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].

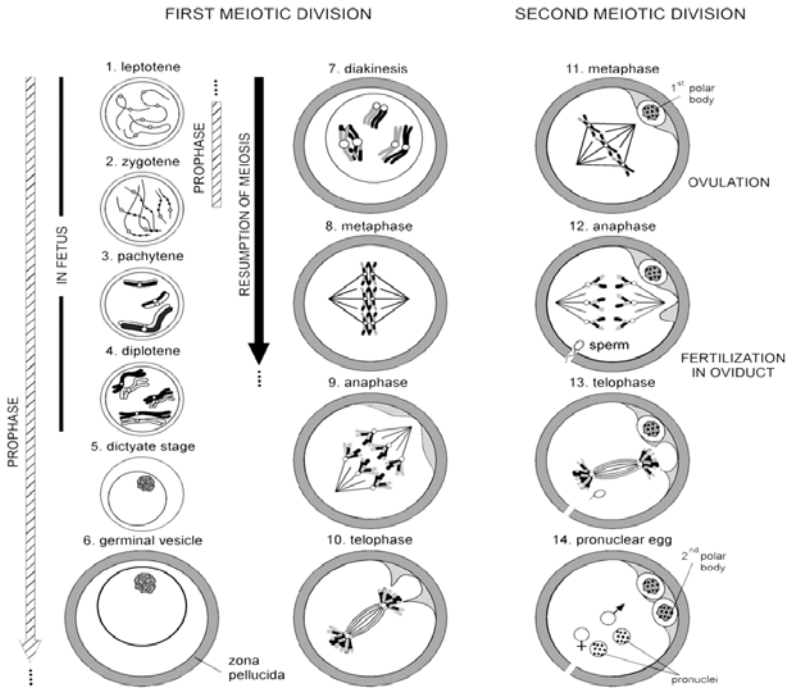

Fig. 1: Schematic representation of meiosis in the mammalian oocyte. Adapted from [61]. Prophase I: 1-5 in fetal ovary, 6-7 germinal vesicle formation. After the LH surge, oocyte meiosis resumes, Metaphase I: 8, Anaphase I: 9, Telophase I: 10, and arrest at Metaphase II: 11untill fertilization. After 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).

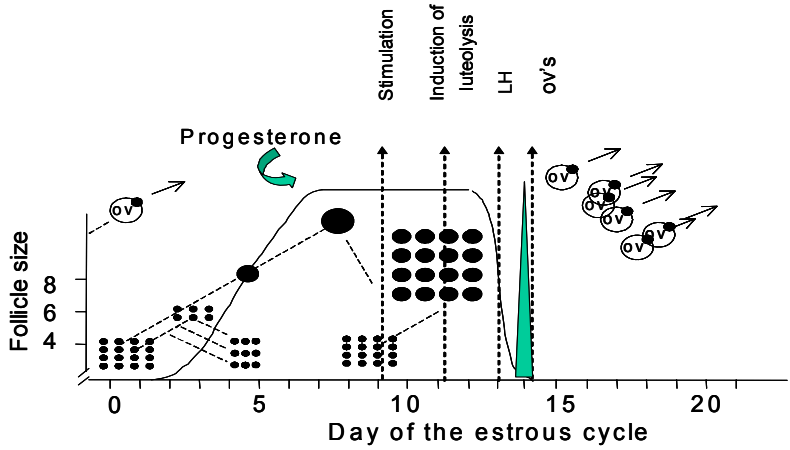

Figure 2. Schematic model of follicular dynamics on the ovary and changes in progesterone and LH concentrations in the peripheral blood during the estrous cycle in the cow. A commonly used scheme for superovulation is depicted at the top of the figure. (Adapted from van de Leemput, E. E, Thesis, 1998)

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.

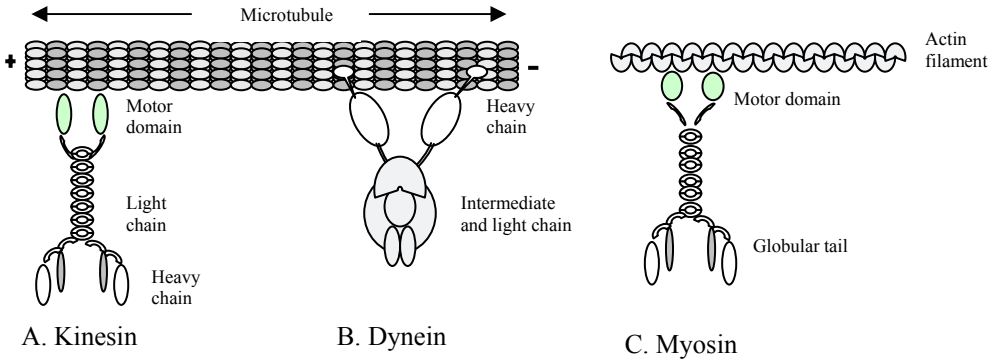

Fig. 3. A basic overview of the structure of the molecular motors; kinesin, dynein and myosin. All of the molecular motors are large multiprotein complexes with a dimeric structure. They each consist of a globular head domain, which contacts the cytoskeleton and provides the force for motility by hydrolysing 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.

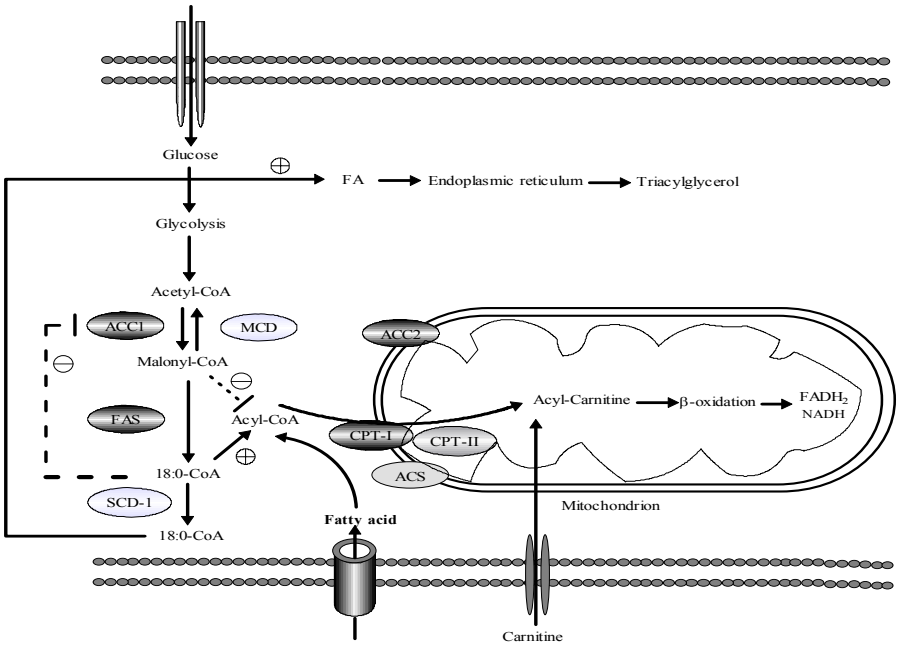

Figure 4. Metabolic pathways involved in lipid metabolism of liver and skeletal muscle. Among the lipid metabolic enzymes, acetyl-CoA carboxylase (ACC), fatty-acid synthase (FAS) and carnitine palmitoyl transferase (CPT) are the three that primarily regulate the synthesis of malonyl-CoA, which is the 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.

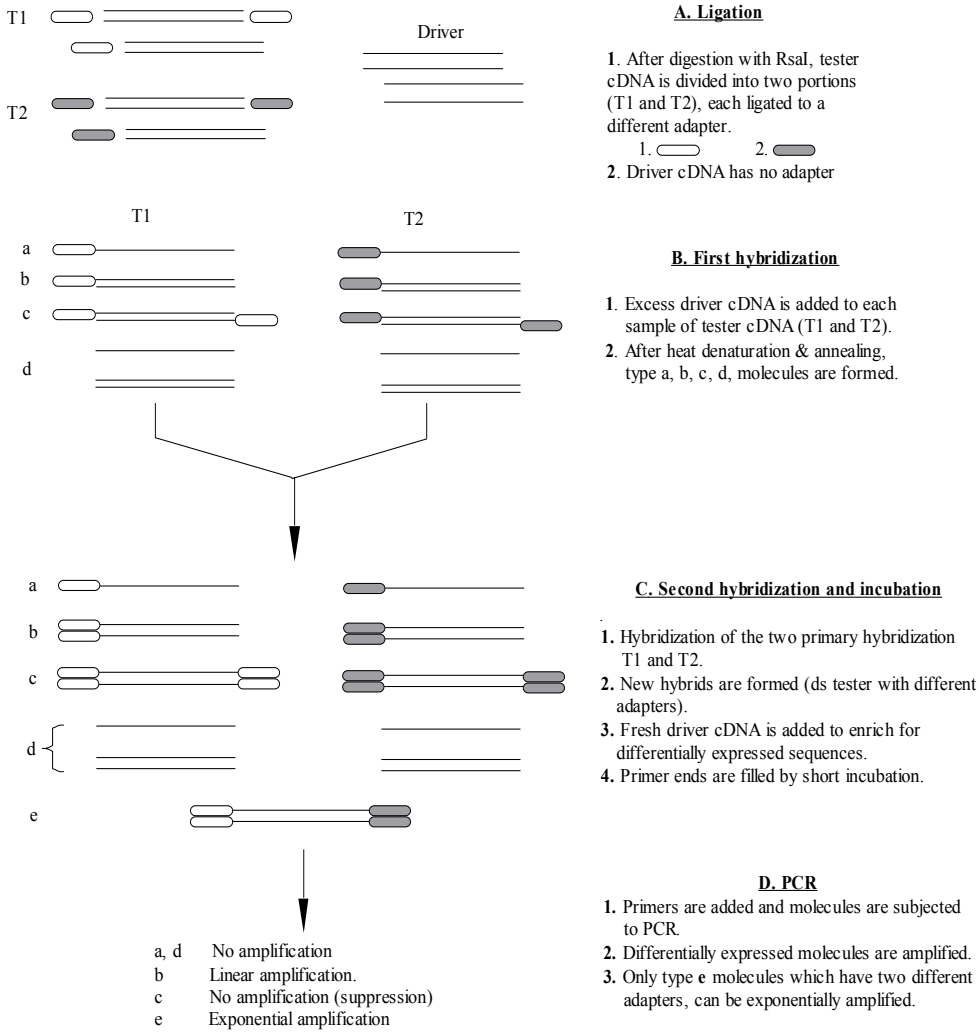

Fig. 5. Schematic diagram of the cDNA library construction procedure using suppression subtractive hybridization. Modified from [200]

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.

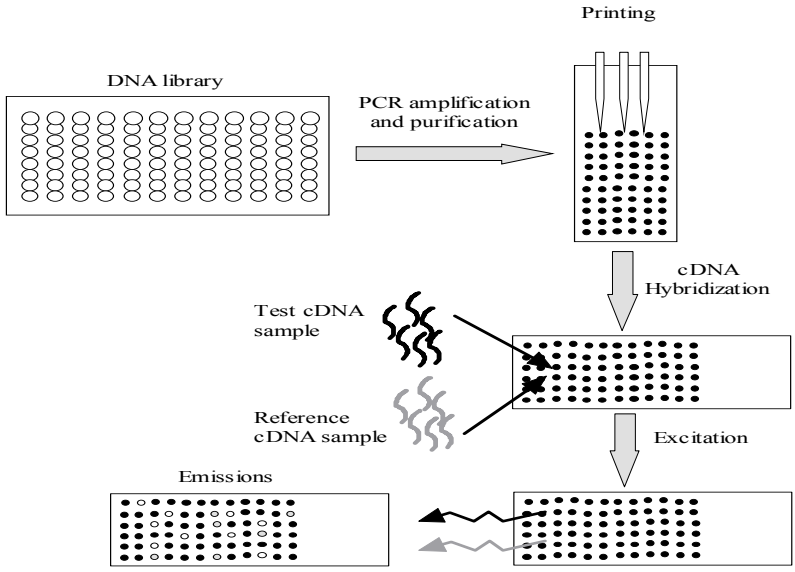

Fig. 6. Schematic diagram of a microarray procedure. Modified from [212]

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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