SWI/SNF is preferentially bound on an acetylated template, implying that acetylation stabilizes SWI/SNF association [100, 101].

One of the most significant findings in the present study was the up-regulation of several transcripts playing a role in the cellular energy generation. During maturation, the oocyte undergoes a series of modifications like GVBD, chromosome separations, organelle translocation, receptor phosphorylation and de-phosphorylation. All these modifications are ATP-consuming process. Based on the identified transcripts, it appears that the oocytes evolve adaptive responses to compensate for the energy requirements caused by meiotic resumption, and one of these regulatory systems could be through cytchrome c oxidase (COX), which is a terminal component of the mitochondrial respiratory chain, encoded by mitochondrial and nuclear genes, playing a vital role in the cellular energy generation (mainly heat and adenosine triphosphate, ATP) [102]. However, due to limited coding capacity of mtDNA, nuclear genes make a major contribution to mitochondrial metabolic system [103]. In our study, we have identified nuclear transcript COX8. In yeast, it has been shown that the level of COX8 transcripts expression increases with increasing oxygen concentration and falls off rapidly at lower oxygen concentrations [104]. Therefore, their activity is proportional to oxygen availability. Furthermore, AMP-activated protein kinase (AMPK) a metabolic sensing protein kinase is another interesting transcript identified in our study and also reported in mouse oocytes [105]. In various cell types, AMPK plays a fundamental role in cellular homeostasis by monitoring the availability of energy stores and is activated by the factors that cause energy depletion, AMPK activation shuts down the energy consuming processes like glucose and fatty acid synthesis and facilitates energy producing processes such as fatty acid breakdown and glucose uptake [106]. Most cell types, under normal aerobic conditions, 50 to 70% of the total energy is obtained from fatty acids, while the majority of the rest is obtained from carbohydrates (mainly glucose and lactate). All of the ATP produced from fatty acid oxidation is dependent on the presence of

oxygen. In contrast, ATP production from glucose originates from both glycolysis (which is not dependent on oxygen) and glucose oxidation (which is dependent on the presence of oxygen). It is clear that during oocyte maturation higher energy and oxygen supply are needed and fatty acids oxidation probably constitutes a large portion of energy supply.

In conclusion, by using SSH we have been able to enrich and normalize rare mRNAs using the microarray technique, which detects a greater number of genes, by which we were able to identify a set of genes previously not known to be involved in oocyte maturation. The physiological significance of these genes during maturation has not been entirely characterized.

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Chapter 4

Quantification of mRNAs encoding molecular motors and genes involved in chromosome segregation during final maturation of bovine oocytes in vivo

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Abstract

In the bovine as in many mammalian species, successful pregnancy following maturation, fertilization and culture in vitro of bovine oocytes remains relatively low compared to their in vivo counterparts. Moreover, it is well documented that not all oocytes obtained when using exogenous FSH hormone stimulation have a normal final maturation. The objective of this study was to investigate whether such differences in the oocytes and blastocysts developmental competence are associated with changes in oocyte mRNA levels of genes related to molecular motors, spindle formation and accurate chromosomal segregation throughout oocyte maturation (2 h pre LH, 6 and 22 h after LH) and at the blastocyst stage (day 7) in vivo. These genes include, 1) kinesin family member 3A 2) cytoplasmic dynein intermediate chain 3) Myosin regulatory light chain, 4) Formin-2, 5) partitioning defective protein 3, 6) Aurora-A. For this purpose, presumptive competent oocytes were selected after oFSH treatment on the basis of the steroid profile in the enclosing follicle. Moreover, to determine the role of these transcripts in developmental competence, mRNA expression was compared with that of non-competent oocytes from preovulatory follicles of the same group of cows, and to that of cultured oocytes from slaughterhouse ovaries at corresponding times of maturation and in vitro derived blastocysts.

Using quantitative real-time PCR, we detected for all above mentioned genes mRNA in the oocytes throughout the maturation process and the blastocyst stage. Formin-2 and Aurora-2 mRNA however, was only detected in the oocytes. Analysis of the mRNA levels within the oocytes scored as normal during the maturation did not reveal statistically differences. Relative abundance of all the mRNAs tested were significantly higher in the normal oocytes compared to that scored as deviant in the prematuration group. In the prematuration group, the mRNA for cytoplasmic dynein, Formin-2 and par-3 were significantly lower in the oocytes collected from slaughterhouse ovaries than those matured in vivo and scored as normal. At MII, Formin-2 and Aurora-A mRNA levels were significantly higher in the normal in vivo matured oocytes compared to the deviant or those matured in vitro. The level of par-3 mRNA was significantly higher ($P < 0.05$) in the in vivo derived blastocysts than in vitro produced blastocysts. The present results suggest an important role for these genes in spindle assembly, position and chromosome segregation. Furthermore it may be suggested that both the exogenous FSH induced intrafollicular environment and in vitro maturation and culture conditions may affect the developmental competence by affecting the expression of these genes.

***Key words*:** gene expression; bovine oocyte; blastocyst; KIF3A; par-3; Aurora-A; Formin-2

Introduction

Meiotic and developmental competence is acquired progressively during follicular and oocyte growth and is associated with a series of nuclear and cytoplasmic changes [1, 2]. The rate of successful pregnancy following maturation, fertilization and culture in vitro of bovine oocytes remains relatively low compared to their in vivo counterparts [3-5]. Moreover, it is well known that the hormonal environment of the follicles is altered in cows stimulated with exogenous gonadotrophin to a varying degree, depending partially on the type of protocol and the hormonal treatment used [6, 7]. Ovarian stimulation in mice has been shown to result in a significant increase in the frequency of oocyte spindle defects resulting in chromosomal errors [8-10]. Reduced fertility and pre- and post-implantation mortality have also been indicated as a consequence of using standard doses of gonadotrophins [11-15]. Clear evidence from several species indicated compromised developmental competence of an embryo to give rise to a viable healthy offspring, reflecting intrinsic oocyte defects that may include nuclear abnormality and suboptimal cytoplasmic maturation [16-20].

The intracellular trafficking of organelles complexes plays a major role in many cell and developmental processes. During maturation, oocyte ultrastructure studies have consistently demonstrated that translocation and transport organelle defects are likely to predominate in oocytes matured in vitro or obtained from animals stimulated with exogenous gonadotrophins [21-23]. Furthermore, the orientation and integrity of the meiotic spindle must be strictly controlled for proper chromosomal segregation. Misorientation of the spindle and errors in this process severely impairs chromosome segregation and may result in chromosomal aneuploidy. Therefore, the contribution of molecular motors and proteins involved in spindle formation and chromosome segregation to molecular events important for oocyte nuclear and cytoplasmic maturation and hence oocyte developmental competence is possible.

Organelle translocations in diverse organisms have some common molecular requirements, namely microtubule, actin, dynein, myosin, and kinesin. The directed transport of cellular components along the polarized microtubule and actin arrays use three major classes of motor proteins, kinesins [24], dyneins [25] and myosin which each move unidirectionally along the microtubule and actin tracks. These motors are required for multiple cellular tasks, such as the transport and positioning of organelles, the assembly of the meiotic spindle, chromosome movement [26] and mRNA translocation [27, 28].

Microtubules and actin filaments, the two key components of the cellular cytoskeleton, play important roles in all aspects of spindle assembly [29-33]. In somatic cells, several microtubule-dependent motors influence chromosome movement and positioning, in addition to its role in organelles translocation. These include a plus end directed motor, kinesin (KIF) [34, 35], and a minus end directed motor, cytoplasmic dynein. KIF3A is a member of the kinesin superfamily of motor proteins, producing movement necessary for transport of vesicles [36], positioning of nuclei, and proper segregation of genetic material. Targeted disruption of the KIF3A gene in mice uncovered an important role for KIF3 during development. The kif3A-/- mice do not survive beyond midgestation and exhibit apparent morphological abnormalities [37]. Similarly, the cytoplasmic dynein motor is a large complex composed of two heavy chain polypeptides, three intermediate chains and four light intermediate chains [38]. The cytoplasmic dynein motor has been implicated in vesicle transport, the perinuclear positioning of Golgi apparatus, chromosome movement on the mitotic spindle, separation of the mitotic spindle poles and nuclear positioning during cell division [39, 40]. In mammalian cells, dynein activity is proposed to control spindle length by transporting the MT-depolymerizing kinesin-13 KLP10A to the spindle poles [41]. Although the catalytic heavy chain is responsible for force production and dynamic interaction with microtubules, the intermediate chains have been implicated in linking the motor complex to membranous organelles [42, 43].

Actin-based motor myosin is central to the molecular events leading to proper cytoplasmic organelle translocation [44, 45]. Cytoplasmic myosin (Myosin II) has been demonstrated to participate in many cellular movements [46] in addition to its well-established role in cytokinesis [47, 48]. Myosin is a hetero-hexamer composed of two copies each of the heavy chain, essential light chain and regulatory light chain (MRLC) [49]. Biochemical and genetic studies have shown that MRLC regulates myosin activity by phosphorylating certain amino acids of myosin (for review see [50]) and play a direct role in force generating properties of myosin [46]. In Drosophila, mutations in the gene encoding MRLC disrupt cytokinesis in female germ cells [51] and showed defects in tissue movement during embryogenesis [52]. A recent study showed that in fertilized mouse oocytes, inhibition of myosin II or myosin light chain kinase (MLCK), which belongs to the family of Ca^{2+} /calmodulin dependent protein kinases and specifically phosphorylate myosin regulatory light chain inhibited second polar body formation and reduced cortical granules exocytosis [53].

Assembly of actin cables depend on two formin homologues, Bni1p and Bnr1p [54], members of a family of cytoskeletal regulatory proteins defined by conserved formin

homology (FH)1 and FH2 domains, for reviews, see [55]. Loss of Formin-2 (FMN2) function causes an oocyte maturation arrest at metaphase I, polyploidy and asymmetrical spindle positioning [56].

Partitioning defective protein (PAR-3) is known as an essential regulator of egg polarity, and to orient and position mitotic spindles relative to the anterior axis in the *C. elegans* embryo, [57]. Recent findings in mice oocytes showed, that Par-3 asymmetrically localized during meiosis and that its staining intensity increased on the cortical side of the MI spindle after germinal vesicle breakdown, which suggests an important role in defining the future site of the polar body extrusion [58].

Aurora-A is also an important regulator of spindle formation which plays an essential role for accurate chromosome segregation, and is known to be involved in the induction of centrosome duplication-distribution abnormalities and aneuploidy in mammalian cells [59, 60]. Mutation or disruption of the Aurora-A gene in various species leads to mitotic abnormalities, including centrosome separation, spindle aberrations, and chromosome segregation defects [61]. During maturation in mice oocytes, Aurora-A takes part in not only the initiation but also maintenance of meiotic spindle microtubule organization [62].

In vivo, a wide variety of growth factors, steroid and proteins are produced by granulosa cells in response to the LH surge [63]. Consequently, it seems logical that in vitro culture initiates a cascade of altered mRNA activity that leads to reduced oocyte developmental competence. Moreover, there is clear evidence indicating misrepresentation of mRNA expression being involved in molecular mechanisms of oocyte maturation [64] and early embryonic development due to the effect of in vitro culture conditions [65, 66]. Therefore, in this study, we used oocytes collected from cows stimulated with FSH classified as deviant and normal based on the steroid hormones profile, and oocytes collected from slaughterhouse ovaries and matured in vitro, to investigate whether the divergent culture conditions is accompanied by altered mRNA levels of the different selected genes playing important roles in organelles translocation and spindle integrity and formation.

Materials and Methods

Experimental design

At each time point of maturation, oocyte mRNA expression was compared with that of non-competent oocytes from preovulatory follicles of the same group of cows and with that of cultured oocytes from slaughterhouse ovaries at corresponding times of in vitro

maturation. Secondly, mRNA expression of the competent oocytes was compared with that of in-vivo derived blastocysts. Finally, mRNA expression of day 7 in-vitro produced blastocysts was compared to in-vivo derived counterparts.

Follicle development was stimulated in Holstein-Friesian cows using our standard protocol [65] with oFSH and a Crestar/GnRH-controlled LH surge. Cows were allocated at random to three experimental groups for ovariectomy (OVX): 1) at onset (2 h before LH), 2) after initiation (6 h after LH), and 3) at completion (22 h after LH) of final maturation to determine changes in mRNA expression throughout maturation in vivo. Oocytes were selected on the basis of follicle size, the steroid profile in the enclosing follicle as shown in table 2 and, were assigned to replicates for QPCR analysis in such a way that within a group replicates were equivalent with regards to steroid profile and represented maximum numbers of cows. In vivo blastocysts were collected from superovulated cows by flushing the uterus at day 7 after insemination. In-vitro oocytes were produced after maturation in vitro (IVM) using oocytes obtained from an abattoir, while in-vitro produced blastocysts were collected after fertilization and culture in-vitro of the in-vitro matured oocytes till the blastocysts stage. The experiment was carried out as approved by the Ethical Committee of the Veterinary Faculty of Utrecht University.

Animals and treatment

Normally cyclic Holstein-Friesian cows (n=36) were selected and treated for superovulation using the protocol as described before [65] with oFSH (Ovagen ICP, Auckland, New Zealand), prostaglandin (PG; Prosolvin; Intervet International B.V., The Netherlands) and a timed LH surge controlled by norgestomet/GnRH (Crestar ear implant/Receptal; Intervet International B.V.). Cyclicity was verified by measuring the concentration of progesterone in peripheral plasma during at least 6 weeks before FSH-stimulation [67] and the LH surge was monitored in plasma from 38 h after PG until OVX using a validated RIA with bovine LH (bLH-7981) for iodination and standards, and rabbit anti-bLH (8101) as antiserum [68]. Oocytes and follicular fluids were collected following OVX at 50, 58 and 74 h after PG corresponding with 2 h before, 6 and 22 h after the maximum of the LH surge, respectively.

Collection and selection of in-vivo preovulatory oocytes

For every treatment run with a group of 4 cows OVX was performed at 1 h intervals, the time needed to collect all oocytes and follicles from one cow by laparotomy through flank incision under local infiltration anesthesia [69]. Ovaries were collected in 0.9% (w/v)

NaCl at 37°C and immediately transported to the laboratory. The contents of each follicle > 9 mm were aspirated using an 18-ga winged infusion set needle attached to 15 ml polystyrene conical tube under low pressure by means of a suction pump, and were then immediately stored on ice at 4°C. The size of the follicles was calculated from the volume of follicular fluid after collection. After retrieval of the cumulus oocyte complexes (COC) under a stereo microscope, the follicular fluids were centrifuged 3,000 *g* for 10 min at 4°C and stored at -25°C until analysis for steroids. Collected COCs were rinsed twice in 700 µl PBS (Sigma-Aldrich, St. Louis, MO, U.S.A.) supplemented with 5% (w/v) polyvinyl alcohol (PVA; Sigma). Cumulus cells were removed by continuous pipetting after incubation for one minute with 100 µl 0.1% (w/v) hyaluronidase (Sigma-Aldrich, St. Louis, MO, U.S.A.) and denuded oocytes were checked for remaining cumulus cells and washed three times with PBS-PVA and stored individually in at -80°C until RNA extraction.

For each collection group relative to the LH surge, oocytes were sub-divided on the basis of the concentration of steroids in the fluid of the enclosing follicle. Briefly, oocytes from follicles with estradiol 17β > 0.9 µmol/L before LH, > 0.5 µmol/L 6 h after LH, and progesterone > 0.5 µmol/L 22 h after LH were considered to be competent (Table 2). Oocytes from follicles with unmistakably deviating steroid concentrations were assigned to the respective non-competent sub-groups, that is with estradiol < 0.37 µmol/L before and 6 h after LH, and with progesterone < 0.38 µmol/L 22 h after LH. The few oocytes that had heavy atretic features (expanded cumulus scattered in dark clumps in a jelly-like matrix) were excluded.

Blood sampling

Heparinized blood samples were collected from the jugular vein every day during the experimental cycle, every 3 h starting 12 h before removal of the second implant and every hour thereafter for 6 h. After immediate centrifugation at 4ºC, plasma was stored at -25ºC.

RIA of steroids in follicular fluid

Concentrations of the steroid hormones estradiol 17β and progesterone in follicular fluid were determined in aliquots of 1 to 25 µL fluid dependent of the hormone and the size of the follicle by solid-phase ^{125}I RIA methods (Coat-A-Count, Diagnostic Products Corporation, Los Angeles, CA, U.S.A; estradiol 17β: TKE2; progesterone: TKPG) as validated for blood plasma of cows [67] with slight modifications such as extraction with diethyl ether (BDH Laboratory Supplies, Poole, England).

In vitro maturation (IVM) of immature oocytes

Bovine ovaries were collected from abattoirs, and transported in a thermos flask within 3 hours of collection. After washing with water and saline solution, cumulus oocyte complexes (COCs) were aspirated from follicles 3-6 mm in diameter, washed in HEPES-buffered TCM-199 (Gibco BRL, Paisley, UK), and selected on the basis of their morphology for in vitro maturation according to the density of their cumulus cell layers, and randomly allocated in groups of 50 COCs per well to a 4-well culture plate (Nunc A/S, Roskilde, Denmark). In vitro maturation of the COCs was performed in 500 μl TCM-199 per well, supplemented with 10% (v/v) fetal calf serum, 4 μg FSH/ml, and 6 μg LH/ml (Sioux Biochemical Inc., Iowa, USA), and 0.1 mM cysteamine (Sigma Chemical Co., St louis, USA) for 22 h at 38.5ºC under an atmosphere of 5% CO2 in air with maximum humidity. At the beginning (0 h), 6 h and 22 h of the maturation, 13 oocytes from three different batches were denuded from their cumulus cells by vortexing and stored at -80ºC.

In vitro embryo production (IVP)

Procedures for in vitro maturation were performed as described previously. After maturation, oocytes in COCs were fertilized in vitro according to the procedure described by Parrish et al.[70] with minor modifications [71] using frozen-thawed semen from a bull of proven fertility. The presumptive zygotes were freed from cumulus cells 20 h after IVF by vortexing, and a maximum of ten zygotes was placed in a 20 μl droplet of synthetic oviductal fluid (SOF) medium [72] supplemented with essential and non-essential amino acids (Sigma-Aldrich, St. Louis, MO, U.S.A.), 0.1% (w/v) BSA (Sigma-Aldrich), under oil (Reproline medical GmBH, Rheinbach, Germany) and cultured at 39°C, in humidified air containing 5% CO_2 and 7% O_2. On day 4 after IVF the number of cells per cleaved embryo was scored and all cleaved embryos were transferred to fresh SOF droplets. The developmental stage of the embryos was assessed at day 7 after IVF. Four groups of 5 expanded blastocysts were rinsed in PBS and stored at -80°°C until RNA extraction.

Collection of in-vivo derived blastocysts

Superovulation was induced using an eCG/monoclonal anti-eCG/PG treatment scheme [67]. Cows were inseminated with one straw into each uterine horn, 10 h after the LH peak. Seven days later the embryos were non-surgically recovered, embryonic developmental stage and general morphological appearance were assessed by stereo microscopy, once

qualified to those in morphological grades I and II [73], frozen for storage in liquid nitrogen until use.

RNA isolation, precipitation

Total RNA was prepared from at least three replicates at each maturation stage, except for 22 h normal, 6 and 22 h deviant oocytes, of which 20, 18 and 11 oocytes were collected, resulting in 2, 2 and 1 replicate, respectively. Each replicate containing from 9 to 13 pooled oocytes. Four replicates of day 7 blastocysts were used, each containing 5 blastocysts. The RNA was then isolated using microspin column and DNA was digested with Dnase1 to eliminate possible genomic DNA contamination according to manufacturer's instruction (Absolutely RNA Microprep Kit, Stratagene, San Diego, CA, USA), the RNA was recovered by two subsequent 50-µl elutions with warmed (60°°C) elution buffer provided in the kit.
RNA was then precipitated with 250 µl of 100% ethanol (EtOH) and 10 µl of 3 M sodium acetate pH 5.2, using 1 µl of 1 mg/ml linear acrylamide (Ambion, Austin, TX) as co-precipitant. The mixture was chilled at -80^{o}C for 30 min, centrifuged for 20 min at 4^{o}C at 16000 g. The pellet was then washed with 75% EtOH and resuspended in 15 µl of water for real-time PCR analysis.

Real-time Polymerase Chain Reaction

Reverse Transcription and primers design

Total RNA was reverse-transcribed in a total volume of 20 µl using the iScript cDNA Synthesis Kit (Bio-Rad Laboratories, Hercules, CA, U.S.A.) containing oligo-dT and random hexamer primers. Reactions were incubated for 5 min at 25°°C, 30 min at 42°C, and 5 min at 85°C.
Primer sets were designed by using Beacon Designer 4 software (PREMIER Biosoft International, Palo Alto, CA, U.S.A.), from bovine sequences from NCBI, the primers used in the study are shown in table 2. The specificity of the primers was confirmed by sequencing and confirmation of the PCR product size on a standard 2% agarose gel with ethidiumbromide (EtBr). The PCR products were sequenced after purification with the QIaquick PCR Purification Kit (Qiagen, Valencia, CA, U.S.A.) and quantification with a spectrophotometer (Nanodrop ND 1000, Isogen, IJsselstein, the Netherlands). PCR products were then diluted from 100 to 0.01 fg as standards to construct the standard curve.

Real-time Polymerase Chain Reaction

Real-time PCR was performed on a Bio-Rad MyiQ system using the 2X iQ SYBR Green Supermix reagents, following the manufacturer's protocols. Reactions were performed using 25 µl duplicate reactions with the quantity of diluted cDNA corresponding to 0.1 to 0.3 of an oocyte, depending on the mRNA abundance as determined using oocytes from slaughterhouse ovaries matured in vitro, and 0.5 µM of each primer. Each transcript was amplified from at least three different replicates. Each transcript was amplified from at least three different groups of pooled oocytes, except 22 h normal, 6 and 22 h deviant oocytes, where 2, 2 and 1 group were used, respectively (see table 2). Samples and standard curves were amplified on the same run with the same PCR master mix. The thermal cycling program starts with an initial denaturation step at 95°°C for 3 min, and is followed by 45 PCR cycles (dissociation for 5 sec at 95^{o}C, annealing for 5 sec at temperature showed in table 2, and elongation for 20 sec at 72^{o}C), one melting cycle consisting of 5 sec at 95^{o}C, 30 sec at 72^{o}C, and a step cycle up to 95^{o}C (0.3^{o}C/sec transition rate), and finally a cooling down cycle at 40^{o}C. Amplification of the GAPDH mRNA [74, 75] was performed for each reverse transcribed sample as an endogenous quantification standard. These raw CT values were then analyzed with a modified delta-Ct method using a PCR data analysis program, qBase (version 1.3.2) (http://medgen.ugent.be/qbase/) to obtain relative quantification values. PCR product was analyzed on a 1.5% agarose gel with EtBr to confirm amplification. Product sizes and annealing temperatures for each gene are presented in table 1.

Statistical analysis

Data are presented as the mean ± SEM. Initially, Expression analysis data of the oocytes from follicles with a normal profile were subjected to one-way ANOVA and LSD test was used as a post hoc for comparison of individual means to assess the effect of maturation stages (-2, 6, 22). Differences within each oocyte time group (follicles with a normal profile, deviant profile and oocytes matured in vitro) were analyzed using one-way ANOVA. Difference between in vivo and in vitro derived blastocysts was analyzed using Student's t-test. Differences were considered statistically significant at the 95% confidence level ($P < 0.05$).

Table 1. Information on the primers used for real-time PCR

Genes	GeneBank accession number	Oligos sequences	Product size (bp)	Annealing temperature (°C)
KIF3A	NM_007054	F 5'-ggtgtttgggtagtctctg -3' R 5'-cggcacctaacaacaacct-3'	162	56
Dynein	BC015038	F 5'-gagtgaagaaggttctgtgtac-3' R 5'-aggcttgttattcttagttgtcc-3'	198	58
MRLC	BT020978	F 5'-aaagagcaaagaccaagacc-3' R 5'-cctggagcctcgttcatc-3'	226	56
FMNL2	NM_052905	F 5'-tgaccataacacgctgctg-3' R 5'-cctcttctgcttgcttatatgc-3'	200	54
Par3	AF467002	F 5'-ggcttcctcaagcagaacac-3' R 5'-cgagcactgaaaggcactac-3'	350	55
Aurora-A	NM_001038028	F 5'-agagacattaagccagagaa-3' F 5'-atccgaccttcaatcatttcag-3'	152	55
GAPDH	U43284	F 5'-ccacgagaagtataacaacacc-3' R 5'-gccagtagaagcagggatg-3'	229	56

KIF3A, *Homo sapiens* kinesin family member 3A; Dynein, *Homo sapiens* cytoplasmic dynein intermediate polypeptide 2; MRLC, *Bos taurus* myosin regulatory light chain 2; FMNL2, *Homo sapiens* formin-like 2; Par-3, *Homo sapiens* partitioning-defective 3 ; Aurora-A, *Bos taurus* Aurora-A; GAPDH, *Bos taurus* glyceraldehyde-3-phosphate dehydrogenase

Results

The number of oocytes classified as normal and deviant based on steroid and follicle size is presented in table 2.

The mRNAs for all the genes studied were amplified in all of the total RNA samples isolated from the oocytes. The mRNAs for Aurora-A and formin-2 were not detected at the blastocyst stage. DNA sequencing confirmed the PCR products are indeed from the same genes.

Messenger RNA levels in Fig. 1, A represent relative values of KIF3A; QPCR analysis showed that KIF3A mRNA level was highly expressed during maturation but decreases sharply at the blastocyst stage. No significant change was observed during maturation between the three maturation stages. However, in the prematuration group oocytes, a significant lower level of mRNA was found in the oocytes scored as deviant after FSH stimulation compared to those scored as normal or those matured in vitro.

Table 2. Steroid concentrations in follicles with presumptive competent and non-competent oocytes recovered during maturation in FSH-stimulated cows with a controlled LH surge

	notes	2 h before LH (Pre)	6 h after LH (Post)	22 h after LH (Post)
Normal follicles	[1]			
- number		47	50	20
- estradiol 17β	[2]	1.64 ± 0.12	0.84 ± 0.04	0.11 ± 0.01
- progesterone		0.15 ± 0.01	0.51 ± 0.01	0.75 ± 0.05
- follicle diameter	[3]	13 ± 0.4	13.5 ± 0.3	13 ± 0.5
- number of cows	[4]	13	9	8
Deviant follicles				
- number		52	18	11
- estradiol 17β		0.21 ± 0.01	0.23 ± 0.03	0.19 ± 0.02
- progesterone		0.09 ± 0.004	0.58 ± 0.06	0.16 ± 0.03
- follicle diameter		12 ± 0.3	12 ± 0.4	12 ± 0.8
- number of cows		7	7	3

[1] competent: oocytes from functional follicles on the basis of steroid profile
[2] steroid concentrations ± SEM in μmol/L follicular fluid
[3] mean diameter in mm as calculated from volume of fluid of follicles from which oocytes were used
[4] number of cows from which oocytes were retrieved

Messenger RNA levels for cytoplasmic dynein (Fig. 1, B) tended to decrease during oocyte maturation. Low levels of mRNA were detected at the blastocyst stage. However, due to the large variation in the values, the decrease was not significant during maturation. In the prematuration group and oocytes collected 6 h after maturation, the highest levels of mRNA among all oocytes analyzed was found in the FSH oocytes scored as normal.

Messenger RNA of the MRLC (Fig. 1, C) was detected in all stages of the oocyte maturation as well as at the blastocyst stage. No differences were observed between oocytes scored as normal during maturation. Among the prematuration group oocytes, the mRNA level was expressed highly in the oocytes scored as normal compared to those scored as deviant as well as those matured in vitro were no significant difference observed. No statistically significant differences ($P < 0.05$) were observed between the in vivo derived blastocysts and in vitro produced blastocysts.

The oocytes express Formin-2 mRNA throughout the maturation, whereas Formin-2 mRNA was not expressed by the day 7 blastocysts (Fig. 1, D). This expression did not appear to change significantly during maturation. Within the prematuration group, oocytes scored as normal expressed approximately 18 fold more mRNA than oocytes scored as

deviant. Similarly, at 22 h of maturation, normal oocytes expressed approximately 8 fold mRNA more than the deviant ones.

The mRNA of the par-3 was present through the maturation period and at the blastocyst stage. However, as shown in Figure 1, E, levels of par-3 mRNA were not statistically different among oocytes classified as normal at different maturation stages. In vivo derived blastocysts showed significantly ($P < 0.05$) higher mRNA levels than their in vitro counterparts.

The Aurora-A mRNA levels were detected throughout the maturation but, were not statistically different among oocytes classified as normal (Fig. 1, F). During the prematuration period, the mRNA levels for Aurora-A significantly ($P < 0.05$) decreased in the oocytes scored as deviant compared to the normal oocytes. After 22 h of maturation, the same pattern of mRNA expression could be seen, but a significant decrease was also observed in the mRNA level of the in vitro compared to deviant oocytes.

Figure 1. Messenger RNA levels of the molecular motors proteins and genes involved in chromosome segregation (A to F) in bovine oocytes collected from cows undergoing superovulation with FSH, scored as normal or deviant based on steroid profile and follicle sizes and oocytes collected from slaughterhouse ovaries and matured in vitro.
a, b, c: significantly different within the time groups (in vivo normal, in vivo deviant and in vitro oocytes) ($P < 0.05$).
* Significantly different from the oocytes

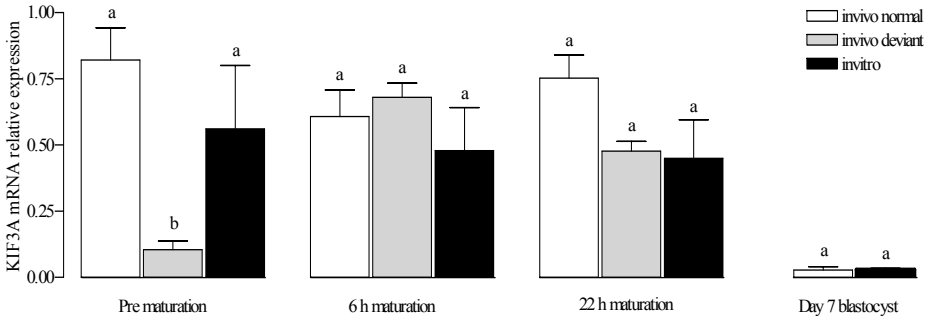

Fig. 1, A. KIF3A mRNA expression

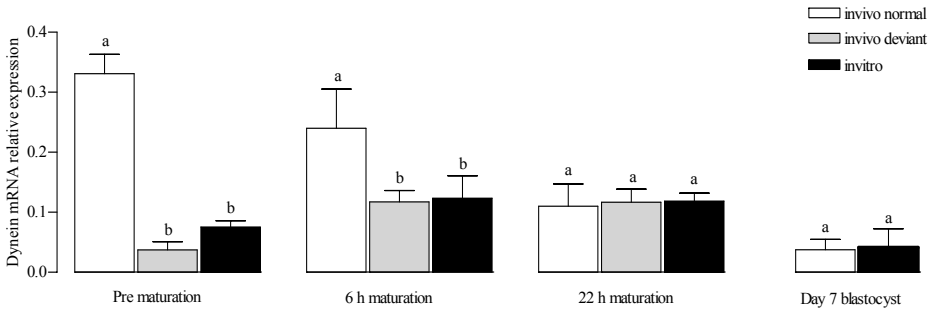

Fig. 1, B. Cytoplasmic dynein

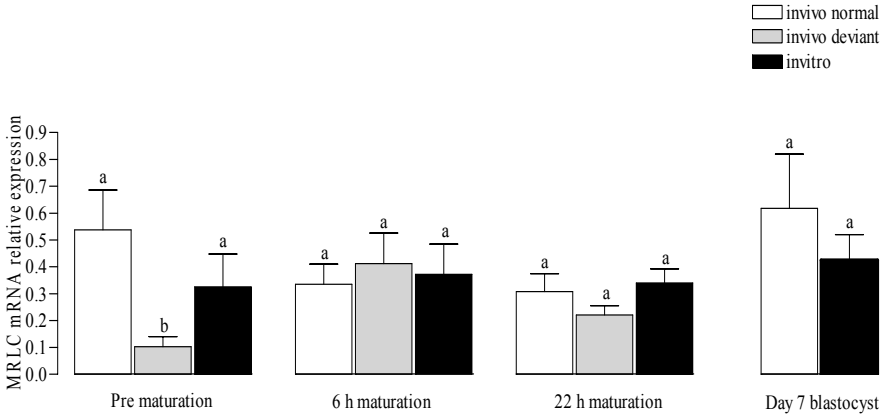

Fig. 1, C. Myosin regulatory light chain mRNA relative expression

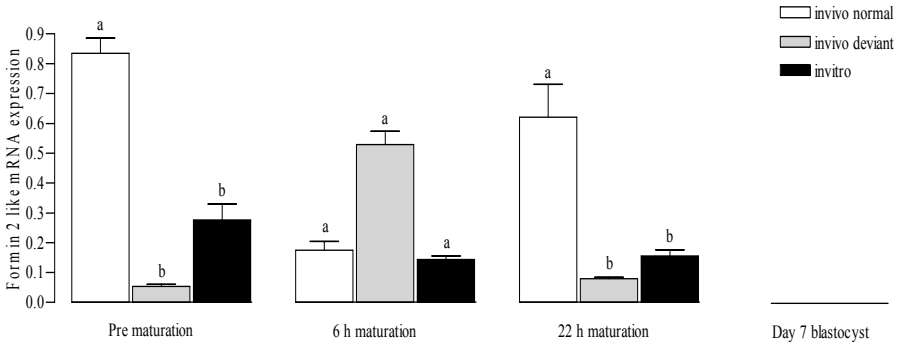

Fig. 1, D. Formin 2 like mRNA expression

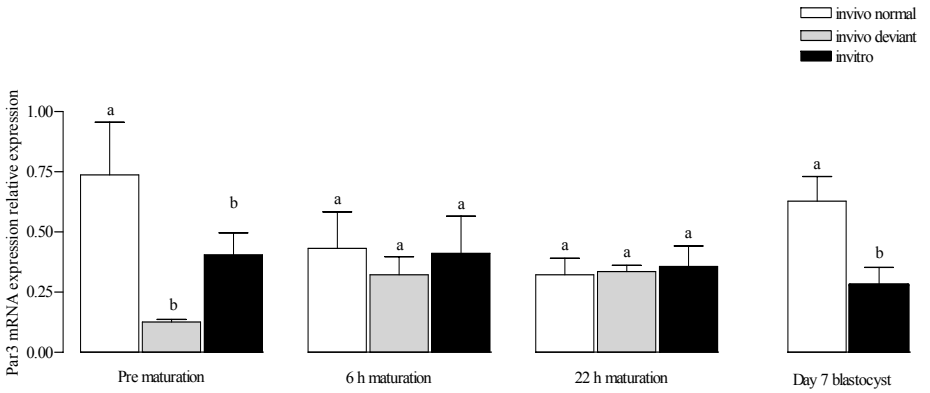

Fig. 1, E. Par3 mRNA expression

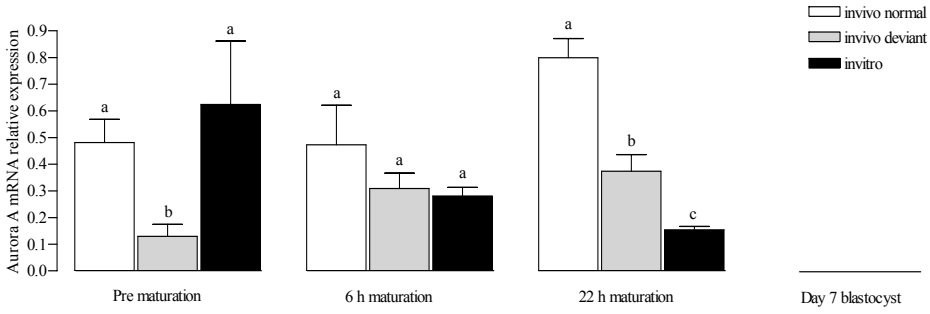

Fig. 1, F. Aurora-A mRNA expression

Discussion

At present, the nuclear and cytoplasmic pathways that contribute to the developmental competence of the bovine oocyte during maturation have not yet been resolved. In the current study, the identification of mRNAs of numerous molecular motors protein and genes involved in organelles translocation, spindle formation and chromosomal segregation in the oocyte have led to the suggestion that the expression of these mRNAs are needed to maintain proper nuclear and cytoplasmic maturation. In vitro and in vivo studies using somatic cells and knockout mouse models support that these genes have a conserved functional obligatory requirement to support nuclear and cytoplasmic maturation. The existence of these mRNAs in significant amount during maturation prompts the question which specific task(s) each individual mRNA performs in the oocyte because most of the studies came from experiments using somatic cells. However, meiotic tissues exhibit unique subcellular movements that may require specialized motors. Chromosome behavior during meiosis, particularly during the first division, differs markedly from that seen during mitosis, alignment and disjunction of chromosomes during meiosis I occurs by orientation of bivalents to the pole, not sister chromatids as in mitosis [76]. Therefore, due to cell type differences, considerable function diversity may exist. Further identification of mRNA for specific proteins in the different pathways should allow a more exact assessment of the role of these genes in oocyte maturation. Moreover, elucidating the functions and the mechanism by which these genes are regulated in the mammalian oocyte may contribute to our understanding of the molecular mechanisms of oocyte maturation.

It is well documented that not all oocytes in a stimulated cycle have a normal final maturation. Aberrations in cytoplasmic maturation as well as in timing of nuclear maturation were seen by Hyttel et al [77] in the bovine oocyte. It has been reported that only 72% of the oocytes collected at the end of maturation reach the MII stage of meiosis after exogenous gonadotrophins stimulation [78], while this percentage increases to more than 90% in the oocytes matured in vitro. The mechanism by which the superovulation techniques increase the incidence of meiotic arrest and aneuploidy is not known [14]. However, different possibilities can explain the higher incidence of aneuploidy in mammalian oocytes stimulated with exogenous gonadotrophins. Compromised follicular vascularity around the follicle may result in an under-developed capillary bed with reduced blood flow. An adequate blood supply in tissues is required to supply oxygen for aerobic metabolism, nutrients and hormones, and to eliminate CO_2 and other metabolic byproducts. Follicle diameter, estradiol concentration in follicular fluid and the vascular area have been shown to be closely associated with each other [79]

In the process of oocyte maturation there is a fundamental requirement for stable distribution of cellular components to different specific destinations at specific times. To achieve this task, cells employ molecular motors and two sets of tracks: microtubules and actin filaments. The motor domains of kinesins and dyneins have ATP-binding and microtubule-binding sites [80].

Kinesins (KIFs) are responsible for intracellular trafficking of vesicles and organelles along microtubules and for the dynamics of chromosomes and microtubules in mitosis and meiosis [81, 82]. All KIFs have a globular motor domain that contains a microtubule binding sequence and an ATP-binding sequence [24]. Kinesin-2 family is a heterotrimeric complex composed of a KIF3A/3B heterodimer and kinesin associated protein 3 (KAP3) [83]. The importance of KIF3A mRNA expression was shown in *Drosophila melanogaster* females that were homozygous for KIF3A mutations. In these females, the sperm and embryo nucleus did not fuse [84] and consequently no further development could take place. In mammals, Marszalek and others [37] demonstrated that mouse null- embryos die at 10 days post coitum. KIF3A null mouse embryos apparently do have some competence, but fail to develop any further. Recently, immunostaining analysis of Hela cells revealed intense KIF3A staining localized at the centrosomes in interphase and, when chromosomes began to condense and the mitotic spindle was formed in prometaphase, KIF3A was localized mainly at the spindle microtubules and was also localized around the cellular cortex [34]. Our results showed that KIF3A was predominately expressed in the oocyte during maturation implying an important function in the molecular events which are important for the progression of meiosis. Moreover, within the prematuration group, deviant oocytes showed significantly low levels of KIF3A mRNA, as discussed previously, it is possible that these oocytes represent the group of the oocytes that lack the ability to resume meiosis. Interestingly, KIF3A has been reported to participate in neuronal cell polarity through the transport of the par-3-aPKC complex at the tip of the neurites [85]. The coexpression of KIF3A and par-3 in the oocytes of the present study may indicate that they are associated with each other, and thus KIF3A may play an important role in polar body extrusion through correct positioning of the par-3.

Cytoplasmic dynein, the main microtubule minus-end-directed motor proteins, is involved in spindle pole organization, nuclear migration during mitosis, the positioning and functioning of the endoplasmic reticulum, Golgi apparatus, mRNA transport [80, 86] and bidirectional motion of lipid droplets [87]. In contrast to kinesin and myosin heads, dynein has multiple ATP binding sites in each head (for review see [80], therefore its mechanics is strongly altered by the ATP concentrations [88]. Homozygous *C. elegans* are sterile and

many postembryonic cell lineages undergo stochastic failed division. In the same experiment, using RNAi specifically to inhibit function of the dynein light intermediate chain 1 (DLIC-1) gene product, the majority of embryos from wild-type injections underwent failed pronuclear migration [89]. In the present study, cytoplasmic dynein intermediate chain (DIC) mRNA was decreasing during final maturation of the oocytes scored as normal. These results are in agreement with previous finding using cDNA array hybridization [90], that demonstrated a two-fold decrease in the mRNA of cytoplasmic dynein (light polypeptide) during in vitro maturation of bovine oocytes. Moreover, near the germinal vesicle breakdown during *Xenopus* oocyte maturation (approx. 6 h post LH surge in bovine), phosphorylation has been shown to increase dramatically, indicating that previously synthesized mRNA had been present in large quantity [91].
A role for decreasing oocyte DIC mRNA in mitochondrial translocation is also possible. During maturation of mice oocytes, mitochondria translocate to the perinuclear region during the resumption of meiosis and formation of the first metaphase plate, cytoplasmic dynein has been shown to play a role in mitochondrial translocation, disruption of dynein function in Hela cells, which leads to the retreat of mitochondria from the cell periphery towards the nucleus, and the formation of long, interconnected mitochondria [92]. In vitro matured oocytes and vivo matured oocytes classified as deviant collected pre and 6 h after the LH surge have revealed significantly lower mRNA levels compared to the normal oocytes. In the present study, estradiol levels within the preovulatory follicles pre and 6 h after LH were used as the main criteria to select presumptive competent from deviant oocytes, in addition to follicle size. Interestingly, estrogen has been shown to induce the transcription and expression of DLC-1 in breast cancer cells in a dose dependent manner. Further, DLC-1 interacts directly with estrogen receptors (ER) and such interaction is required for the transactivation-promoting activity of DLC-1, suggesting an important role for dynein in the action of ER. Conversely, DLC-1 downregulation compromised the ER-transactivation activity and also its nuclear accumulation [93]. Together, these data may explain the lower abundance of cytoplasmic dynein in the in vitro and in vivo deviant oocytes. Moreover, because of the complex structure of dynein, comparing to other motors, it' is possible that its function requires extensive regulation.

Myosin is a motor protein, which has actin-binding sites and ATP-binding sites and is primarily regulated by phosphorylation of the MRLC in mammalian cells [94]. The protein kinase responsible for phosphorylation of MRLC is Ca2+/calmodulin-dependent myosin light chain kinase (MLCK) [95]. Inhibition of MLCK and myosin II activity in mice oocytes revealed important roles in the position of the meiotic spindles, second polar body formation and cortical granule exocytosis [53]. Moreover, at the blastocyst stage, inhibition

of MLCK suggests a regulatory role in compaction, formation of the blastocyst and maintenance of the differentiated state during preimplantation development of mouse [96]. In the present investigation, the higher expression of this mRNA during bovine oocyte maturation and the blastocyst stages suggests important functions during both stages. Moreover, oocytes scored as deviant from the stimulated cows at the prematuration stage, had relatively low amounts of MRLC compared to those scored as normal and oocytes collected from the slaughterhouse ovaries and matured in vitro. Therefore, it may suggest that ovarian factors did not appear to be necessary for maintaining MRLC mRNA levels or, effect of superovulation treatment may affect the stability of the mRNA. However, as mammalian and non mammalian MRLC motor activity is regulated by MLCK phosphorylation, it is reasonable to assume that MRLC function is not regulated at the transcription level.

Formins are a family of conserved proteins and play an important role in actin nucleation processes, the rate-limiting step for actin filaments assembly. All formins of animals, plants and fungi contain a unique, highly conserved formin homology domain, FH2, which interacts with actin, and a domain rich in Pro-FH1 located N terminally to the FH2 domain, which binds to the actin-associated protein profilin, (for review see, [55, 97]). Formins also participate in MT processes, although their role is less clear. During spindle alignment in budding yeast, actin filaments nucleated by the formin Bni1p (Bud neck involved) are thought to provide tracks for the transport of MTs bearing Bim1p and Kar9p at their plus ends to the cortex by a myosin motor, Myo2p [98]. The *S. cerevisiae* Bni1 mutant, exhibit defects in cytoplasmic MT alignment and the positioning of mitotic spindle [99]. Recent analysis of Bni1 in *C. albicans*, by Li et al.[100] confirmed the role of Bni1 in the regulation of cell polarity. Their report shows that Bni1 is required for correct alignment and positioning of the mitotic spindle and that Bni1 defects correlate with the mislocalization of the *C. albicans* Kar9 homolog, the protein that can link cortical actin to astral microtubules. In the present study, we quantified FMNL2 which consist of FDD, FH1 and FH2 domains [101]. Moreover, because formins, myosins and *S. cerevisiae* Kar9p, which has homology with adenomatous polyposis coli (APC) (detected in bovine oocytes, our unpublished results), are all identified in the oocyte in the present study, it is plausible that bovine oocytes have a conserved pathway for delivering MT ends to the cortex, a process needed for correct orientation and positioning of the spindle. It will be interesting to elucidate the mechanisms that ensure proper spindle position and chromosome segregation. In addition, the expression of formin during oocyte maturation and the lack of the expression at the blastocyst stage suggests a distinct regulatory role during meiosis.

One of the most striking findings of the present study was the lower expression of formin both in the oocytes scored as deviant from stimulated cows and those matured in vitro compared to the normal oocytes. Females *Fmn2*-/- mice oocytes have failed to progress through MI and to correctly position the MI spindle at the cortex of the oocyte. Fertilization of the ovulated oocytes from the formin-2 lacking mice resulted in 74% polyploidy embryos, with similar spontaneous abortions [56]. In contrast to the somatic cells, the oocytes have much larger nuclei and, chromosomes at MI have to travel longer distance to the spindle, therefore capture of the chromosomes by MT is likely to be inefficient [29]. In starfish oocytes, it has been shown that MT alone are not able to capture chromosomes more than 40 μm away from the centrosomes, and therefore, polymerized actin network is necessary for delivering chromosomes within the capture distance of microtubule asters, given that actin-depolymerizing or stabilizing drugs cause chromosome loss and aneuploid eggs [29]. Together, the lower level of formin in the present study in deviant oocytes from stimulated cows and in vitro matured oocytes may explain partly the higher incidence of aneuploidy using both systems. However, future analysis of mammalian oocyte actin and formin function should provide a better understanding whether a similar mechanism exists in mammalian oocytes.

Par-3 gene is a major component of an important complex regulating cell polarity. Orientation and positioning of the mitotic spindle relative to the anterior-posterior axis is a well known function of par3-par6-atypical protein kinase C (aPKC) complex in *C. elegans*. These changes are believed to originate from asymmetric forces applied by par proteins through the microtubules [102, 103]. Mutations in the par3 gene affect the asymmetric distribution of the protein involved in cell fate determination and the orientation of mitotic spindles in successive cell cycles in *C. elegans* [104]. In mammalian oocytes, par-3 protein has been shown to localize to the anterior half of the mouse oocyte and, at MI and MII, par-3 is associated with the spindle and enriched in the central subdomain of the actin cap overlying the meiotic spindles [58]. The findings in the present study of decreased par-3 mRNA levels especially in oocytes classified as deviant from stimulated cows may indicate a disturbed function of polar body emission, because most of these oocytes defined by our selection criteria may not be able to resume meiosis [78].
Results from mammalian epithelial cells have shown that par-3, par-6 and aPKC complex are asymmetrically localized to tight junctions (TJ) [105], and this complex in addition to Cdc42 may provide an essential cue for the formation of the TJ complex [106]. During embryonic development, par-3, par-6 and aPKC have been shown to be localized only at the blastocyst stage in TJ. The same study found that par-6 and aPKC are targeted at TJ as soon as they become established while par-3 is targeted at them only when the blastocoels

appears, suggesting that par-3 is involved in the maintenance of tight junction (TJ) [107]. The significantly lower mRNA levels of par-3 in the in vitro produced blastocysts were the major finding that differed from the in vivo derived blastocysts in the current investigation. Cell adhesion is critically important for the cellular development as well as for the integrity of tissue. It is known that establishment of cell contacts during compaction in mouse embryo is mediated by E-cadherin, one component of TJ [108], that has been shown to perturbed in fragmented mouse embryos [109]. Accordingly, lower Par-3 mRNA levels in the in vitro produced blastocysts may contribute to the loss of TJ integrity, higher embryo fragmentation, defects in cell-cell adhesion and lose of the normal cellular polarity and probably also in the initial attachment of the embryo. Additionally, given the conserved function of the genes involved in polarity in eukaryotic and known conservation between polarization of the mammalian embryos and other cells, it seems likely that this system will be used in mammalian oocytes and early embryonic development [110].

The Aurora-A protein kinase, encoded by the STK15 oncogene is of major interest since it is located at the centrosome from S-phase to mitosis [111], involved in centrosome separation [112] and maturation as well as in bipolar spindle assembly and stability [113]. Increased levels of Aurora-A mRNA have been shown to correlate with chromosome instability in tumors [114]. In the mice oocytes, a higher and stable quantity of Aurora-A protein has been shown throughout maturation stages, which was localized to the spindle poles of meiotic spindle from MI to MII stage [62]. This indicates an important role for Aurora-A in the regulation of spindle formation and accurate chromosome segregation and may explain the lower levels of Aurora-A mRNA levels in MII oocytes matured in vitro. Moreover, in breast tumor cells, it has been shown that poly(A) tails of cyclin B1 and cyclin dependent kinase 1 (Cdk1) mRNA were synergistically elongated by Aurora-A and cytoplasmic polyadenylation element binding protein (CPEB). Therefore, the higher level of Aurora-A mRNA during maturation might strongly indicate an important role in meiotic resumption pathway, similar to the egg maturation process in frogs [115].

In conclusion, we studied a set of important genes expected to be involved in developmental competence of bovine oocytes and early embryonic development. Its apparent expression in the oocyte indicates that these genes have an important developmental role. One such role may be to enhance nuclear maturation by supporting spindle formation and accurate chromosomal segregation, the other, by supporting proper cytoplasmic maturation through correct organelles translocation. The detailed physiological significance of mRNA expression of most of these genes is largely unknown in the oocyte.

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Chapter 5

Alterations in the expression of genes involved in lipid metabolism during bovine oocyte maturation and at the blastocyst stage in vivo vs. in vitro

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Abstract

Lipids, mainly long chain fatty acids, are in addition to their role as energy source implicated in many physiological processes such as precursors for the biosynthesis of membrane lipids, signaling transduction molecules and ligands for transcription factors. Fatty acids (FAs) and lipid transport, catabolism and synthesis require the correct temporal activity of several gene products. While the control of FAs transport and metabolism has been examined in different mammalian cells, regulation and coordination of lipid metabolism throughout maturation of mammalian oocytes and culture of early embryonic development is widely unknown. Therefore, transcript abundance of mRNAs involved in, 1) FAs transport, including FA translocase (FAT/CD36), FA transport proteins 1 (FATP1), 2) FAs β-oxidation like AMP-activated protein kinase (AMPK), carnitine palmitoyltransferase 1 (CPT-1), peroxisome proliferator-activated receptors αα (PPARα) and acetyl CoA carboxylase β (ACCβ), and 3) *de novo* FA synthesis including acetyl CoA carboxylase α (ACCα) and fatty acid synthase (FAS), were analyzed throughout oocyte maturation (2 h pre LH, 6 and 22 h after LH) and at the blastocyst stage (day 7) in vivo, using quantitative real time PCR. Presumptive competent oocytes were selected after oFSH treatment on the basis of the steroid profile in the enclosing follicle. Moreover, to determine the role of these transcripts in developmental competence, mRNA expression was compared with that of non-competent oocytes from preovulatory follicles of the same group of cows, and to that of cultured oocytes from slaughterhouse ovaries at corresponding times of maturation and in vitro derived blastocysts. Taken together, our results showed an increase in mRNA implicated in FAs transport (CD36 and FATP1) and catabolism (AMPK, CPT-1) during oocyte maturation compared with the blastocysts, where there is a marked decrease or lack of these transcripts. In contrast, a sharp and significant increase was observed in mRNAs involved in de novo FA synthesis (FAS and ACCα) at the blastocyst stage compared to the oocytes. Moreover, our results showed that different oocyte microenvironments can have a profound effect on mRNA encoding components of FA utilization, catabolism and synthesis. In vitro produced blastocysts showed a significant decrease in ACCα compared to the in vivo derived ones. Our results suggest that an aberrant expression of these transcripts might contribute to oocytes and blastocyst developmental competence.

***Key words*:** gene expression; lipid metabolism; CD36; FATP1; CPT-1; ACCα

Introduction

Lipids, as well as carbohydrates and amino acids, are the three basic materials providing an important source of energy for all mammalian cells. Beside their crucial role as energy source, lipids, mainly long chain fatty acids (LCFAs) are precursors for the biosynthesis of membrane lipids, lipid signaling molecules and ligands for transcription factors that control metabolic activity. Depending on the cell type, and the energy needs, LCFAs can be imported into the cell from exogenous source, or generated endogenously from de novo synthesis, hydrolysis of triglycerides. Although, energy contained in lipids exceeds that in carbohydrates and proteins, much research relevant to energy needs during mammalian oocyte maturation and early embryonic development in vitro has been directed towards carbohydrates and amino acids and we know much less about the possible regulatory roles of lipids during mammalian oocyte maturation and early embryonic development.

Structurally, lipid content in bovine oocytes tends to increase progressively with oocyte growth [1], In addition, ranking the oocytes based on the commonly used indices for COCs quality [2] demonstrated a difference in fatty acids (FAs) composition and indicates that the appearance of the cytoplasm may reflect its lipid, FAs content and developmental competence [3, 4]. Biochemically, higher lipase activity [5] and a decrease in triglyceride content recorded during in vitro maturation of bovine oocytes [4] indicate that lipid may act as energy source during bovine oocyte maturation.

Oocyte and embryo FAs profiles and their rate of uptake have been proposed as a tool to predict oocytes and embryo quality. Haggarty et al [6] showed a reduction in the concentrations of linoleic and oleic acid in human embryos that failed to develop beyond 4-cell stage compared to that developed normally. Similarly, Zeron et al [7] found evidence that the seasonal changes in the bovine oocyte developmental competence might arise primarily due to changes in FAs profiles of ovarian follicular fluid and oocytes. Moreover, marked changes in linoleic acid levels of NEFA and phospholipids concentrations have also been reported between estradiol active and inactive bovine preovulatory follicles [8]. On the other hand, lipid accumulation during in vitro culture of early bovine embryos in medium containing FCS compared to serum free medium remains a source of debate for a number of studies. Earlier studies had indicated that culture in the presence of serum results in intracellular lipid accumulation and greater sensitivity to freezing [9-11] but more recent work suggests no difference in the post-cryopreservation survival [12]. Regardless of the presence or absence of serum, it is clear that culture of bovine embryos in vitro is

associated with a greater sensitivity to freezing during cryopreservation comparing to those matured in vivo [13]. Despite this evidence, the necessity of FAs and the pathways involved in regulation and coordination of lipid metabolism at the molecular level throughout maturation of oocytes and culture of early embryonic development remains largely unknown.

Examining some of the key genes controlling the major pathways of 1) FAs transport like FA translocase (FAT/CD36), FA transport proteins 1 (FATP1), and 2) FAs β-oxidation like AMP-activated protein kinase (AMPK), carnitine palmitoyltransferase 1 (CPT-1), peroxisome proliferator-activated receptors α (PPARα) and acetyl CoA carboxylase β (ACCβ), and 3) *de novo* FA synthesis including acetyl CoA carboxylase α (ACCα) and fatty acid synthase (FAS), may elucidate the possible role of FAs during oocyte maturation and early embryonic development, and the tasks played by these genes with regard to the control of lipid metabolism. Although the Free FAs can be taken up by diffusion through the plasma membrane [14], different proteins integrally associated with the plasma membrane that have been identified [15] can facilitate uptake and coordinate the import of Free FA in harmony with metabolic demands. The best characterized, including FAT/CD36 [16] and FATPs [17], changes in LCFA uptake have been observed to occur in parallel with changes in FAT/CD36 expression in rat heart and skeletal muscle [18], and a null mutation in FAT/CD36 reduced the uptake of FAs [19]. FATP1 is a well characterized member of a large family of plasma membrane proteins that increase FA import when expressed in cultured cells [17], disruption of the yeast *Saccharomyces cerevisiae* FATP1 has been shown to impair LCFA uptake growth [20].

Within most mammalian cells, controlling energy demands in the form of ATP are done through β oxidation of FAs in the mitochondria. Different genes have been involved in the transcriptional control of β oxidation. AMPK is an attractive potential candidate, and its role in this process has been suggested [21, 22]. AMPK is activated by low ATP/AMP ratios to serve as a fuel gauge for mammalian cells to protect against energy deprivation [23], its activation is associated with increased mitochondrial biogenesis [24] and enzyme content [25] in rat skeletal muscle. Activation of this enzyme in muscle increases FAs oxidation [26-28] and inhibits acetyl CoA carboxylase [25, 29]. Within the cytoplasm, FAs are activated to long-chain acyl-CoA by an acyl-CoA synthetase. The acyl part is then transferred into the mitochondria for oxidation by a complex of enzymes involving CPT-1 on the outer aspect of the inner mitochondrial membrane. Inside the mitochondrial matrix, long-chain acyl-CoA passes through the β-oxidation enzyme system to produce acetyl-CoA. It is generally accepted that in different mammalian cells β oxidation is regulated

through CPT 1 through different mechanisms, including changes in the activity and transcription rate of CPT 1, or its inhibitor malonyl-CoA the end product of ACCβ associated with mitochondrial membrane [30]. The transcription factor PPARα is a member of PPAR family that functions as a ligand-dependent transcription factor [31], its expression is more abundantly in tissues that are characterized by high rates of FAs oxidation (FAO), and is considered as the main subtype that mediates lipid-induced activation of FAO genes [32].

FAs are synthesized by a common biochemical pathway in all mammalian cells involving two key enzymatic reactions, the first is the carboxylation of acetyl-CoA in the cytosol to form malonyl CoA by ACCα [30], then FAS catalyzes the synthesis of LCFAs, by using acetyl-CoA as a primer, malonyl-CoA as a two-carbon donor for chain elongation, and NADPH for the reduction reactions [33].

While the control of fatty acid transport and metabolism has been examined in different mammalian cells, little is known about the oocyte control of fatty acid entry during the final stages of maturation. It is also well documented that oocyte quality obtained from gonadotropin-stimulated cows or matured in vitro can be variable due to many factors, among these factors which have been clearly demonstrated are the ATP content [34, 35] and lipid composition of the oocyte [7]. To adjust to changes in the demand of lipids during maturation and early embryonic development, oocytes and early embryos must precisely regulate and coordinate the expression of several genes. Therefore, this study investigates the expression of key mRNAs involved in lipid metabolism throughout bovine oocyte maturation and blastocysts stage in vivo. Additionally, we also analyzed the expression of these genes under in vivo and in vitro culture conditions to unravel part of the signals and mechanisms that participate in developmental competence.

Materials and Methods

Experimental design

At each time point of maturation, oocyte mRNA expression was compared with that of non-competent oocytes from preovulatory follicles of the same group of cows and with that of cultured oocytes from slaughterhouse ovaries at corresponding times of in vitro maturation. Secondly, mRNA expression of the competent oocytes was compared with that

of in-vivo derived blastocysts. Finally, mRNA expression of day 7 in-vitro produced blastocysts was compared to in-vivo derived counterparts.

Follicle development was stimulated in Holstein-Friesian cows using our standard protocol [36] with oFSH and a Crestar/GnRH-controlled LH surge. Cows were allocated at random to three experimental groups for ovariectomy (OVX): 1) at onset (2 h before LH), 2) after initiation (6 h after LH), and 3) at completion (22 h after LH) of final maturation to determine changes in mRNA expression throughout maturation in vivo. Oocytes were selected on the basis of follicle size, the steroid profile in the enclosing follicle as shown in table 2 and, were assigned to replicates for QPCR analysis in such a way that within a group replicates were equivalent with regards to steroid profile and represented maximum numbers of cows. In vivo blastocysts were collected from superovulated cows by flushing the uterus at day 7 after insemination. In-vitro oocytes were produced after maturation in vitro (IVM) using oocytes obtained from an abattoir, while in-vitro produced blastocysts were collected after fertilization and culture in-vitro of the in-vitro matured oocytes till the blastocysts stage. The experiment was carried out as approved by the Ethical Committee of the Veterinary Faculty of Utrecht University.

Animals and treatment

Normally cyclic Holstein-Friesian cows (n=36) were selected and treated for superovulation using the protocol as described before [36] with oFSH (Ovagen ICP, Auckland, New Zealand), prostaglandin (PG; Prosolvin; Intervet International B.V., The Netherlands) and a timed LH surge controlled by norgestomet/GnRH (Crestar ear implant/Receptal; Intervet International B.V.). Cyclicity was verified by measuring the concentration of progesterone in peripheral plasma during at least 6 weeks before FSH-stimulation [37] and the LH surge was monitored in plasma from 38 h after PG until OVX using a validated RIA with bovine LH (bLH-7981) for iodination and standards, and rabbit anti-bLH (8101) as antiserum [38]. Oocytes and follicular fluids were collected following OVX at 50, 58 and 74 h after PG corresponding with 2 h before, 6 and 22 h after the maximum of the LH surge, respectively.

Collection and selection of in-vivo preovulatory oocytes

For every treatment run with a group of 4 cows OVX was performed at 1 h intervals, the time needed to collect all oocytes and follicles from one cow by laparotomy through flank incision under local infiltration anesthesia [39]. Ovaries were collected in 0.9% (w/v) NaCl at 37^{o}C and immediately transported to the laboratory. The contents of each follicle >

9 mm were aspirated using an 18-ga winged infusion set needle attached to 15 ml polystyrene conical tube under low pressure by means of a suction pump, and were then immediately stored on ice at 4°C. The size of the follicles was calculated from the volume of follicular fluid after collection. After retrieval of the cumulus oocyte complexes (COC) under a stereo microscope, the follicular fluids were centrifuged 3,000 *g* for 10 min at 4°C and stored at -25°C until analysis for steroids. Collected COCs were rinsed twice in 700 µl PBS (Sigma-Aldrich, St. Louis, MO, U.S.A.) supplemented with 5% (w/v) polyvinyl alcohol (PVA; Sigma). Cumulus cells were removed by continuous pipetting after incubation for one minute with 100 µl 0.1% (w/v) hyaluronidase (Sigma-Aldrich, St. Louis, MO, U.S.A.) and denuded oocytes were checked for remaining cumulus cells and washed three times with PBS-PVA and stored individually in at -80°C until RNA extraction.

For each collection group relative to the LH surge, oocytes were sub-divided on the basis of the concentration of steroids in the fluid of the enclosing follicle. Briefly, oocytes from follicles with estradiol 17β > 0.9 µmol/L before LH, > 0.5 µmol/L 6 h after LH, and progesterone > 0.5 µmol/L 22 h after LH were considered to be competent (Table 2). Oocytes from follicles with unmistakably deviating steroid concentrations were assigned to the respective non-competent sub-groups, that is with estradiol < 0.37 µmol/L before and 6 h after LH, and with progesterone < 0.38 µmol/L 22 h after LH. The few oocytes that had heavy atretic features (expanded cumulus scattered in dark clumps in a jelly-like matrix) were excluded.

Blood sampling

Heparinized blood samples were collected from the jugular vein every day during the experimental cycle, every 3 h starting 12 h before removal of the second implant and every hour thereafter for 6 h. After immediate centrifugation at 4 ºC, plasma was stored at -25 ºC.

RIA of steroids in follicular fluid

Concentrations of the steroid hormones estradiol 17β and progesterone in follicular fluid were determined in aliquots of 1 to 25 µL fluid dependent of the hormone and the size of the follicle by solid-phase ^{125}I RIA methods (Coat-A-Count, Diagnostic Products Corporation, Los Angeles, CA, U.S.A; estradiol 17β: TKE2; progesterone: TKPG) as validated for blood plasma of cows [37] with slight modifications such as extraction with diethyl ether (BDH Laboratory Supplies, Poole, England).

In vitro maturation (IVM) of immature oocytes

Bovine ovaries were collected from abattoirs, and transported in a thermos flask within 3 hours of collection. After washing with water and saline solution, cumulus oocyte complexes (COCs) were aspirated from follicles 3-6 mm in diameter, washed in HEPES-buffered TCM-199 (Gibco BRL, Paisley, UK), and selected on the basis of their morphology for in vitro maturation according to the density of their cumulus cell layers, and randomly allocated in groups of 50 COCs per well to a 4-well culture plate (Nunc A/S, Roskilde, Denmark). In vitro maturation of the COCs was performed in 500 µl TCM-199 per well, supplemented with 10% (v/v) fetal calf serum, 4 µg FSH/ml, and 6 µg LH/ml (Sioux Biochemical Inc., Iowa, U.S.A.), and 0.1 mM cysteamine (Sigma Chemical Co., St Louis, MO, U.S.A.) for 22 h at 38.5ºC under an atmosphere of 5% CO2 in air with maximum humidity. At the beginning (0 h), 6 h and 22 h of the maturation, cumulus cells of 13 oocytes from three different batches were denuded by vortexing and stored at -80 ºC.

In vitro embryo production (IVP)

Procedures for in vitro maturation were performed as described previously, after maturation, oocytes in COCs were fertilized in vitro according to the procedure described by Parrish et al. [40] with minor modifications [41] using frozen-thawed semen from a bull of proven fertility. The presumptive zygotes were freed from cumulus cells 20 h after IVF by vortexing, and a maximum of ten zygotes was placed in a 20 µl droplet of synthetic oviductal fluid (SOF) medium [42] supplemented with essential and non-essential amino acids (Sigma-Aldrich, St. Louis, MO, U.S.A.) and 0.1% (w/v) BSA (Sigma-Aldrich), under oil (Reproline medical GmBH, Rheinbach, Germany) and cultured at 39°C, in humidified air containing 5% CO_2 and 7% O_2. On day 4 after IVF the number of cells per cleaved embryo was scored and all cleaved embryos were transferred to fresh SOF droplets. The developmental stage of the embryos was assessed at day 7 after IVF. Four groups of 5 expanded blastocysts were rinsed in PBS and stored at -80°C until RNA extraction.

Collection of in-vivo derived blastocysts

Superovulation was induced using an eCG/monoclonal anti-eCG/PG treatment scheme [37]. Cows were inseminated with one straw into each uterine horn, 10 h after the LH peak. Seven days later the embryos were non-surgically recovered, embryonic developmental stage and general morphological appearance were assessed by stereo microscopy, once

qualified to those in morphological grades I and II [43], frozen for storage in liquid nitrogen until use.

RNA isolation, precipitation

Total RNA was prepared from at least three replicates at each maturation stage, except 22 h normal, 6 and 22 h deviant oocytes, where 20, 18 and 11 oocytes were collected, respectively. Each replicate containing from 9 to 13 pooled oocytes. Four replicates of day 7 blastocysts were used each containing 5 blastocysts, the RNA was then isolated using microspin column and DNA was digested with Dnase1 to eliminate possible genomic DNA contamination according to manufacturer's instruction (Absolutely RNA Microprep Kit, Stratagene, San Diego, CA, U.S.A.). The RNA was recovered by two subsequent 50-µl elutions with warmed (60°C) elution buffer provided in the kit.
RNA was then precipitated with 250 µl of 100% ethanol (EtOH) and 10 µl of 3 M sodium acetate pH 5.2, using 1 µl of 1 mg/ml linear acrylamide (Ambion, Austin, TX, U.S.A.) as co-precipitant. The mixture was chilled at -80^{o}C for 30 min, centrifuged for 20 min at 4^{o}C at 16000 g. The pellet was then washed with 75% EtOH and resuspended in 15 µl of water for real-time PCR analysis.

Real-time Polymerase Chain Reaction

Reverse Transcription and primers design

Total RNA was reverse-transcribed in a total volume of 20 µl using the iScript cDNA Synthesis Kit (Bio-Rad Laboratories, Hercules, CA, U.S.A.) containing oligo-dT and random hexamer primers. Reactions were incubated for 5 min at 25 °C, 30 min at 42°C, and 5 min at 85°C.
Primer sets were designed by using Beacon Designer 4 software (PREMIER Biosoft International, Palo Alto, CA, U.S.A.), from bovine sequences from NCBI, the primers used in the study are shown in table 1. The specificity of the primers was confirmed by sequencing and confirmation of the PCR product size on a standard 2% agarose gel with ethidiumbromide (EtBr). The PCR products were sequenced after purification with the QIaquick PCR Purification Kit (Qiagen, Valencia, CA, U.S.A.) and quantification with a spectrophotometer (Nanodrop ND 1000, Isogen, IJsselstein, the Netherlands). PCR products were then diluted from 100 to 0.01 fg as standards to construct the standard curve.

Real-time Polymerase Chain Reaction

Real-time PCR was performed on a Bio-Rad MyiQ system using the 2X iQ SYBR Green Supermix reagents, following the manufacturer's protocols. Reactions were performed using 25 µl duplicate reactions with the quantity of diluted cDNA corresponding to 0.1 to 0.3 of an oocyte, depending on the mRNA abundance as determined using oocytes from slaughter house ovaries matured in vitro, and 0.5 µM of each primer. Each transcript was amplified from at least three different groups of pooled oocytes, except 22 h normal, 6 and 22 h deviant oocytes, where 2, 2 and 1 group were used, respectively (see table 2).

Table 1. Information on the primers used for real-time PCR

Genes	GeneBank accession number	Oligos sequences	Product size (bp)	Annealing temperature (^{o}C)
CD36	NM_174010	F 5'-tcattgctggtgctgtcattgg-3' R 5'-aactgtcacttcatctggattctgc-3'	209	59
FATP1	NM_001033625	F 5'-caagagcctggtcaagttc-3' R 5'-cgtgtagatgtagaagagtcg-3'	157	57
AMPAKγ1	BT025456	F 5'-aagatagaaacttggagagaggtg-3' R 5'-aacttgaggaacttgaggatgc-3'	197	59
CPT-I	NM_001034349	F 5'-ggtcaacagcaactactacg-3' R 5'-tgaacatcctctccatctgg-3'	188	52
PPARα	NM_001034036	F 5'-tcgtttcctcctttaccttc-3' R 5'-tctgtgtccaccatctcc-3'	170	54
ACCβ	AJ966324	F 5'-tacctgcgggcgtctgag-3' R 5'-gctgattctggtgtagtcttgg-3'	192	56
ACCα	NM_174224	F 5'-aagcaatggatgaaccttcttc-3' R 5'-gatgcccaagtcagagagc-3'	197	58
FAS	NM_001012669	F 5'-ctctccctcagccgttcg-3' R 5'-gcctgtcatcatctgtcacc-3'	168	63
GAPDH	BTU85042	F 5'-ccacgagaagtataacaacacc-3' R 5'-gccagtagaagcagggatg-3'	229	56

Bos taurus CD36, Fatty acid translocase; FATP1, *Bos taurus* fatty acid transport protein 1; AMPAKγ1, *Bos taurus* AMP-activated protein kinase gamma 1; CPT-1, *Bos taurus* carnitine palmitoyltransferase 1; PPARαα, *Bos taurus* peroxisome proliferator-activated receptors α; ACCβ, *Bos taurus* acetyl-CoA carboxylase β; ACCα, *Bos taurus* acetyl-CoA carboxylase α; FAS, *Bos taurus* fatty acid synthase; GAPDH, *Bos taurus* glyceraldehyde-3-phosphate dehydrogenase

Samples and standard curves were amplified on the same run with the same PCR master mix.

The thermal cycling program starts with an initial denaturation step at 95°C for 3 min, and is followed by 45 PCR cycles (dissociation for 5 sec at 95°C, annealing for 5 sec at temperature showed in table 1, and elongation for 20 sec at 72°C), one melting cycle consisting of 5 sec at 95°C, 30 sec at 72°C, and a step cycle up to 95°C (0.3°C/sec transition rate), and finally a cooling down cycle at 40°C. Amplification of the GAPDH mRNA [44, 45] was performed for each reverse transcribed sample as an endogenous quantification standard. These raw CT values were then analyzed with a modified delta-Ct method using a PCR data analysis program, qBase (version 1.3.2) (http://medgen.ugent.be/qbase/) to obtain relative quantification values. PCR product was analyzed on a 1.5% agarose gel with EtBr to confirm amplification. Product sizes and annealing temperatures for each gene are presented in table 1.

Statistical analysis

Data are presented as the mean ± SEM. Initially, expression analysis data of the oocytes from follicles with a normal profile were subjected to one-way ANOVA and Bonferroni test was used as a post hoc for comparison of individual means to assess the effect of maturation stages (-2, 6, 22). Secondly, data from oocytes classified normal in the prematuration stages (-2) were compared using un-paired Student's t-test to the in vivo derived blastocysts to study the difference in gene expression between oocytes and blastocysts. Thirdly, differences within each oocyte time group (follicles with a normal profile, deviant profile and oocytes matured in vitro) were analyzed using one-way ANOVA. Difference between in vivo and in vitro derived blastocysts was analyzed using Student's t-test. Differences were considered statistically significant at the 95% confidence level ($P < 0.05$).

Results

In total 171/241 before LH, 135/183 at 6 h after LH and 60 oocytes/ 93 follicles were retrieved resulting in 71, 74 and 65% recovery, respectively. The number of oocytes classified as competent and non-competent based on steroid and follicle size are presented in table 2.

Table 2.: Steroid concentrations in follicles with competent and non-competent oocytes recovered during maturation in FSH-stimulated cows with a controlled LH surge

	notes	2 h before LH (Pre)	6 h after LH (Post)	22 h after LH (Post)
Normal follicles	[1]			
- number		47	50	20
- estradiol 17β	[2]	1.64 ± 0.12	0.84 ± 0.04	0.11 ± 0.01
- progesterone		0.15 ± 0.01	0.51 ± 0.01	0.75 ± 0.05
- follicle diameter	[3]	13 ± 0.4	13.5 ± 0.3	13 ± 0.5
- number of cows	[4]	13	9	8
Deviant follicles				
- number		52	18	11
- estradiol 17β		0.21 ± 0.01	0.23 ± 0.03	0.19 ± 0.02
- progesterone		0.09 ± 0.004	0.58 ± 0.06	0.16 ± 0.03
- follicle diameter		12 ± 0.3	12 ± 0.4	12 ± 0.8
- number of cows		7	7	3

[1] competent: oocytes from functional follicles on the basis of steroid profile
[2] steroid concentrations ± SEM in μmol/L follicular fluid
[3] mean diameter in mm as calculated from volume of fluid of follicles from which oocytes were used
[4] number of cows from which oocytes were retrieved

Results of gene expression were used to organize group of genes according to their function.

Group 1. Fatty acid transport: within the oocytes selected from normal follicles, levels of CD36 (fig. 1, A) and FATP1 (fig. 1, B) mRNA were relatively high before, 6 and 22 h after maturation, but no significant ($P < 0.05$) differences were observed between the different time groups (from normal follicles). The expression pattern of this group at the blastocysts stage characterized by the absence (CD36) or low levels of mRNA (FATP1). Within each time group, there were marked changes in the expression between the groups but it was significant only between oocytes collected from FSH stimulated cows classified as normal and deviant in the pre-maturation groups for both mRNA.

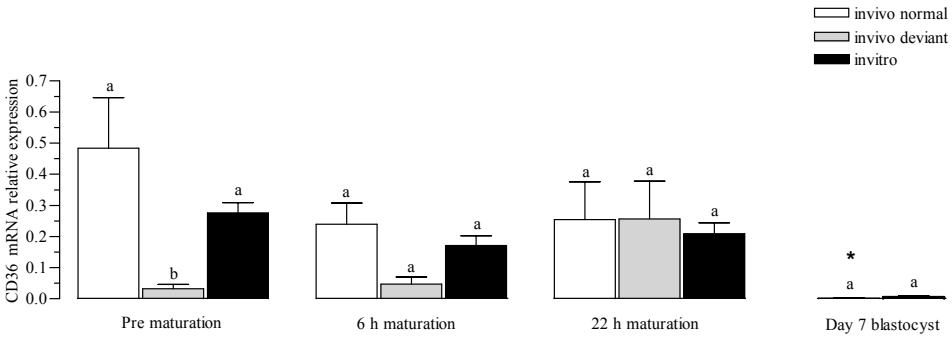
Group 2. Fatty acid β-oxidation: mRNA levels varied across this group, with the level of AMPK (Fig.1, C) and CPT-I (fig. 1, D) high during the pre-maturation and 6, 22 h after maturation but there were no statistical significant differences between the time groups ($P < 0.05$). A marked reduction in the mRNA expression for both mRNA at the blastocyst stage,

CPT-1 mRNA was not detected in in vivo derived blastocysts. The gene expression of AMPK and CPT-1 was significantly ($P < 0.05$) reduced in the oocyte scored as bad during pre-maturation compared to the good one, CPT-1 expression in the oocyte matured in vitro was significantly altered compared to the oocytes classified as normal, whereas both transcripts remained unchanged during 6 h of maturation. A significant difference within the 22 h group in the expression of AMPK, between in vivo normal, deviant, and those matured in vitro was observed.

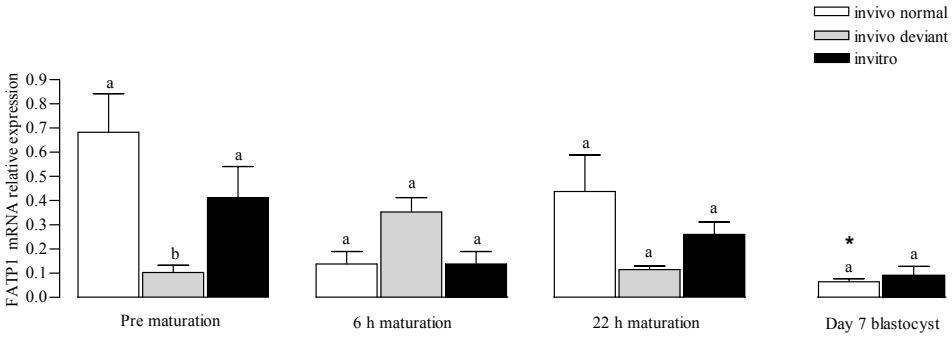
The levels of PPARα (Fig. 1, E) mRNA remained unchanged between the different time groups and also between oocytes and blastocysts. A significant difference between the oocytes collected from follicles with normal profiles was seen compared to those scored as deviant and matured in vitro ($P < 0.05$). Whereas no significant alterations in PPARα mRNA expression between in vivo and in vitro blastocysts were detected. Similarly, ACCβ (Fig. 1, F) mRNA levels were not significantly different ($P < 0.05$) within the oocyte time groups (-2, 6, 22) and also between the oocytes and the blastocysts.

Group 3. Fatty acid synthesis: A similar pattern of changes characterize both member of this group, ACCα (Fig. 1, G) and FAS (Fig. 1, H). No differences between different time groups during oocyte maturation and a sharp and significant increase in both mRNA levels during the blastocysts stages compared to oocyte maturation. A stastically significant difference ($P < 0.05$) was observed in the expression of ACCα between in vivo derived blastoysts and those cultured in vitro.

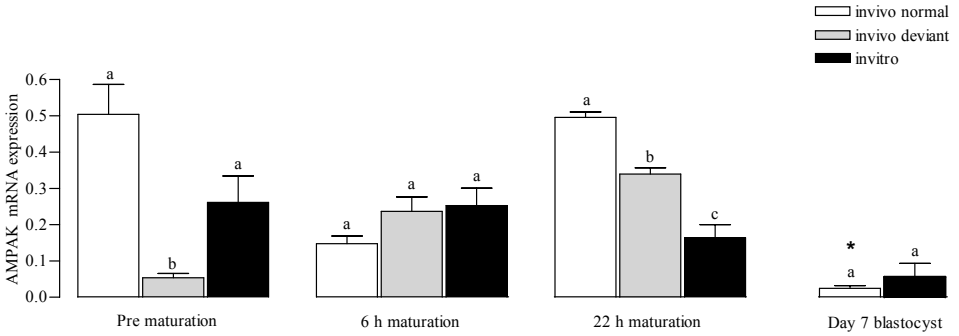
Figure 1. Quantification of mRNA levels by real-time PCR of genes involved in lipid metabolism (A to H) in bovine oocytes from cows undergoing superovulation with FSH scored as normal or deviant based on steroid profile and follicle sizes and oocytes collected from slaughterhouse ovaries and matured in vitro.
a, b, c: significantly different within the time groups (in vivo normal, in vivo deviant and in vitro oocytes) ($P < 0.05$).
* Significantly different from the oocytes



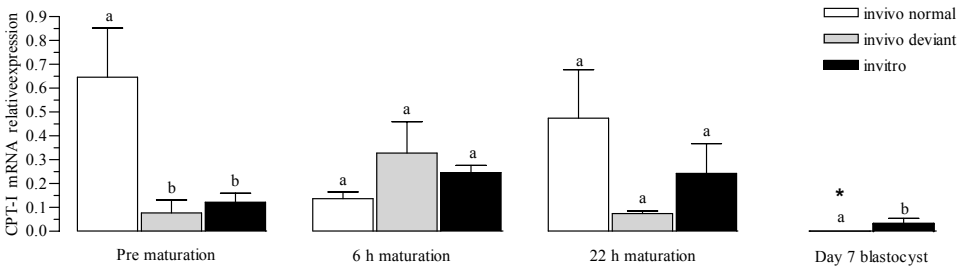
A. Fatty acid translocase/CD36 mRNA



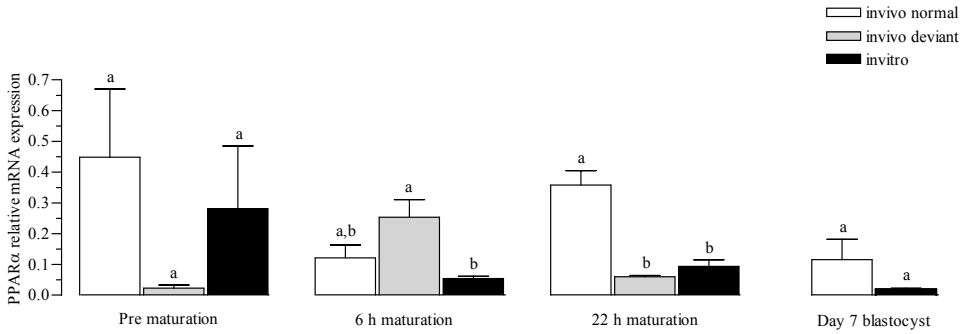
B. Fatty acid transport protein1



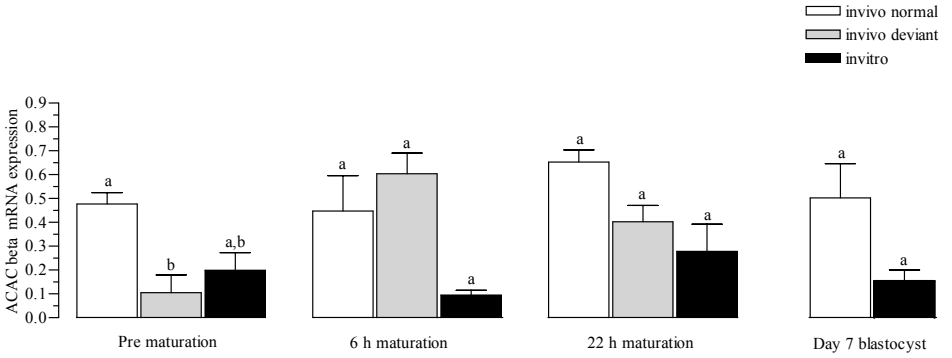
C. AMP-activated protein kinase γ1



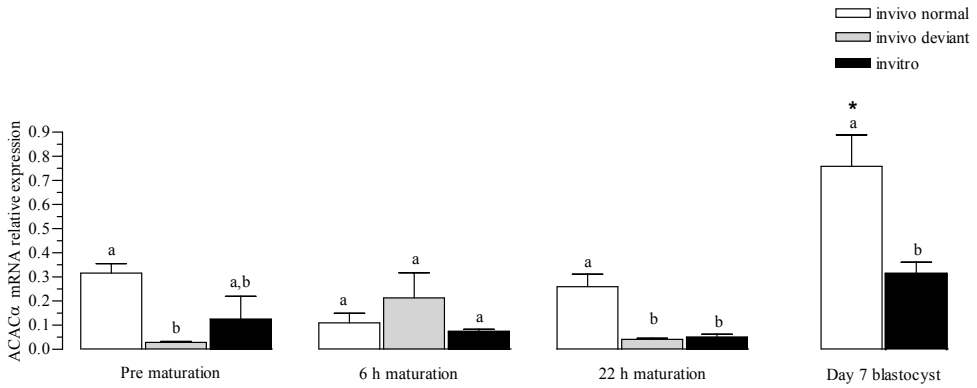
D. Carnitine palmitoyltransferase I



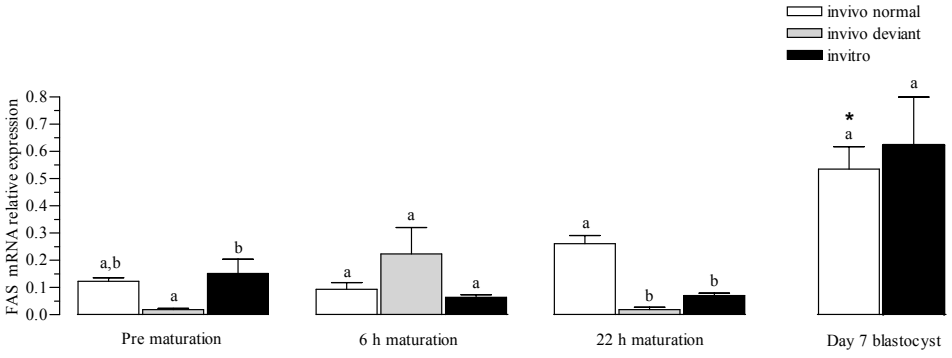
E. Peroxisome proliferative activated receptor alpha



F. Acetyl CoA carboxylase beta



G. Acetyl CoA carboxylase Alpha



H. Fatty acid synthase

Discussion

Our data suggest that, under physiological conditions, 1) during oocyte maturation, exogenous FAs supply is probably the preferential source of LCFA and energy source, based on the higher expression of transcripts involved in FAs transport and key regulatory enzymes involved in β-oxidation of FAs. 2) At the blastocysts stage, the parallel increase in the mRNA abundance of FAS and ACCα suggests an increased rate of lipogenesis which may be needed to support earlier embryogenesis. 3) A lower reliance on FAs as a source of energy at the blastocysts stage as shown by a lack or sharp decrease of mRNAs implicated in FAs catabolism. 4) Exogenous FSH stimulation and in-vitro culture conditions can results in extreme deviations in several transcripts involved in lipid metabolism during oocyte maturation and blastocysts formation.

CD36 expression favors tissues with high metabolic capacity for LCFA, such as adipose, heart and muscle, whereas it is absent from tissues like brain that do not utilize LCFA [16], its mRNA expression is induced by LCFA and not by short chain FAs [46]. The CD36 mRNA has been reported previously in mouse oocytes using the microarray technique [47]. The mechanisms by which oocyte competence is affected by FAs are not completely understood. However, the higher abundance of plasma membrane fatty acid transport mRNAs (CD36 and FATP1) suggests that the effect of LCFAs on oocyte maturation is direct and not mediated by cumulus cells. It also may indicate that the oocyte can not synthesize certain LCFA and that exogenous FAs are needed to support certain oocyte requirements. It is well known that animals can synthesize most fatty acids, with the notable exceptions of polyunsaturated fatty acids (PUFA) like linoleic and linolenic acids, since animals must have a dietary source of these FAs and therefore they are considered as essential FAs. The essential FAs have different important functions, the most important is structural contribution to the maintenance and function of the cellular membranes [48]. Alterations in FAs composition and lipid contents during oocyte growth are a well documented phenomenon. Zeron et al [7] reported that the decline in the developmental competence of oocytes Holstein cows was mainly associated with a significantly lower linoleic acid content in the oocyte membrane. The same author reported changes in the ewes oocyte's FAs profile by PUFA diet supplementation which associated with better oocyte quality and chilling resistance [49]. As a result, it appears likely that the reduction in the synthesis of mRNA for the genes encoding CD36 and FATP1 observed in oocytes classified as deviant and oocytes matured in vitro may reflect later reduced LCFAs import into the oocyte that may affect fundamental cellular processes such as FA incorporation into triglycerides, membrane components, signal transduction pathways, gene expression

and FAs generated energy. Moreover, the co-expression of both CD36 and FATP1 in the oocyte may indicate that they have a different function since a number of studies using 293 cells [50] and FATP1 knock-out mice have reported that LCFAs taken by FATP1 were channeled into triglycerides synthesis. This synthesis exceeded oxidation in the mitochondria [51], whereas FA/CD36 was identified recently on mitochondria isolated from rat and human skeletal muscle and found to be involved in LCFA oxidation [52, 53].

Little is known about the nutritional requirement of the bovine oocyte. Using FCS as IVM medium supplement can promote oocyte developmental competence. Its efficacy has been ascribed to its superior protein quality, amino acids, vitamins, heavy-metal chelators and unknown growth factors, making it the most effective IVM component. Oocyte triglyceride and the total lipid content have been shown to increase in oocytes matured in vitro in a medium containing FCS compared to those matured in serum free medium [4]. This may strongly indicate indirectly that the beneficial effect of FCS as a major component of bovine oocyte IVM medium may be also due to FAs contents. It has been suggested that decrease or increase in the expression of different lipid metabolic genes are modulated by direct contact with FA, that alter chromatin structure and transcription through direct effect on histone deacetylase activity, for instance, FATPs, CPT-1, PPAR has been shown to be upregulated by FAs whereas FAS, ACC are down-regulated (for review see [54]. Based on the above, decreased FA oxidation may reflect a defect in fatty acid oxidation genes activation due to alterations in CD36 and FATP1 abundance as a result of poor culture conditions.

AMPK activity depends on the AMP/ATP ratio that is changed during oocyte maturation. Cyclic AMP degradation results in the accumulation of AMP which binds and activate AMPK directly by binding to its γ subunit, mechanisms other than changes in the cellular AMP-to-ATP ratio have also been reported to activate AMPK [55]. However, our results showed a higher expression of AMPK mRNA even in the oocytes collected before LH surge and maturation in vitro, which is consistent with the recent finding using mouse oocytes [56]. AMPK is a stress-activated enzyme, and even gentle physical manipulation can stimulate the kinase [57, 58]. In mouse oocytes, AMPK activation has been shown to precede GVBD and to play an important role in meiotic resumption [56]. The simultaneous increase of AMPK, CD36, FATP1, and CPT-1 suggests that AMPK is associated with FAs catabolism as shown previously in most mammalian cells.
Cyclic AMP-dependent protein kinase (PKA) and AMPK phosphorylates and inactivates ACC, decreases malonyl-CoA levels and increases fatty acid oxidation both in vivo and in vitro [59, 60]. This phosphorylation regulation may be also an important regulatory aspect

of fatty acid metabolism during oocyte growth and maturation. Higher cAMP within the oocytes activates PKA before maturation and maintain meiotic arrest while a decrease in intra-oocyte cAMP initiate oocyte maturation [61]. The expression of both ACC and AMPK may suggest that the oocyte uses the same mechanism to phosphorylate and inactivate ACC, which stimulates fatty acid oxidation by different pathways as for other somatic cells through stimulation of PKA following elevation of cAMP levels. Thus, cAMP signaling is required not only for meiotic arrest and resumption but also for their intimate coordination of the several oocyte metabolic processes.

CPT-1 resides on the outer mitochondrial membrane and catalyses the conversion of palmitoyl-CoA to palmitoylcarnitine, which is the rate limiting step in the transfer of long-chain fatty-acylCoAs from the cytosol to the mitochondria for oxidation [30, 62]. Its activity is inhibited by malonyl-CoA, therefore, a decrease in the malonyl-CoA removes inhibition of CPT-1 and allows fatty acid oxidation to increase to meet the increased energy requirement. In mammals, two different CPT-1 enzymes are encoded by two different genes, liver CPT-1 and muscle CPT-1 [63]. Muscle CPT1 has a crucial role in controlling the rate of β-oxidation in heart and skeletal muscle and is more sensitive to malonyl-CoA inhibition [64]. Therefore, we decided to specifically amplify the muscle isoform. In the present study, we found that a general increase of CPT-1 mRNA levels takes place during early oocyte maturation or results from the polyadenylation of stored pools and lack of expression at the blastocysts stage. These findings together with the results published recently, using methyl palmoxirate (MP), the inhibitor of CPT-1 enzyme activity [65], indicate an increase in the rate of β-oxidation and important role of fatty acids as a source of energy during oocyte maturation. The lower expression of CPT-1 during prematuration in the oocytes matured in vitro and oocytes from deviant follicles may indicate a dysregulation of fatty acid oxidation activity causing a shortage of ATP and hence lower oocyte developmental competence [34]. These results are not surprising, steroid hormones have a significant control on cellular lipid in metabolically active tissues [66]. In skeletal muscle, ovariectomy suppresses the maximal activity of CPT-1 and the activity of this enzyme is up-regulated following treatment with E2. Moreover, In aromatase deficient mice (ArKO), exogenous E2 is necessary to maintain the gene expression and enzyme activity of the hepatic β-oxidation pathway [67] suggesting a capacity of E2 to regulate the expression of genes implicated in lipid metabolism. Low mitochondrial β-oxidation could also change the fatty acid composition within the oocyte since a marked change has been reported in the linoleic acid levels occuring between E2 active and inactive bovine preovulatory follicles [8]. In addition to its role in β-oxidation, lower CPT-1 activity has also been reported to enhance palmitic acid (PA) induced cell death and PA anti-

proliferative effect [68]. Possibly, this is also the case in the oocytes from deviant follicles and in those matured in vitro, the decrease in CD36 and FATP1 may result in the preferent use of FAs other than LCFA through passive diffusion, and lower CPT-1 mRNA expression may decrease the utilization of LCFA. This altered mRNA expression may affect the balance in the lipid FAs composition ratios at the physiological level. Thus, understanding of the import of FAs, the specific metabolic fates of different FAs and the manner in which these are regulated may contribute to the better design of oocyte IVM medium.

At the blastocysts stage, we found a small increase of a CPT-1 mRNA in the ones cultured in vitro compared to the in vivo counterparts. This may contribute to incomplete blockage of β-oxidation pathway in blastocyst cultured in vitro. It has been hypothesized that metabolically active blastocysts have less chance to develop to term after transfer and the metabolically inactive are more likely to develop after transfer [69, 70]. This slight increase in CPT-1 mRNA in the blastocysts cultured in vitro may be also related to the lower levels of ACCβ mRNA expression in in vitro blastocysts that probably was not enough to block CPT-1 activity. The block of CPT-1 activity probably induces more acetyl-CoA to go to lipid synthesis instead of β-oxidation. Our finding that the oocytes express CPT-1 mRNA before and during maturation and the lack of the expression at the blastocysts stage, support and explain recent findings that blocking of β-oxidation using CPT-1 inhibitor methyl palmoxirate before or during maturation of bovine oocytes significantly decreases oxygen consumption, while no difference was reported in the oxygen consumption of day 7 blastocysts [65]. Furthermore, based on the lack of CPT-1 mRNA expression in blastocysts, it appears that the lipids synthesized during early embryonic development were not used as a source of energy, and may be directed for membrane structure and organogenesis. Therefore, energy requirements at this stage are preferentially supplied by carbohydrates and amino acids as shown previously [71, 72]

The potential role of PPARα in regulating fatty acid oxidation has been well demonstrated. Activation of PPARα results in increase in the gene expression of many FAO enzymes [73, 74]. In the present study, the synergistically up-regulation of fatty acid transport and oxidation genes and PPARα mRNA may indicate that these genes are targets for the activation by PPARα. Fatty acids especially highly unsaturated fatty acids have profound effects on gene expression, acting on the genome through PPAR leading to changes in metabolism, growth and cell differentiation [75, 76]. Fats rich in PUFA like fish oil increase activity and mRNA levels of hepatic FAO enzymes [77]. Polyunsaturated fatty acids have also been considered as a natural ligands or activators of PPAR [78]. A study

using PPARα-deficient mice suggested that PUFA (fish oil) up-regulates FAO enzyme gene expression through a PPAR dependent mechanism [79]. In the same experiment, it has been shown that PUFA-mediated suppression of lipogenic gene expression does not require PPARα. Therefore, the lack of certain FAs may explain the difference in the abundance between oocytes from normal or deviant and those matured in vitro.

In the present study, the expression of both isoforms of ACC was identified in the oocyte and the blastocyst stage. The malonyl-CoA, the product of ACCα and ACCβ, exists in two separate compartments in the cell, cytosol and mitochondria, respectively [80] and do not mix. The cytosolic malonyl-CoA is used in FAs synthesis, while the mitochondrial part regulates CPT-1 and hence, FAs oxidation. ACCα is the dominant isoform and contributes to most of the malonyl-CoA. A study using ACCα knockout mice reported an essential role in embryo development [80] whereas ACCβ works as a regulator of FAs oxidation [81]. Oocytes from deviant follicles and those matured in vitro, showed a lower mRNA level of ACCα at the GV and MII than those classified as normal. Since ACCα is involved in fatty acid elongation, this indicated that the activity of ACCα is not completely blocked and certain enzyme activity is needed. A *Saccharomyces cerevisiae* mutant of ACCα grown at restrictive temperature developed an altered nuclear envelope and showed severe abnormalities in spindle formation to become arrested in the G2-M phase of the cell cycle loosing viability [82]. In addition, our study showed a statistical significant decrease in ACCα mRNA abundance in the blastocysts obtained using IVP system. This may indicate a change in the quantity of synthesized lipid, resulting in a lack of certain LCFAs needed for membrane integrity and structure. In yeast, inactivation of ACCα completely inhibits growth and cause cell death even after supplementation of FAs [83]. Moreover, inhibition of ACCα in a prostate tumor cell line has been shown to induce a growth arrest and apoptosis [84]. LCFAs and the degree of unsaturation of membrane lipids are the major factors determining the structure and fluidity of lipid membranes and hence chilling sensitivity [85, 86]. Thus, membranes with unsaturated acyl chains in phospholipids remain fluid at lower temperatures than do membranes with saturated lipids [87, 88]. In view of the high degree of conservation of ACCα in most living organisms, a similar function of this gene is possible in the oocytes and early embryos. Collectively, these findings may explain partly the lower developmental competence of the in-vitro matured oocytes and those from deviant follicles with FSH stimulated cows. They probably explain the sensitivity of IVP blastocysts to chilling compared to the in-vivo derived ones, and represent strong candidate markers for developmental competence of oocytes and blastocysts.

FAS, catalyses the *de novo* synthesis of saturated FAs such as, myristate, palmitate and stearate using acetyl- and malonyl-CoA. Targeted deletion of the FAS gene results in mice that die in utero [89], and more than 50% of heterozygous FAS knockout mice fail to survive embryonic development [89]. Expression of FAS is controlled primarily at the level of transcription and in response to both hormonal and nutritional signals [90, 91]. In the present study, contrary to our anticipations, there was a marked increase of FAS mRNA abundance at the blastocysts stage compared to that in oocytes suggesting an important role in the FAs or triglycerides synthesis. However, the absence of difference in the abundance of FAS mRNA between the in vivo derived blastocysts and those obtained from in vitro, may suggest a less critical role in the developmental competence of early embryos.

Changes in transcription cannot be equated to changes in functional pathways because transcripts have to be translated, and proteins may require post-translation modification in order to become functional, [92]. Minimal polyadenylation is required for reverse transcription but it does not ensure translation. To validate translation, protein levels have to be measured and often they increase as the RNA level drops before the maternal-zygote transition (MZT) in bovine while it is the opposite after the MZT [93]. Therefore, changes in mRNA levels needed the support of functional biochemical assays. Unfortunately, current protein and enzymatic activity evaluation techniques will require a large quantity of oocytes, which are very difficult to obtain and limit such assays in the case of oocytes from in vivo material. Identification of mRNA expression will provide the basis to define the complex regulation of lipid metabolism.

In conclusion, the increase in mRNA levels implicated in FAs transport and catabolism during oocyte maturation compared to that during early embryonic development is probably due to an increased metabolic activity resulting from higher energy requirements. Moreover, we have shown that different oocyte and blastocyst microenvironment and culture conditions can have a profound effect on mRNA encoding components of FA utilization and synthesis. We propose that the resultant changes in the oocyte and blastocyst gene expression in vivo vs. in vitro contribute to the observed decrease in the developmental competence of embryos by alteration in lipid metabolism.

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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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Samenvatting

De inductie van de rijping van de eicel hangt af van de voltooiing en integratie van een aantal essentiële factoren. Ten eerste moet de eicel zijn groeicyclus hebben voltooid en alle moleculen hebben verworven voor de inductie van de rijping aanvangt. Een tweede sleutelvoorwaarde is het genereren van essentiële endocriene en paracriene signalen op het juiste tijdstip en in voldoende mate om alle noodzakelijke intracellulaire veranderingen te induceren die samenhangen met zowel de kern- als de cytoplasmatische rijping en daarmee met de ontwikkelingscompetentie van de eicel. Het concept van de ontwikkelingscompetentie is echter niet eenduidig bepaald omdat er geen specifiek mechanisme mee is geassocieerd. Algemeen wordt aangenomen dat het verwerven van ontwikkelingscompetentie verband houdt met verschillende veranderingen zoals de synthese en opstapeling van specifieke RNA moleculen en eiwitten, en de herschikking van cytoplasmatische organellen bijvoorbeeld van corticale granula, vetten en mitochondriën. De gevolgen van feilen in één van de processen resulteert in een falen van de ontwikkeling.

Dit proefschrift omvat onderzoek naar de expressie van genen in eicellen van het rund tijdens de hervatting van de meiose op 6 uur na de LH piek samenvallend met de afbraak van het kernmembraan de zogenaamde “germinal vesicle breakdown”. Het werd verondersteld dat deze opzet een beeld zou geven van de belangrijkste veranderingen in de regulatie van de celcyclus, de herschikking van het cytoskelet en het uitlijnen van de chromosomen. De eerste studie, **(Hoofdstuk 2)**, verklaart de methodiek die is toegepast voor het verzamelen van het materiaal dat werd gebruikt in dit proefschrift en de criteria voor de selectie van functionele pre-ovulatoire follikels, waarmee de verondersteld competente en niet-competente eicellen konden worden onderscheiden op de verscheidene stadia van rijping. In de tweede studie, **(Hoofdstuk 3)**, werden de verzamelde eicellen gebruikt om een DNA microarray samen te stellen en te hybridiseren met als doel op te helderen welke transcripten in potentie betrokken zouden kunnen zijn bij de hervatting van de meiose en eventueel de ontwikkelingscompetentie. De hieruit afgeleide identificatie van verscheidene transcripten samenhangende met de opbouw van de spindel en de scheiding van de chromosomen leidde tot de volgende studie, **(Hoofdstuk 4)**, waarin de mate van

transcriptie met betrekking tot deze genen werd onderzocht in eicellen die in vivo waren betrokken bij het rijpingsproces in functionele en afwijkende pre-ovulatoire follikels zoals bepaald in de eerste studie. Ter vergelijking werden eicellen gebruikt die afkomstig waren uit kleine niet-preovulatoire follikels tijdens rijping in een kweekmedium (: in vitro).

Omdat de werking van de producten van de diverse transcripten zoals geïdentificeerd in de Hoofdstukken 3 en 4 afhankelijk is van ATP, en omdat het vaststaat dat de ontwikkelingscompetentie van een eicel is gecorreleerd met het gehalte aan ATP in de eicel, is de voorziening van energie een vereiste voor het bewerkstelligen van competentie. Om deze reden werd in de vierde studie, **(Hoofdstuk 5)**, de betrokkenheid bij de rijping onderzocht van verscheidene routes voor transport van lipiden, β-oxidatie en *de novo* synthese van vetzuren. De mate van expressie van genen waarvan bij somatische cellen bekend is dat zij een sleutelrol spelen in het lipid-metabolisme, werd evenals in de voorgaande hoofdstukken bestudeerd in eicellen tijdens het rijpingsproces in vivo in vergelijking tot in vitro.

Deze samenvattende discussie bespreekt de belangrijkste bevindingen en voorziet in suggesties voor toepassingen in de praktijk en voor toekomstig onderzoek.

Het effect van de grootte van de follikel op de concentratie van steroid-hormonen in pre-ovulatoire follikels van het rund na stimulatie met FSH

Moderne voortplantingstechnieken gebruiken routinematig stimulatie met gonadotrope hormonen voor het werven van eicellen en de inductie van ovulatie. Aldus kan een groter aantal eicellen worden verkregen waarbij echter wel het risico aanwezig is dat niet alle gameten over een zelfde ontwikkelingscompetentie beschikken [1, 2]. Deze heterogeniteit is waarschijnlijk toe te schrijven aan intrinsieke verschillen tussen de eicellen. Het is algemeen bekend dat het hormonale milieu in follikels in bepaalde mate is gewijzigd in runderen die zijn behandeld met exogeen gonadotropine deels afhankelijk van het toegepaste hormoon en de behandelwijze [3-7]. Tot op heden is er echter weinig bekend over de relatie tussen de grootte en de gezondheid van de follikels. In **Hoofdstuk 2** hebben wij aangetoond dat de concentraties van steroiden in de follikel worden beïnvloed door de grootte van de pre-ovulatoire follikel na stimulatie met oFSH. Één van de voornaamste bevindingen is dat de concentratie van progesteron toeneemt met de grootte in follikels tijdens de rijping na de endogene LH piek. Tot dusver blijft het echter onbekend in hoeverre het intra-folliculaire milieu samenhangt met de ontwikkelingscompetentie van de eicel die zich daar bevindt. Toch zijn er aanwijzingen dat de fysiologische staat van

follikels in schaap [8] en mens [9] de daarop volgende rijping en competentie van de eicel in vivo beïnvloedt. Asynchrone rijping van follikel en eicel treedt op na superovulatie en kan de ontwikkelingscompetentie van de eicel beperken [1, 2].

Anders gezegd hebben wij in het hier beschreven onderzoek niet beschikt over bewezen criteria om duidelijk onderscheid te maken tussen follikels met competente en niet-competente eicellen. Echter, in een eerder onderzoek in ons laboratorium werd aangetoond dat er in eicellen verkregen na stimulatie van runderen met recombinant humaan FSH een verstoring van de rangschikking optreedt van de corticale granula ten tijde van de voltooiing van de rijping terwijl aan het begin van de rijping de functionaliteit van de follikels was verminderd afgemeten aan een sterk verlaagde concentratie van oestradiol in de follikels [10]. Normale rangschikking van deze granula langs de periferie van de rijpe eicel is een algemeen bekend teken van ontwikkelingscompetentie. Op grond hiervan werd aangenomen dat competente eicellen hoofdzakelijk voorkomen in functionele follikels van pre-ovulatoire grootte die dezelfde veranderingen in concentraties van steroiden vertonen als beschreven voor onbehandelde, normaal cyclische runderen. Steroid-hormonen zijn betrokken bij een scala van fysiologische processen, o.a. de regulatie van glucose [11] en lipiden. Zo is bijvoorbeeld exogeen oestradiol noodzakelijk in muizen die deficiënt zijn aan aromatase (ArKO muizen) om de expressie van genen en de activiteit van daarbij behorende enzymen te handhaven die een rol spelen in het lipide metabolisme van de lever. Steroid-hormonen reguleren de voortgang van de celcyclus [12, 13], remmen het proces van apoptose [14], en moduleren de afgifte van calcium [15, 16]. In het ovarium van zoogdieren is de follikel de belangrijkste plaats voor synthese en secretie van steroid-hormonen tijdens pre-ovulatoire ontwikkeling en rijping van de eicel. De regulatie van de productie van steroiden door de ovariële, folliculaire cellen varieert opvallend met het stadium van ontwikkeling. Tijdens de pre-ovulatoire periode wordt de geselecteerde dominante follikel gekarakteriseerd door veranderingen in de concentraties van deze hormonen [17]. Voorafgaand aan de pre-ovulatoire LH piek synthetiseren de granulosa cellen oestrogeen en scheiden dit af, terwijl na LH de granulosa cellen luteïniseren en meer progesteron afscheiden in samenhang met afname van mRNA voor 17alpha-hydroxylase en P450 aromatase [18]. De specificiteit van de acties van de steroiden is toe te schrijven aan de aanwezigheid van intracellulaire receptor eiwitten. Ondanks de overvloed aan informatie aangaande steroid receptoren in verschillende soorten weefsel en hun belang in de voortplanting is tot dusverre alleen de receptor voor oestradiol en ERβ mRNA aangetoond in de eicel van het rund [19]. Het mRNA voor de receptor van progesteron wordt kortstondig geïnduceerd in granulosa cellen van pre-ovulatoire follikels van het rund tussen 5 à 7 uur na de LH piek [20-22]. Er is echter niets bekend aangaande de expressie van kern

of membraan receptoren voor progesteron in de eicel van enige zoogdiersoort. In primaten is de activiteit van mRNA voor androgeen receptoren (AR) essentieel voor follikelontwikkeling in een vroeg stadium en voor eicel kwaliteit [23], en in ratten is de volledige afbraak van AR activiteit geassocieerd met intensieve apoptose van de granulosa cellen in pre-ovulatoire follikels en met een povere kwaliteit van de cumulus eicel complexen (COCs) [24]. Verder is beschreven dat androgeen receptoren worden verplaatst van het cytoplasma naar de kernenvelop en vervolgens naar de nucleolus wat een rol doet veronderstellen als een door ligande geactiveerde transcriptiefactor [25]. In het licht van deze waarnemingen zullen de identificatie en karakterisering van de patronen van veranderingen in mRNA, en functionele analyse van steroidreceptoren die tot expressie worden gebracht in de eicel kunnen leiden tot een fundamenteel begrip van het belang van de rol van steroiden tijdens eicelrijping in vivo. In de klinische praktijk bestaat duidelijk behoefte om het stimulatie protocol van ovaria te optimaliseren. Een geschikt ontwerp van superovulatie behandeling zou de concentratie van LH en zijn halfwaardetijd in het FSH preparaat in overweging moeten nemen, en daarnaast de concentratie van steroiden en de expressie van enzymen betrokken bij de synthese van steroiden in de pre-ovulatoire follikels.

De expressie van boodschapper RNA in de eicel van het rund tijdens hervatting van de meiose

Hoewel de groeiende eicel van zoogdieren morfologisch gezien eenvoudig is, ondergaat deze een serie van afzonderlijke stappen van differentiatie. Een relatief groot aantal genen is benodigd om zijn gehele ontwikkeling te programmeren. Een klein deel van deze genen zijn specifiek voor de eicel [26] terwijl het overgrote merendeel zowel in de eicel als in somatische cellen tot expressie komt. Transcriptie en repressie van genen is een dynamisch proces waarvan kan worden verwacht dat het in de eicel in vitro varieert met de kweekomstandigheden. Om vast te stellen welke genen door specifieke stimuli kunnen worden gereguleerd is het noodzakelijk te beschikken over de mogelijkheid genen te onderzoeken in een scala van omstandigheden waaraan de eicel wordt blootgesteld. De triggers voor verandering in genexpressie in de eicel zijn van groot belang voor het doorzien van het moleculaire mechanisme van eicelrijping. Feitelijk is de huidige kennis met betrekking tot moleculaire mechanismen grotendeels verworven in onderzoek van eicelrijping in vitro. Mogelijk van nog groter belang is het feit dat de meeste in vitro studies voor de koe zijn uitgevoerd met eicellen uit kleine of middelgrote follikels (3 tot 6 mm in diameter) die geen prematuratie hebben ondergaan [27, 28]. Prematuratie vangt aan vanaf het bereiken van een grootte van 8,5 mm dat wil zeggen vanaf het begin van verschil in

groei tussen de twee grootste follikels [29], en dit proces hangt samen met een differentiatie in de concentratie van oestradiol [30].

Omdat de eicelrijping in vivo berust op een subtiel evenwicht in de follikel tussen de verschillende bij de regulatie betrokken stoffen en waarschijnlijk ook tussen verschillende receptoren van de eicel, worden de normale processen zoals die zich in vivo afspelen niet noodzakelijkerwijs weerspiegeld door moleculaire en biochemische veranderingen veroorzaakt door kunstmatige liganden in vitro. De complexiteit van de regelmechanismen van de hervatting van de meiose is duidelijk aangetoond in eicellen van het rund [31]. De pre-ovulatoire follikel van het rund blijkt een aantrekkelijk experimenteel model op te leveren voor onderzoek naar de regulatie van eicelrijping en de competentie van de eicel tot verdere ontwikkeling na bevruchting. De pre-ovulatoire follikel bevat voldoende vloeistof om steroiden, eiwitten en diverse bij de regulatie betrokken stoffen te analyseren. De follikel bevat eveneens voldoende hoeveelheden granulosa en cumulus cellen wat een uitmuntende mogelijkheid biedt om de functionele interactie tussen de diverse bij de regulatie betrokken stoffen te onderzoeken.

Differentiële expressie van genen tussen eicellen al dan niet blootgesteld in vivo aan LH kan ons inzicht vergroten in de moleculaire grondslag van de hervatting van de meiose in vivo. De identificatie en karakterisering van genen die uitsluitend of bij voorkeur tot expressie komen in de eicel die 6 uur in vivo aan het rijpingsproces is onderworpen zullen hopelijk de mechanismen van het rijpingsproces verhelderen en bruikbare informatie verschaffen ten behoeve van de ontwikkeling van efficiënte kweekmedia voor eicelrijping. De suppressie subtractieve hybridisatie (SSH) methode maakt het mogelijk om genen te identificeren die in overexpressie zijn (zg. “forward” +SSH) maar ook die in onderexpressie zijn (zg. “reverse” –SSH) door de zogenaamde “driver” en “tester” populaties te verwisselen tijdens de procedure (Clontech, Palo Alto, VS) [32-37]. De SSH methode is een wijdverbreide techniek doordat daarmee de verzameling mogelijk is van zowel overvloedig aanwezig mRNA als van mRNA waarvan slechts een beperkt aantal kopieën present zijn. De methode benodigd echter een behoorlijk grote hoeveelheid initiërend RNA wat toepassing bij in vivo gerijpte eicellen bemoeilijkt. Door daarbij gebruik te maken van de SMART amplificatie methode (:“*S*witch *M*echanism *A*t the 5’ end of the *R*everse *T*ranscript”) kan deze beperking worden voorkomen.

De ontwikkeling van microarray technieken maakt het mogelijk om duizenden genen te screenen in één enkel experiment waarmee differentiële genexpressie kan worden vastgesteld in behandelde versus controle populaties van cellen. Zo zou het gebruik van

DNA microarray aanzienlijk kunnen bijdragen in beperken van de inspanning die nodig is om de vele variabelen te screenen die een rol spelen bij het effectief onderzoeken van genexpressie patronen. Er zijn microarrays ontwikkeld om geëxpresseerde mRNA transcripten af te spiegelen (cDNA arrays), of om een herkenbaar deel van een mRNA transcript weer te geven (oligonucleotide arrays). De aantrekkelijkheid van deze technieken wordt weerspiegeld in de exponentiële toename aan publicaties sinds de introductie van de techniek in 1995. Het gebruik van microarrays is wijdverbreid in onderzoek op het gebied van de pathologie, farmacologie, oncologie, celbiologie en recentelijk eicellen [26, 38, 39].

Verscheidene technieken zijn gebruikt voor de ontdekking van genen waarbij microarrays zijn ontworpen voor specifiek cDNA van de eicel ten behoeve van mogelijke toepassing om het succes van voortplantingstechnieken te taxeren. Twee van deze technieken maken gebruik van een methode om selectief cDNA klonen te scheiden of fragmenten die in de ene maar niet de andere populatie van cellen of weefsel aanwezig zijn. Op het ogenblik is te voorzien dat de gehele database aan genen voor het rund beschikbaar wordt. Om deze reden zou genoom informatie moeten worden gebruikt om een microarray te construeren voor het screenen van transcripten in de eicel van het rund. De ideale aanpak om genexpressie te profileren bestaat uit het gebruik van microarrays die het volledige genoom betreffen. Hiermee kunnen genen worden geïdentificeerd die zijn up of down gereguleerd in response op een bepaalde behandeling. Omdat het echter niet aannemelijk is dat microarrays een routine test worden in de nabije toekomst, zal onderzoek op het gebied van de voortplantingsbiotechnologie waarschijnlijk de identificatie verlangen van een kleine subset aan genen waarvan de expressie kan worden toegepast in de ontwikkeling van een kwaliteitstest die op genen is gebaseerd. Daarom gebruikten wij in **Hoofdstuk 3** de SSH en microarray techniek in combinatie met eicellen die in vivo aan het rijpingsproces waren onderworpen om genen te identificeren die betrokken zijn bij de regulatie van de rijping van de eicel van het rund in de veronderstelling dat LH en rijping in vivo bevorderlijk zijn voor de regulatie van meerdere aspecten van de eicel functie.

Gebruik makend van de SSH techniek waarbij een verschilfactor van 1,3 als drempelwaarde werd gedefinieerd konden 115 genen worden geïdentificeerd uitgaande van 945 DNA klonen in het begin. Dit relatief kleine verschil in genexpressie patroon ten gevolge van de LH piek wijst mogelijk op het feit dat slechts een kleine subset aan genen nodig is om de hervatting van de meiose en de ontwikkelingscompetentie te reguleren. De microarray analyse zoals hier toegepast heeft nieuwe mRNAs aan het licht gebracht die in potentie een rol spelen in de juiste functie van de eicel, de rijping en/of de meiotische competentie. Wij hebben belangrijke veranderingen onderkend in genen die betrokken zijn

bij regulatie van de celcyclus, signaal transductie, transcriptie en mRNA verwerking, het cytoskelet, celadhesie, en metabolisme. In vervolg op het identificeren van deze genen op mRNA niveau is het de uitdaging om deze informatie efficiënt toe te passen om een beter begrip te verwerven van het mechanisme van de hervatting van de meiose. De benadering via de zogenaamde proteomics zal informatie kunnen opleveren die niet kan worden verkregen op het RNA niveau wat toe te schrijven valt aan ofwel een betrekkelijke correlatie tussen mRNA en eiwit concentraties of aan modificaties van eiwitten die optreden na de translatie wat kan leiden tot verscheidene iso-vormen gegenereerd vanuit één mRNA. Voor veel van de geïdentificeerde genen blijven de vermeende liganden die de afgeleide eiwitten zouden activeren en ook hun doelwit voor actie onbekend. Dit vormt een uitdaging voor toekomstig onderzoek om zo het mechanisme van eicelrijping te onthullen en daarmee een efficiënt in vitro maturatie (IVM) systeem te ontwikkelen.

De expressie van boodschapper RNA van genen betrokken bij het transport van organellen en de scheiding van chromosomen

Hoewel de morfologie van de spindel en chromosomen tijdens de meiose al meer dan een eeuw bekend is zijn de basale moleculaire mechanismen merendeels onbekend die de scheiding van chromosomen in de eicel van zoogdieren reguleren. Meiose in de eicel moet nauwgezet worden gereguleerd om een juiste deling van het genetische materiaal zeker te stellen. Falen van de scheiding van chromosomen leidt tot aneuploïdie en kan leiden tot verlies van levensvatbaarheid. Naar schatting hebben 10 tot 30% van bevruchte eicellen bij de mens een verkeerd aantal chromosomen waarbij trisomie en monosomie het meeste voorkomen. Dit fenomeen heeft vergaande klinische gevolgen: bij benadering treedt aneuploïdie op bij eenderde van de miskramen waardoor dit de omvangrijkste bekende oorzaak voor verlies van zwangerschap uitmaakt. Bij bevruchtingen die overleven tot de geboorte betreft aneuploïdie de belangrijkste genetische oorzaak van onvermogen tot ontwikkeling en zwakbegaafdheid [40].

De analyse zoals hier uitgevoerd met “real-time” kwantitatieve PCR van zes van de genen die een significante rol spelen bij de vorming van de spindel, bij het onderhouden van een accurate scheiding van de chromosomen en bij de opbouw van het cytoskelet toonde de aanwezigheid aan van ontregelde en afwijkende niveaus van mRNA in eicellen afkomstig uit als afwijkend gedefinieerde follikels in vergelijking tot eicellen uit normaal functionele follikels. Deze bevinding zou een verklaring kunnen verschaffen voor abnormale chromosomen samenstelling zoals die frequent wordt waargenomen in de eicel en vroege pre-implantatie embryo’s na kweek in vitro. Dit fenomeen wordt doorgaans in

verband gebracht met beschadiging bij klieving, slechte embryo kwaliteit en een toename van fragmentatie; al deze verschijnsels kunnen de potentie tot implantatie van het embryo in gevaar brengen [41-43].

In het embryo van zoogdieren is polariteit een welbekend verschijnsel. In de eicel echter is het bestaan van polariteit nog controversieel [44-46] en er is weinig bekend betreffende de genen die polariteit en de daarmee samenhangende activiteit reguleren. In **Hoofdstuk 3** en **4** hebben we verscheidene transcripten geïdentificeerd in de eicel waarvan bekend is dat zij een rol spelen in de vorming van de polariteitsas zoals par-3, formine, KIF3, β-catenine en CDC42 (niet gepubliceerde resultaten). Organellen en corticaal actine worden asymmetrisch verdeeld in de eicel van vele soorten wanneer de dorsale/ventrale as wordt gevormd [46]. Onze identificatie van genen in de eicel die de polariteit reguleren en de recente bevinding dat Par-3 eiwit is geassocieerd met de meiotische spindel [47] kunnen wijzen op een belangrijke functie tijdens de hervatting van de meiose en een mogelijke rol in de polariteit van de eicel.

Hoewel de meeste genen die in de studie van hoofdstuk 4 in de eicel zijn geïdentificeerd ook algemeen zijn tijdens mitose in somatische cellen is er bovendien een fundamenteel verschil. Tijdens de prometafase van meiose II worden de chromatiden alleen via de centromeren bijeen gehouden in tegenstelling tot de prometafase van de mitose waar zij tenminste initieel zijn verenigd over de gehele lengte. Dit roept de interessante mogelijkheid op dat disjunctie van de chromosomen tijdens mitose ook twee verschillende sets aan machinerie nodig heeft: namelijk één die normaal aanwezig is gedurende meiose I wat de armen van de chromatiden scheidt, en een andere die normaal wordt gevonden bij meiose II (of tijdens de voorafgaande interfase) wat leidt tot scheiding in het gebied van de centromeren. In de toekomst zal het belangrijk zijn de verschillen vast te stellen tussen de twee mechanismen die operationeel zijn tijdens meiose om de chromosomen te scheiden. De scheiding van zusterchromosomen is een complex proces en er zijn zeker andere factoren betrokken bij de regulatie van de aanhechting en scheiding van de zusterchromatiden.

Regulatie van het lipide metabolisme tijdens finale eicelrijping en vroeg-embryonale ontwikkeling

Een volledig inzicht in het fysiologische effect van rijping in vivo op de expressie van genen vereist de identificatie van transcripten die invloed hebben op metabole routes, de opheldering van hun werkingsmechanisme, en de gevolgen voor groei, differentiatie en overleven. Vanuit praktisch oogpunt is het identificeren van genen van groot belang bij het

samenstellen van succesvolle media voor maturatie om de ontwikkeling te ondersteunen van eicellen na bevruchting.

Veranderingen in de ultrastructuur van groeiende eicellen die verband houden met de opstapeling van voedingsstoffen zoals lipiden zijn een noodzakelijke voorwaarde voor de energievoorziening ten behoeve van de hervatting van de meiose en daaropvolgende embryonale ontwikkeling. De oorsprong van lipiden die in de eicel terecht komen wordt niet volledig begrepen. Van lipiden die zijn opgeslagen in de eicel is aangetoond dat ze zijn opgestapeld in de eicel gedurende de ontwikkeling van follikels [48] en dat de voorraad ervan afneemt tijdens het rijpingsproces [49]. Kim et al. [50] toonden aan dat de voorraad aan lipiden in de eicel van het rund de lipiden voorraad in het maturatie medium weerspiegelen wat erop wijst dat de lipiden die worden opgestapeld in de eicel van oorsprong afkomstig zijn uit het medium. Het is niet bekend of deze lipiden passeren via de verbindingen tussen de eicel en de omringende cumuluscellen of dat ze direct worden opgenomen vanuit de follikelvloeistof. Aangezien vele genen zijn geconserveerd over de zoogdiersoorten heen inclusief de mens kan de functie van bepaalde genen worden geëxtrapoleerd. Op grond hiervan werden in **Hoofdstuk 5** de mRNAs onderzocht in normale eicellen in vivo die de voornaamste metabole routes vertegenwoordigen in het lipide metabolisme. Uit de resultaten kan een model worden voorgesteld betreffende het transport van lange keten vetzuren (LCFA) tot in de eicel. De vetzuren worden verplaatst vanuit de extracellulaire omgeving naar het cytoplasma door vetzuur-translocase (FAT/CD36) om vervolgens na solubilisatie te worden getransporteerd door vetzuur bindende eiwitten (FABPs) naar de plaats waar ze worden gemetaboliseerd [51, 52]. Eenmaal na transport over het membraan worden de LCFA bestemd voor specifieke metabole doelen. Deze bevindingen samen met het hogere niveau aan CPT-1 mRNA wijzen erop dat vetzuren direct nodig zijn voor de hervatting van de meiose en dat β-oxidatie de voornaamste route is die bijdraagt aan de behoefte aan energie tijdens eicelrijping, en aan de toename in de mate van lipogenese tijdens het blastocyst stadium die nodig zou kunnen zijn om vroeg-embryonale ontwikkeling te ondersteunen. Vervolgens werd het niveau van mRNAs betrokken bij het lipide metabolisme in de normale eicellen vergeleken met het niveau in afwijkende eicellen verzameld uit de gestimuleerde runderen en met die van eicellen die in vitro waren gerijpt om nauwkeuriger te kunnen vaststellen op welke specifieke routes van het lipide metabolisme ontsporingen optreden. De afwijkende niveaus van verscheidene mRNAs wijzen er mogelijk op dat de intracellulaire vetzuursamenstelling niet juist is, o.m. afgenomen β-oxidatie, en verschaffen mogelijk een verklaring voor een beperktere voortgang van de meiose, lagere niveaus van ATP en een afgenomen ontwikkelingscompetentie voor deze eicellen. De significant lagere mRNA niveaus voor

acetyl CoA carboxylase α (ACCα) wat het belangrijkste enzym is dat de vetzuursynthese *de novo* controleert, kunnen deels een verklaring zijn voor de lagere ontwikkelingscompetentie van in vitro geproduceerde blastocysten. Dit wijst op een verandering in de hoeveelheid gesynthetiseerd lipide wat op zijn beurt leidt tot een tekort aan bepaalde LCFA die nodig zijn voor membraanintegriteit en structuur.

Hoewel ons onderzoek mRNAs heeft bepaald van de hoofdroute van het lipide metabolisme, zijn er een aantal beperkingen met betrekking tot de interpretatie van de data in relatie tot vetzuurmetabolisme. Als bijvoorbeeld in de beschouwing wordt betrokken dat er meer dan 30 reactiestappen nodig zijn om acetyl-CoA in triglyceriden om te zetten, dan zouden er vele stappen of genen kunnen zijn die de opbrengst aan eindproduct controleren. Bovendien is glucose naast de vetzuren een ander belangrijk te oxideren metabool substraat waarvan echter de rol als energiebron tijdens de eicelrijping wordt betwist [53-55]. Interactie tussen deze substraten worden verondersteld de mate te controleren waarin zij ieder worden geoxideerd, d.w.z. controle van de reciproque relatie tussen glucose en vetzuur oxidatie. Welke van de twee substraten de primaire regulator van energie in de eicel betreft is niet helder en vraagt om verder onderzoek.

CONCLUSIES EN VOORUITZICHTEN

Dit proefschrift heeft veel nieuwe basale eigenschappen onthuld van de eicel van het rund, en een aannemelijk model mogelijk gemaakt voor de regulatie van de hervatting van de meiose en juist functioneren van de eicel. Zo weten wij nu uit het onderzoek beschreven in **Hoofdstuk 3** dat de mechanismen van meiotisch arrest en hervatting in de eicel van het rund verschillende fosfodiesterases (PDE7) vereisen, en verder de betrokkenheid van G-eiwitten, de regulatoren van G-eiwit signaalvoorziening (RGS) in meiotische hervatting, de moleculaire componenten betrokken bij de scheiding van chromatiden, de regulatie van Ca^{2+} oscillatie activiteit en van de celcyclus. Het merendeel van deze genen werd voor het eerst in de zoogdiereicel geïdentificeerd. De uitdaging is echter om nu de potentiële liganden te onderkennen die deze genen activeren waarmee een antwoord kan worden verkregen op de vraag hoe het meiotisch arrest wordt opgeheven. Toekomstig onderzoek zal zeker helderheid verschaffen met betrekking tot deze openstaande kwesties. Bovendien werden in **Hoofdstuk 4** variaties beschreven in het niveau van verschillende mRNAs die van doorslaggevend belang zijn voor een juiste verplaatsing van organellen en scheiding van de chromosomen, waaruit de verstoorde functie van afscheiding van het poollichaampje en defecten in de cytoplasmatische rijping kunnen worden verklaard die

doorgaans worden waargenomen in de afwijkende eicellen van gestimuleerde dieren en in eicellen na in vitro rijping. Tenslotte, hebben wij zoals beschreven in **Hoofdstuk 5** nieuwe kennis verworven betreffende het transport van lange keten vetzuren naar binnen de eicel en betreffende de regulatie van de behoefte aan energie tijdens rijping en het blastocyst stadium op basis van lipide als substraat. Verder doet een afwijkende expressie van mRNAs betrokken bij het lipide metabolisme veronderstellen dat er een defect is in het vetzuurmetabolisme bij eicellen die in vitro worden gerijpt en eveneens in afwijkende eicellen uit gestimuleerde dieren en in blastocysten die in vitro zijn geproduceerd.

Hoewel de kennis op het gebied van eicelrijping snel is toegenomen gedurende de laatste paar jaren, dienen de moleculaire details nog te worden gecompleteerd zoals het bepalen van de sleutelmoleculen. Bovendien moeten fysiologische functies en regelmechanismen worden toegekend en opgehelderd, en moeten de raakvlakken met andere cellulaire systemen worden geëxploreerd. Vele nu ontbrekende onderdelen zullen worden gevonden met beschikbare biochemische methoden en met moleculaire en klassieke genetica samen met het vaststellen van de volgorde van het hele genoom. Functionele analyse zal niet alleen van groot belang zijn om de betreffende mechanismen te begrijpen maar zal ook de volledige opheldering versnellen van het regelsysteem van de eicel. Tenslotte voegt de ontdekking dat microRNA genen behoren tot de meer overvloedig voorkomende regelgenen in dieren [56] toe aan de complexiteit van ons inzicht in de regulatie van eicelrijping. De uitdaging is nu hoe de voordelen te plukken van de talrijke technieken en de biologische kennis in het veld van de voortplantingsbiotechnologie.

Referenties

[1-56] zie de Engelse tekst van de samenvattende discussie.

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في هذه المساحة اود ان اعبر عن خالص الشكر والأمتنان لكل من ساهم بشكل او بأخر في اظهار هذه الأطروحة بالشكل المطلوب, وأخص بالذكر, الأستاذ/ محمدعبدالنور الفالح المستشار الثقافي بالمكتب الشعبي الليبي الذي اثبت وبشهادة الجميع ان متابعة الطلبة فن يتطلب مهارة خاصة, فبأسمي و بأسم الطلبة الليبيين الدارسين بهولندا اثناء فترة قيامه بمهامه نتقدم له بأسمى عبارات الشكر و التقدير. شكر خاص ايضا للدكتور سليمان الغريب على المتابعة و الأتصال الدائم.

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ألأستظافة الكريمة لي من الدكتور وجدي المشري اثناء زيارتي لكندا ليست بالأمر المستغرب, فله مني كل الشكر و التقدير. للأخوة معمر ابوغرارة, عمران ابوعبود, عمران عبدالسلام , عبدالمجيد الخبولي, عزالدين علي فرج و محمد ابراهيم تقدير خاص للتواصل الدائم.

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Curriculum Vitae

Omran Algriany was born on November 17th 1966 in Soukna, Libya, he studied veterinary medicine at he Veterinary Faculty in Tripoli, Al-Fateh University, Libya and was awarded of Degree of Doctor of Veterinary Medicine (DVM) in 1990. Shortly after his graduation, he began work as a demonstrator at the same faculty, and after having completed two year national service, in 1996, he started his three year Master degree study at the lab of reproductive biology, Szent Istvan University, Godollo, Hungary. After graduation he began work as assistant lecturer at the Department of Physiology and Biochemistry at the Faculty of Veterinary Medicine, Al-Fateh University. In 2001, he received a scholarship to perform a PhD programme at the Department of farm Animal Health, Faculty of Veterinary Medicine, Utrecht University, under supervision of Prof. Ben Colenbrander and Dr. Mart Bevers, the research project focused on the effect of follicular fluid on oocyte developmental competence. From September 2003 on word, he has been working under the supervision of Prof Ben Colenbrander, prof Marc-Andre Sirard, Dr. Steph Dieleman and Dr. Peter Vos, resulting in this thesis.