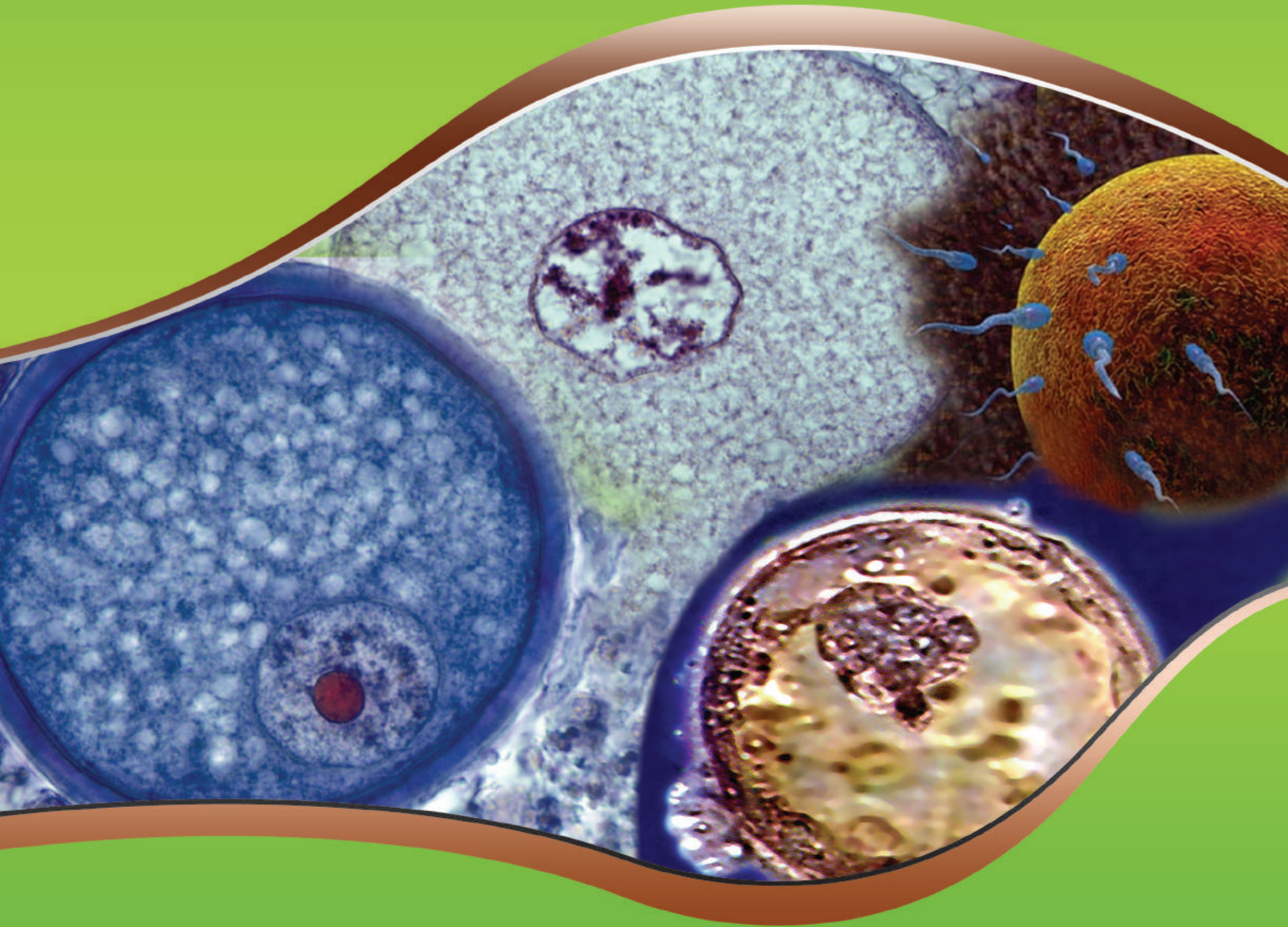Oocyte Maturation and Fertilization

A LONG HISTORY FOR A SHORT EVENT

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Oocyte Maturation and Fertilization: A Long History for a Short Event

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FOREWORD

When I was a student 50 years ago I thought that all the interesting and important things on fertilization had already been discovered and nothing was left for me to study. I was very wrong. Very little was known about what you read in this book. Not known at all or not even imaginable. Today's students should discover what is not written in this book. Fertilization is the event which connects successive generations. Its biological and medical importance cannot be overemphasized. Female and male gametes (egg and sperm) are equally important. Female germ cells were designed and made for male germ cells and *vice versa*. Although the time may come when unlimited numbers of functional gametes are produced *in vitro* from somatic cells and, reproduction without germ cells becomes possible, we must not forget that life on earth would not have flourished without sexual reproduction. I wish I could live 50 years more to see how this book will be revised by successive students.

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PREFACE

Sexual reproduction introduced a wide range of possible life forms into development, with a continuous evolution that is dominated by genetic variability. The main components of this complex biological process are two very special cells, the gametes, which undergo a unique form of division, meiosis. The physiology, biochemistry and reciprocal interaction of these special cells give rise to a new and unique individual.

Although the first documentation of assisted reproduction dates back to 1783, when Lazzaro Spallanzani performed the first artificial insemination in a bitch, the majority of experimental studies on reproduction started only during the second half of 19th century. Studies on fertilization have expanded widely during the last 150 years, from the simple description of shape, size and function of gametes and pre-implantation embryos to the breakthroughs facilitated by advanced biotechnology. A vast amount of information has emerged and continues to expand, in order to describe a "brief event" that covers the time span from the first meiotic arrest to the second meiotic arrest, i.e., oocyte maturation, to completion of meiosis following fertilization. In this brief time span, lasting from minutes to hours according to the species, the female gamete that has been quiescent for potentially many years rescues a complex machinery of events which culminate in fusion with a foreign cell in order to generate a new organism.

Upstream events, such as oocyte maturation, are a fundamental pre-requisite for successful completion of the developmental process. Analogous to the links in a chain, all of the reproductive steps are united by a common target: producing an individual that carries a new and original genome.

This book is an integrated approach to the study of the basic events involved reproduction, and contains recent achievements described by most of the outstanding scientists of this field. General and basic patterns of oocyte maturation and fertilization are described in a modern context of integrated morphological and biochemical methods, up to the practical application of this knowledge. Because of their typical external fertilization, simple marine invertebrate models (sea urchin, ascidians, etc.) initially provided relevant and unique sources of information on reproductive biology. A major impetus for transferring and comparing this information in mammals coincided with the development of protocols that also allowed external fertilization in mammals, first in laboratory animals, and then in livestock and human. In Vitro Fertilization (IVF) technology represents a revolution in the general knowledge of reproductive biology, opening new doors that lead to biomedical applications.

The initiation of a new life and the potential of being able to manipulate this event represent a very attractive subject, a concept that has spread from the biological to philosophical and theological interests. Unfortunately, the combination of ideology and biology led to conflicts that limited the natural pursuit of knowledge.

The journey that this issue embarks upon starts by describing the morphological modifications that occur in the oocyte during maturation and fertilization. The effects of orchestrated dynamics of hormones, growth factors and metabolites that influence the follicle during in vivo maturation is described, and related to in vitro conditions. The analysis of signal transduction by secondary messengers (i.e., Ca^{2+}, cAMP) and effectors (i.e., IP_3, NAADP) offers useful information about mechanisms that trigger oocyte meiotic competence, a necessary condition for efficient fertilization. A detailed view of genetic and epigenetic control of oocyte maturation is provided. The expression profiles of this control mechanism have been widely screened by microarray analysis and related to the correct progress of maturation required to achieve cytoplasmic hereditability.

Fertilization represents a very brief event, but it is responsible for long-lasting mechanisms that affect embryonic and foetal development. The reciprocal activation of gametes is described and highlighted with respect to novel information related to sperm factor and cascade mechanisms that occur in the oocyte as a consequence of sperm penetration. Gametes are electrogenic cells, i.e., capable of responding to electrical stimuli and modifying their electrical properties during the crucial periods of maturation and fertilization. A detailed description of ion currents during fertilization depicts another perspective on mechanisms of oocyte activation, further reinforcing the complexity of these systems. Molecular dynamics involved in meiotic arrest and resumption, as well as cascade mechanisms related to their control are analyzed and integrated with the other events described.

Finally, all of the basic information related to this brief time lapse is considered in relation to clinical application of assisted reproductive technologies (ART), analysing IVF efficiency and follow-up information in the perspectives of this information. New frontiers of ART, such as stem cells and cloning technologies, have been analyzed and future applications and improvements hypothesised. Old dogmas, such as the irreversible differentiation of tissue cells and impossibility of renewing the pool of female germ cells after birth, are now surpassed and new opportunities are presented through research advancements in reproductive biology.

We would like to thank all of the eminent authors who have joined us in devoting time and effort to this venture, and hope that the readership will benefit from their experience and skills.

Elisabetta Tosti and Raffaele Boni

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This book is dedicated to our families

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P. Loi and G. Ptak
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Somatic cell nuclear transfer
Abnormal placentation
Imprinted genes
Nuclear reprogramming

x

Electron Microscopy of Mammalian Oocyte Development, Maturation and Fertilization

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Abstract: The ultrastructure of the oocyte and zygote reveals in great details the processes of oocyte growth, maturation and fertilization. In this Chapter these details are addressed in cattle in comparison with pig, horse, fox, mouse and man. In the growing oocyte a variety of common and oocyte-specific organelles and inclusions are build up resulting in the complex ultrastructure of the oocyte; one of the largest cells of the mammalian body. During development, the oocyte is surrounded by cumulus cells of which the innermost establish gap junctions with the oolemma. However, when oocyte maturation is initiated and meiosis resumed, this intimate contact is broken and the cytoplasm of the oocyte is restructured towards a more independent fate allowing for cytoplasmic oocyte maturation. As one important aspect, cortical granules migrate to solitary positions along the oolemma immediately prior to ovulation. The fertilizing spermatozoon completes acrosome reaction on the surface of the zona pellucida, penetrates the zona, and fuses with the oolemma at the equatorial segment. Consequently, the oocyte is activated resulting in exocytosis of the cortical granules, establishing the block against polyspermic fertilization, and in resumption of meiosis from metaphase II. The maternal and paternal chromatin is gradually surrounded by nuclear envelope developed from the smooth endoplasmic reticulum to form pronuclei that later swell to their typical spherical shape. Later, the pronuclei appose each other, the nuclear envelopes are dissolved, and the maternal and paternal chromosomes are arranged in the center of the zygote forming the metaphase of the first mitosis.

GENERAL INTRODUCTION

All researchers working with stereo microscopical grading of mammalian oocytes, zygotes, and embryos recognize the wish of turning the magnification zoom beyond its physical limit in order to assess the nature of structures that escape the resolution of the equipment. However, non-invasive microscopical techniques for embryo evaluation are hampered by on the one hand the limit of resolution of the stereo microscope normally working within magnifications up to a few hundred times and on the other hand the thickness and lack of transparency of the three dimensional structure of the oocytes and embryo of approaching 150 μm in diameter. Transmission electron microscopy bypasses both of these limitations: The microscopical principle offers a much higher resolution in itself working at many thousands times magnification and the third dimension is almost eliminated by examining extremely thin sections of the specimen. The most obvious disadvantage by the electron microscopical approach is the inherent invasiveness including fixation, embedding and sectioning. Nonetheless, researchers who have had even limited experiences by revealing the electron microscopical structure, i.e. the ultrastructure, of oocytes and embryos by either observing pictures or even sitting at the microscope themselves also recognize that insight to the ultrastructure allows for a much more detailed stereo microscopical assessment. A structure, the nature of which cannot be immediately distinguished by the stereo microscope, may very well reveal itself at this simple method of observation if it has previously been examined in the electron microscope! One could say that the electron microscopical imprint provides the alphabet for reading the stereo microscopical appearance of an oocyte or embryo. In this sense, electron microscopical studies of oocytes and embryos have two main purposes: Firstly, they allow for a direct description and understanding of cell biological phenomena of importance for oocyte, zygote, and embryo development and, secondly, they serve as a bridge to the stereo microscope providing the necessary information to "read" the low power embryo morphology.

INTRODUCTION TO THE ULTRASTRUCTURE OF THE MAMMALIAN CELL

Oocytes, zygotes, and embryos are constructed from the same organelles and inclusions as somatic cells. However,

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upon a closer examination it is clear that they present a number of particular features and modifications which cannot be regarded as general cell biological features. Nevertheless, in order to understand the structural cell biology of the embryo, a short basic introduction to the ultrastructure of the common organelles and inclusions of the somatic cell will be presented (Fig. **1**).
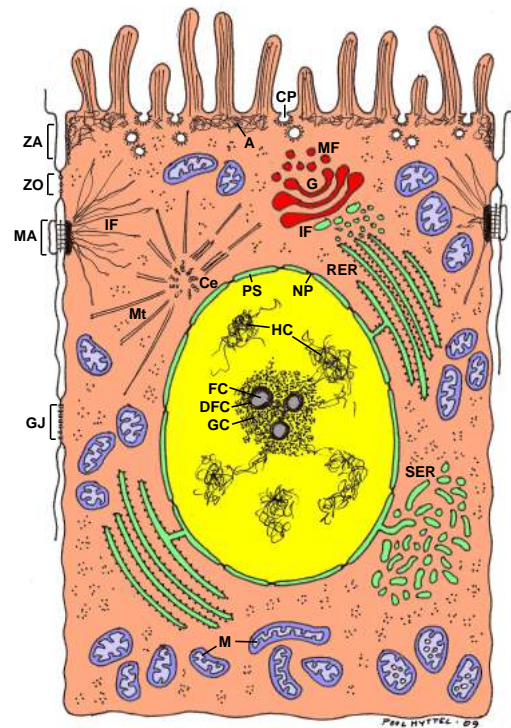
Figure 1: Schematic drawing of a mammalian cell. CP: Coated pit; ZA: Zonula adherens; ZO: Zonula occludens; MA: Macula adherens; GJ: Gap junction; A: Actin filaments; IF: Intermediate filaments; MT: Microtubules; Ce: Centrioles; G: Golgi complex; IF: Immature Golgi face; MF: Mature Golgi face; RER: Rough endoplasmic reticulum; SER: Smooth endoplasmic reticulum; M: Mitochondria; NP: Nuclear pore in nuclear envelope; PS: Perinuclear cisternae; HC: Heterochromatin; FC: Fibrillar center of nucleolus; DFC: Dense fibrillar component of nucleolus; CG: Granular component of nucleolus.

The nucleus of the eukaryote cell is bounded by the nuclear envelope, which isolates the processes of DNA replication and RNA transcription from those of RNA translation and post-translational protein processing occurring in the cytoplasm. The nuclear envelope consists of two parallel trilaminar unit membranes with a perinuclear space between, which is continuous with the lumen of the endoplasmic reticulum (see later). The envelope possesses circular holes into which the nuclear pore complexes are fitted. The pore complexes are involved in exchange of material between the nuclear and cytoplasmic compartments allowing for e.g. mRNA and ribosomal subunits to be exported from the nucleus and transcription factors and other signaling molecules to be imported. Under most circumstances, the nucleus is actively transcribing DNA and this can be recognized at the ultrastructural level: In the interphase nucleus the chromatin is organized as either condensed electron-dense heterochromatin or as uncondensed euchromatin, which is less electron-dense due to a less compact packing of histones allowing for transcription. The most prominent nuclear organelle, also visible at the light microscopical level, is the nucleolus, which is mainly involved in rRNA transcription and ribosome production. The active fibrillo-granular nucleolus consists of three components: The fibrillar component, which can typically be subdivided into the fibrillar centers and the surrounding dense fibrillar component, which, in turn, is surrounded by the granular component. The fibrillar centers contain the enzymatic apparatus required for rRNA transcription, e.g. RNA polymerase I and up-stream binding factor (UBF), and the rRNA-genes have also been localized to this structure as well as to the dense fibrillar component [1]. It is believed that rRNA transcription occurs at the interphase between the fibrillar centre and the dense fibrillar component, and that the latter is formed by the newly synthesized nascent rRNA. This material is subsequently spliced and packed with protein and other non-nucleolar transcripts to form the ribosomal subunits that make up the granular component.

The mitochondria, which are also visible at the light microscopical level, play key roles in the oxidative cellular energy metabolism and their specialized function is reflected in the morphology. The mitochondrion consists of an outer membrane, an inner membrane and two internal compartments, the matrix and the inter-membrane space. The inner membrane is highly folded and carries numerous enzyme systems required for oxidative metabolism. In somatic cells the mitochondria are easily recognized because they are elongated and their inner membrane presents typical transverse cristae, with the exception of steroid producing cells, where the mitochondria present tubular infoldings of the inner membrane instead. The number and structural organization of the cristae change with cell function and activity, and mammalian oocytes, zygotes, and embryos present particular types of mitochondria. It should be mentioned that the mitochondria contain their own specific genome, which at mammalian reproduction is inherited entirely from the oocytes (maternal inheritance).

The smooth (SER) and rough endoplasmic reticulum (RER) are also prominent membranous structures present in the cytoplasm typically forming tubules (SER) or cisternae (RER). The membranes of the endoplasmic reticulum are continuous with the outer nuclear membrane and enclose a continuous internal space. The membranes of the SER carry a multitude of enzyme systems engaged in e.g. synthesis of steroid hormones and detoxification. The RER is morphologically distinct from the SER, and has got its name from the numerous ribosomes that are attached to the external surface of the cisternal membranes giving them a "rough" appearance. Free ribosomes and polysomes can also be observed in the cytoplasm of cells actively engaged in protein synthesis.

Another membranous organelle, which is closely related to the function of the RER, is the Golgi apparatus which is composed of numerous disc-shaped membrane bounded cisternae, resembling a stack of plates. At the immature face of the Golgi apparatus, transport vesicles pinched off from the RER fuse to form the first and most immature Golgi cisterna. Upon reaching a certain size, this cisterna starts its journey towards the mature Golgi face and new immature cisternae will subsequently form. At the mature face, the cisternae are dissolved into secretory vesicles and membrane enclosed inclusions meant for use in the cell as e.g. lysosomes. The Golgi apparatus plays an important role in post-translational modification of proteins by e.g. glycosylation, which occurs during the journey of cisternae from the immature to the mature face of the apparatus.

Cell structure and shape is maintained by the cytoskeleton. In general, the cytoskeleton is composed of microtubules, which are fine tubules of 20-25 nm in diameter, intermediate filaments of about 10 nm in thickness, and actin filaments of about 7 nm thicknesses.

In somatic tissues physical cell-to-cell interactions are mediated through cell junctions. Epithelial cells, in particular, display a number of well-defined junctions, typically referred to as the junctional complex, which are crucial for the cell sheet to function as an epithelium. The resulting cell polarity results in an apical and a baso-lateral compartment of the plasma membrane. Most apically in the junctional complex, epithelial cells are typically connected by tight junctions (*zonula occludens*) that as a continuous belt seal the intercellular space between adjacent cells. The intercellular space is interrupted at the location of the tight junction where the membranes of the two cells share certain membrane proteins. Located just basal to this structure, another belt in the form of adherens junctions (*zonula adherens*) constitutes the second component of the junctional complex and acts as a physical connecting force where the intercellular space is visible. Actin filaments are associated with the adherens junction. As a last, and most baso-laterally located, component of the junctional complex, spot-like desmosomes (*macula adherens*) are found. At the desmosomes, the plasma membrane is coated by electron-dense material which is associated with intermediate filaments. Both adherens junctions and, in particular, desmosomes are associated with intercellular proteins, as e.g. cadherins, physically connecting adjacent cells. Another type of junction, which is normally not included in the junctional complex, is the gap junction where fine intercellular protein channels, referred to as connexons, with a central pore of 1.5 nm allow for communication and transportation of material between adjacent cells.

PHASES OF OOCYTE, ZYGOTE AND EMBRYO DEVELOPMENT

The mammalian embryo inherits most of its structural elements from the oocyte. The ultrastructure of the mature fertilizable oocyte is gradually built up during different phases of oocyte development exemplified in cattle in the following. The first and longest phase comprises the probably several months of oocyte growth accompanying follicular development from the primordial follicle up to the tertiary, i.e. antral, follicle. The second phase is the development of the oocyte in the dominant follicle, a process lasting for one to two weeks. This phase of oocyte development may be referred to as "oocyte capacitation" [2]. The third and last phase is the oocyte maturation
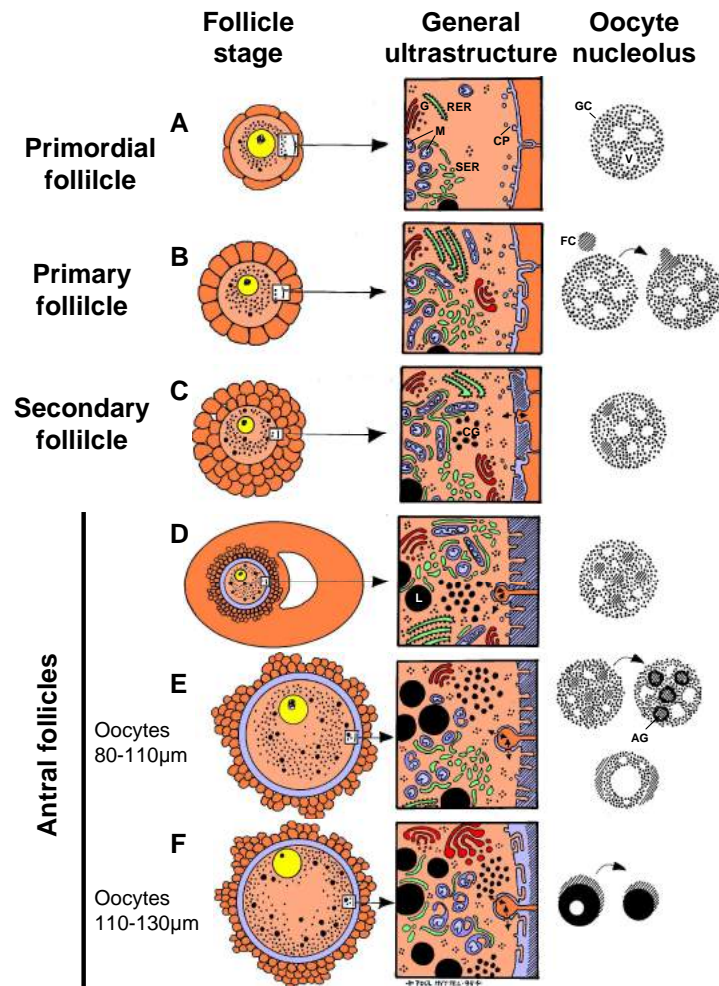
taking place in the ovulatory follicle during the approximately 24 hour long period between the peak of the LH-surge, i.e. the LH-peak, and ovulation. The ultrastructure of the zygote, i.e. the one-cell fertilized egg, strongly reflects what is gradually built during these phases, and with the development of the embryo towards hatching, the ultrastructure gradually changes along with the so-called maternal-embryonic transition when the embryonic genome is activated (Fig. **2**). Consequently, in order to understand the ultrastructure of the zygote and embryo, the ultrastructure of the oocyte during its growth, capacitation and maturation is described in the following. The description is presented in a chronological sequence, and in order to avoid repetitions only the changes noted from one stage to the next are described.
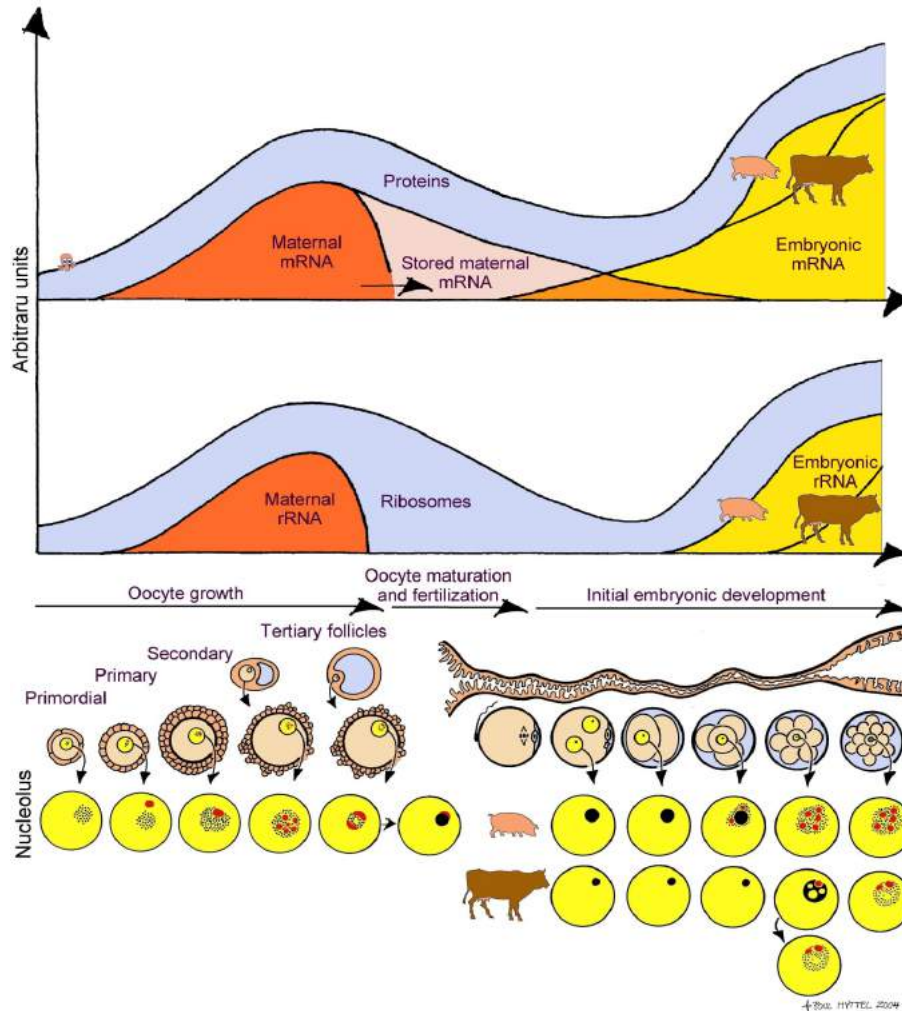
Figure 2: Schematic illustration of transcription, protein synthesis and nucleolar ultrastructure during oocyte growth and maturation, fertilization and initial embryonic development in pigs and cattle. **Upper Panel:** Maternal mRNA (dark red) is synthesized during oocyte growth; some messengers are translated immediately to proteins (blue), whereas others are stored for later translation during initial embryonic development (light red). A minor activation of the transcription of embryonic mRNA (orange and yellow) occurs from the one-cell stage and a major activation occurs at the four- (pigs) or eight-cell stage (cattle). **Mid Panel:** Maternal rRNA (dark red) is synthesized during oocyte growth, resulting in ribosome production (blue). Embryonic rRNA synthesis (yellow) is initiated in parallel with the major activation of embryonic mRNA synthesis contributing to ribosome production. **Lower Panel:** During oocyte growth, the nucleolus is activated in the secondary follicle, when fibrillar centers (red) invade the already existing granular component (black dots). At the end of the growth phase, the nucleolus is inactivated to a dense nucleolar remnant (black) by marginalization of the fibrillar centers. In cattle, remnants of a fibrillar centre (red) remain attached to the nucleolar remnant. Upon fertilization, nucleolus precursor bodies (NPBs, black) are formed in the pronuclei. The NPBs are large in the porcine zygote and smaller in the bovine. Subsequently, embryonic nucleologenesis occurs in parallel with the major activation of embryonic mRNA synthesis according to different models in pigs (external) and cattle (internal). In pigs, the fibrillar centers and the fibrillar component (red) and the granular component (dots) of the nucleolus develop on the surface of the NPBs, whereas in cattle this process occurs inside the precursor body.

In other species the chronology of oocyte maturation, i.e. the time required for the oocyte to resume meiosis from the diplotene stage of prophase I and to reach metaphase II differs from the 24 hours mentioned in cattle. Hence, in the pig, horse, mouse and human, approximately 42, 36, 15, and 40-48 hours, respectively, are required for this process to be completed [3-5]. Interestingly, in the fox most of the progression from the diplotene stage to metaphase II occurs in the oviduct after ovulation. Hence, in this species the oocytes are ovulated at one to 2 days after the LH-peak, at a stage of development when the initially spherical oocyte nucleus has attained a more flattened appearance [6], and the metaphase II is reached at 2-3 days after ovulation.

Among the large domestic animal species, the ultrastructure of the oocyte, zygote, and pre-hatching embryo has received greatest attention in cattle. Hence, the following description will be based on this species as a model, and some comparative aspects of oocyte and zygote ultrastructure in pig, horse, fox, mouse and man will be presented at the end.

OOCYTE, ZYGOTE AND PRE-HATCHING EMBRYONIC ULTRASTRUCTURE IN CATTLE

The basic ultrastructure of the oocyte is generated during its growth accompanying follicular growth from the primordial to the tertiary follicle. When the tertiary follicles in a cohort reach a diameter of about 3-5 mm, one dominant follicle is selected in cattle, and the ultrastructure of the oocyte in this particular follicle is modified during its so-called capacitation. The estrous cycle in cattle generally comprises 2 or 3 follicular waves, and only the dominant follicle of the last wave becomes ovulatory. In the ovulatory follicle the oocyte undergoes a third and last phase of ultrastructural changes during an approximately 24 hour period between the peak of the LH-surge and ovulation. During this last phase, referred to as maturation, the oocyte becomes fertilizable.

Oocyte Growth in Cattle

During the growth of the bovine oocyte, the inside zona diameter of the gamete increases from less than 30 μm in the quiescent primordial follicle to more than 120 μm in the tertiary follicle. In the following, the ultrastructure, transcriptional activity and developmental competence of bovine oocytes will be addressed in relation to the sequential stages of follicular development and, for oocytes from the early antral follicles, in relation to their diameter. The general ultrastructure, transcriptional activity and nucleolar ultrastructure of oocyte growth in cattle are summarized in Table **1** and Fig. **3** [7-10].

Table 1: Ultrastructural characteristics of bovine oocytes during growth, capacitation and maturation in relation to follicular growth.

| Feature	Primordial	Primary	Secondary	Early tertiary	Later tertiary			Capacitated	Matured
					Oocytes <100	Oocytes 100-110	Oocytes >110		
Meiosis	Prophase I	Prophase I	Prophase I	Prophase I	Prophase I	Prophase I	Prophase I	Prophase I	Metaphase II
Oocyte-granulosa cell junctions	Intermediate	Intermediate	Intermediate and gap	Intermediate and gap	Intermediate and gap	Intermediate and gap	Intermediate and gap	Superficial and gap	Disconnection retraction
Zona pellucida (ZP)	Absent	Absent	Small portions	Present	Present	Present	Present	Present	Present
Perivitelline Space (PvS)	Absent	Absent	Absent	Absent	Absent	Small	Moderate	Moderate	Wide
Microvilli	Few, bent	Few, bent	More erect	Numerous ZP embedded	Numerous ZP embedded	Numerous bent in PvS	Numerous bent in PvS	Many bent in PvS	Many erect in PvS
Mitochrondria	Round	Round	Round	Round/elongated	Round/elongated and few hooded	Round/hooded	Hooded	Hooded	Hooded
Golgi complexes	Few	Few	Few	Moderate	Moderate	Moderate	Many	Moderate	Few
Free RER	Extensive	Extensive	Extensive	Extensive	Extensive	Moderate	Absent	Absent	Absent
Free SER	Extensive	Extensive	Extensive	Extensive	Extensive	Moderate	Sparse	Sparse	Moderate
Vesicles	Few	Few	Moderate	Many	Many	Many	Abundant	Abundant	Abundant
Lipid droplets	Few	Few	Moderate	Many	Many	Many	Many	Abundant	Abundant
Cortical granules	Absent	Absent	Few	Moderate clustered	Many clustered	Many clustered	Many clustered	Many clustered	Many solitary

Figure 3: Schematic drawing of aspects of bovine oocyte growth with respect to general ultrastructure and nucleolar ultrastructure. **(A)** Primordial follicle. The oocyte is surrounded by a single layer of flattened granulosa cells. The central oocyte nucleus (yellow) is surrounded by round mitochondria (M), smooth (SER) and rough (RER) endoplasmic reticulum and small Golgi complexes (G). The oocyte cortex presents numerous coated pits (CP) and vesicles. The oocyte nucleolus only presents the granular component (GC) interspersed with vacuoles (V). **(B)** Primary follicle. The oocyte is surrounded by a single layer of cuboidal granulosa cells, and it presents some microvilli and some elongated mitochondria. The eccentrical oocyte nucleus displays a granular nucleolus close to which fibrillar centers (FC) appear and gradually start to invade the granular component. **(C)** Secondary follicle. The oocyte is surrounded by more than one layer of cuboidal granulosa cells. Small patches of zona pellucida material (hatched areas) have appeared and gap junctions (small arrows) have developed between the oocyte and the granulosa cells, and the oocyte microvilli have become more erect. The number of coated pits and vesicles at the oocyte cortex has decreased. In the oocyte, the first small clusters of cortical granules (CG) have developed and some membrane bounded vesicles have appeared (not shown). The oocyte nucleus displays a nucleolus in which the fibrillar centers have become completely incorporated into the periphery forming a fibrillo-granular nucleolus. **(D)** Early tertiary follicle up to about 1mm. The antrum of the follicle has developed and the oocyte is located in the cumulus oophorus surrounded by cumulus cells and an innermost layers of corona radiata cells, which possess projections that penetrate the zona pellucida, invaginate the oolemma and make gap junctional contact to it. In the oocyte, the organelles have attained a more even distribution throughout the ooplasm, elongated mitochondria have become more numerous, lipid droplets (L) have become common, and the number and size of the cortical granule clusters have increased as has the number of vesicles (not shown). The erect microvilli have become embedded within the zona pellucida. The oocyte nucleus displays a fibrillo-granular nucleolus presenting numerous evenly distributed fibrillar centers. **(E)** Tertiary follicle up to about 3 mm as represented by oocytes at 80 to 110 μm in diameter. The number of lipid droplets in the oocyte has increased. In oocytes at less than 100 μm, the nucleus presents a fibrillo-granular nucleolus gradually developing aggregations of electron-dense granules (AG) around the fibrillar centers. In oocytes 100 to 110 μm, the fibrillo-granular nucleolus develops a large central vacuole and marginalized fibrillar centers. **(F)** Late tertiary follicles as represented by oocytes at more than 110 μm in diameter. In the oocyte, the organelles have been dislocated to the periphery, the number of lipid droplets and vesicles (not shown) have increased as have the size of the Golgi complexes. The microvilli have been released from the zona pellucida and pile up in stacks in the perivitelline space. The peripheral oocyte nucleus presents a nucleolus which has turned into a remnant consisting of a sphere of densely packed fibrilles with a fibrillar center attached as a halo. In oocytes at 110 to 120 μm, the nucleolar remnant may still be vacuolated.

The quiescent **primordial follicle** is 34.6±3.7 μm (mean±SD) in diameter [11]. The oocyte, which in itself is 27.9±3.3 μm in diameter, is surrounded by a single layer of flattened granulosa cells (Figs. **4** and **5**). Gap and intermediate junctions are present between adjacent granulosa cells, whereas exclusively intermediate junctions, but not gap junctions, are seen between the granulosa cells and the oocyte (Fig. **6**). The oocyte plasma membrane, i.e. the oolemma, forms numerous coated pits (Fig. **6**), and coated vesicles are found in the cortical cytoplasm, i.e. the ooplasm, signaling endocytosis. The oolemma also forms projections, which penetrate between adjacent granulosa cells, and few short bent microvilli lying parallel with the oocyte surface (Fig. **6**). The nucleus of the oocyte occupies a central or slightly off centre position and the organelles are concentrated in the perinuclear region (Fig. **5**). The mitochondria are predominantly round with few peripheral cristae (Fig. **7**). However, a limited number of elongated mitochondria with transverse cristae are also seen. Many mitochondria contain large electron-dense granules. A sparse number of lipid droplets and membrane-bounded vesicles containing a flocculent material are
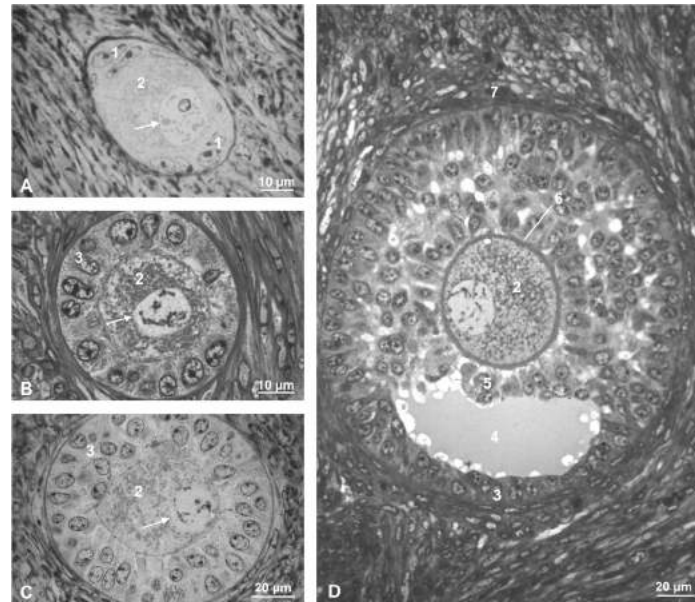
Figure 4: Follicular development in cattle. (**A**) Primordial follicle presenting flat granulosa cells (1), and the oocyte (2) with its nucleus (arrow). (**B**) Primary follicle presenting cuboidal granulosa cells (3). 2: Oocyte; Arrow: Oocyte nucleus. (**C**) Secondary follicle presenting a multilayered granulosa cell layer (3). 2: Oocyte; Arrow: Oocyte nucleus. (**D**) Tertiary follicle presenting the granulosa cell layer (3) and cumulus cells (5) enclosing the oocyte (2). The theca cell layers (7) have started to form. 2: Oocyte; 6: Zona pellucida. From Fair *et al.* [10].
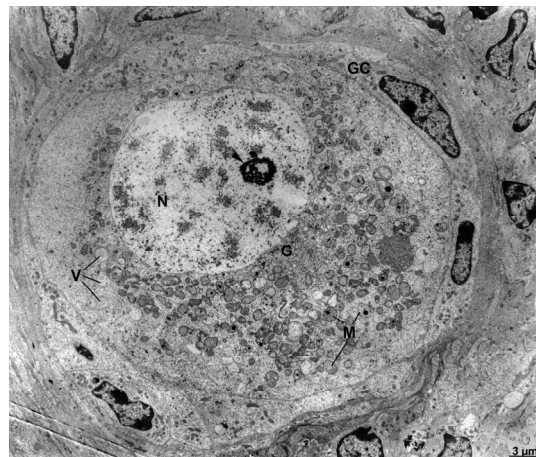
Figure 5: Electron micrograph showing a bovine primordial follicle with flat granulosa cells (GC) and an oocyte presenting the oocyte nucleus (N), with a nucleolus (arrowhead), mitochondria (M), scarce vesicles (V) and Golgi complexes (G). From Fair *et al.* [9].

seen. Both smooth and rough endoplasmic reticulum (SER and RER, respectively) are observed in the ooplasm, either as separated aggregations or as a meshwork spatially connecting mitochondria, lipid droplets and vesicles (Figs. **6** and **7**). The endoplasmic reticulum is often found in close spatial relationship with the nuclear envelope.
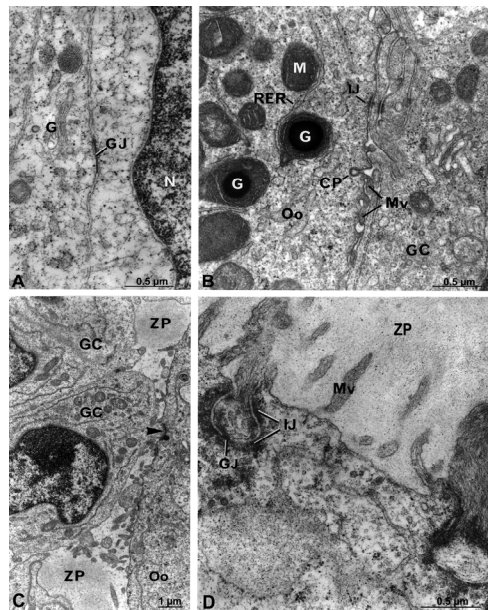

Figure 6: Electron micrographs of intercellular junctions in the developing bovine follicle. (**A**) Detail from a primordial follicle showing a gap junction (GJ) between two adjacent granulosa cells. N: Nucleus; (G): Golgi complex. (**B**) Detail from a primordial follicle showing an intermediate junction (IJ) between a microvillus of a granulosa cell (GC) and the oocyte (Oo). Note the coated pit (CP) at the oocyte surface together with oocyte microvilli (Mv), and in the oocyte, mitochondria (M), of which some present prominent mitochondrial granules (G), and rough endoplasmic reticulum (RER). (**C**) Detail from a secondary follicle showing portions of zona pellucida (ZP) forming between granulosa cells (GC) and the oocyte (Oo). Note that one of the granulosa cells has formed a projection which is connected to the oocyte through an intermediate junction (arrowhead). (**D**) Detail from early antral follicle showing the zona pellucida (ZP) with oocyte microvilli (Mv) embedded in it. Note the cumulus cell projection penetrating the zona and forming an invagination of the oolemma where contact is established through intermediate junction (IJ) and a gap junction (GJ). From Fair *et al.* [10].
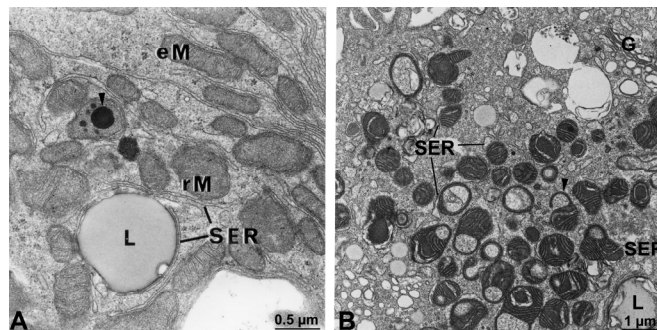
Figure 7: Electron micrograph of mitochondria in bovine oocytes. (**A**) Detail from oocyte from primordial follicle presenting elongated (eM) and rounded (rM) mitochrondria. Note the intimate relationship between the smooth endoplasmic reticulum (SER), mitochondria and lipid droplets (L). Some mitochrondria presents prominent mitochondrial granules (arrowhead). (**B**) Detail from fully grown oocyte presenting typical hooded mitochondria (arowhead). Note the intimate relationship between smooth endoplasmic reticulum (SER) and mitochondria and lipid droplets (L). G: Golgi complex. From Fair *et al.* [10].

Small Golgi complexes (Fig. **8**) are seen and polyribosomes are present on the surface of the RER and free in the ooplasm. The nucleus contains a nucleolus which exclusively consists of the granular component, thus lacking the fibrillar components (Figs. **3** and **9**). Such a nucleolus is, from an ultrastructural point of view, considered as being transcriptionally inactive. Accordingly, as determined by 30 min incubation with 3H-uridine followed by autoradiography, the oocyte is transcriptionally inactive (Fig. **10**). Some primordial follicles present a few cuboidal

granulosa cells, probably representing the first sign of activation of follicular growth. The nuclei of oocytes enclosed in such follicles often present nucleoli in which tentative fibrillar centers are seen either adjacent to or in contact with the granular component (Figs. **3** and **9**). According to the ultrastructural aspects of the nucleolus described previously, the invasion of the tentative fibrillar centers into the granular component of the oocyte nucleolus is thought to mark the initial step towards the assembly of a functional ribosome synthesizing nucleolus in this cell.
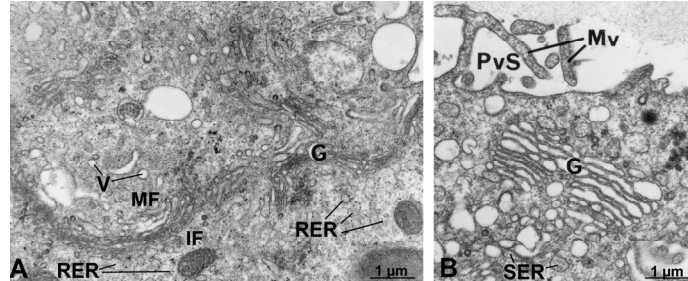
Figure 8: Electron micrographs of Golgi complexes in bovine oocytes. (**A**) Detail from an oocyte from a primordial follicle showing several small Golgi complexes (G). The immature face (IF) is associated with rough endoplasmic reticulum (RER), and vesicles (V) are pinching off at the mature face (MF). (**B**) Detail from the periphery of a fully grown oocyte showing the perivitelline space (PvS) with oocyte microvilli (Mv) extending into it. Note the Golgi complex (G) in the cortical ooplasm closely associated with smooth endoplasmic reticulum (SER). From Fair *et al.* [10].

The mean diameter of the **primary follicle** is 46.1±6.1 μm [11]. The oocyte, which in itself is as a mean 31.6±4.3 μm in diameter, is surrounded by a single layer of cuboidal granulosa cells (Fig. **4**) and occasionally small portions of zona pellucida substance can be observed between the granulosa cells and the oocyte. As in the "activated" primordial follicles, the oocyte nucleoli present tentative fibrillar centers either adjacent to or in contact with the periphery of the granular component (Figs. **3** and **9**).
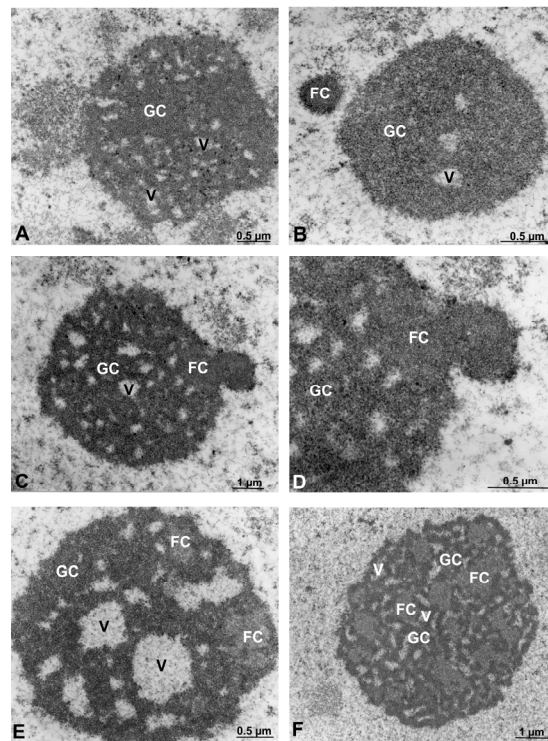
Figure 9: Electron micrographs of the development of the oocyte nucleolus during the initial phases of bovine oocyte growth. (**A**) Detail from oocyte from primordial follicle presenting an oocyte nucleolus consisting of exclusively the granular component (GC) with vacuoles (V). (**B**) Detail from an activated primordial follicle presenting an oocyte nucleolus consisting of a granular component (GC) with vacuoles (V). Note the fibrillar center (FC) associated with chromatin adjacent to the granular component. (**C, D**) Detail from a late primary follicle presenting an oocyte nucleolus consisting of a granular component (GC) with vacuoles

(V) and a fibrillar center (FC) embedded in the periphery. (**E**) Detail from a secondary follicle presenting an oocyte nucleolus consisting of a granular component (GC) with vacuoles (V) and several fibrillar centers (FC) embedded in its periphery. (**F**) Detail from early tertiary follicle presenting an oocyte nucleolus with a reticulated granular component (GC), vacuoles (V), and multiple deeply embedded fibrillar centers (FC). From Fair *et al.* [9].

The **secondary follicle** is as a mean 101.7± 41.8 μm in diameter [11]. The oocyte, which in itself is 45.6±14.0 μm in diameter, is surrounded by more than one layer of cuboidal granulosa cells. Portions of zona pellucida are commonly observed associated with erect microvilli and granulosa cell processes extending towards the oocyte (Fig. **6**). The continued zona-formation is associated with the embedding of these processes as well as the erect oocyte microvilli into the zona pellucida. At the same time, gap junctions are established between the granulosa cell processes and the oocyte. The concomitant decrease in the frequency of coated pits and vesicles in the oocyte
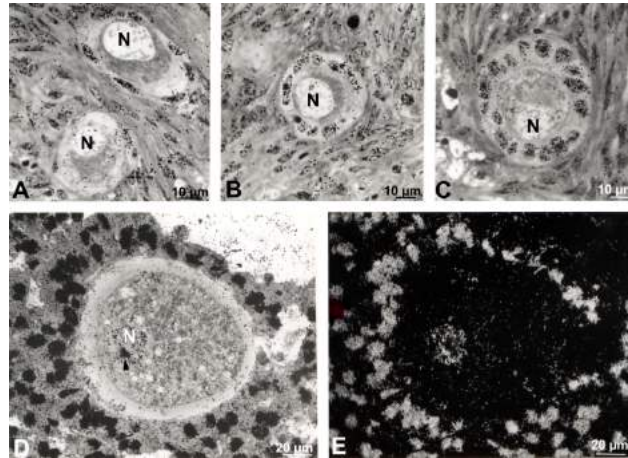
Figure 10: The transcriptional activity in bovine oocytes can be assessed by incubation for 30 min in 3H-uridine, which will become incorporated in potentially synthesized RNA, followed by fixation, sectioning and processing for autoradiography. (**A**) Bright field light micrograph of two primordial follicles. Note the presence of autoradiographic labeling (small black grains) over fibroblast and granulosa cell nuclei, but not over the oocyte nuclei (N). (**B**) Bright field light micrograph of an activated primordial follicle. Note the presence of autoradiographic labeling (small black grains) over the fibroblast and granulosa cell nuclei, but not over the oocyte nucleus (N). (**C**) Bright field light micrograph of a primary follicle. Note the presence of autoradiographic labeling (small black grains) over the fibroblast and granulosa cell nuclei, but not over the oocyte nucleus (N). (**D, E**) Bright field (**D**) and epipolarized light micrograph (**E**) of detail of early tertiary follicle. Note the autoradiographic labeling of the cumulus cells as well of the oocyte nucleus (N) and its nucleolus (arrowhead). At bright field microscopy the autoradiographic labeling appears as small black grains whereas in the epipolarized light, the grains become lucent on a black background. From Fair *et al.* [9].

indicates a shift in the mode of intercellular communication between oocyte and granulosa cells from endocytotic to direct cell-to-cell coupling. Rarely, annulate lamellae (Fig. **11**) are seen, and small clusters of cortical granules may be found in the cortical ooplasm. The cortical granules act in establishing the block against polyspermic fertilization (see later). The oocyte nucleoli present tentative fibrillar centers more or less embedded into the granular component (Figs. **3** and **9**). At this stage of development, the first signs of oocyte transcription can be detected after 30 min incubation with 3H-uridine.
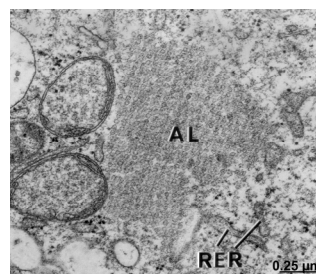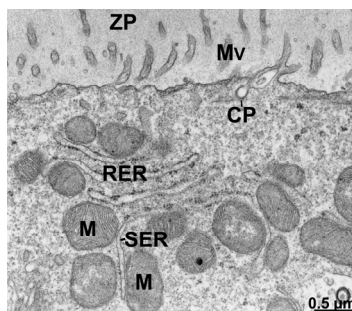

Figure 11: Electron micrograph from oocyte from a secondary follicle. Note the annulate lamellae (AL) stacked in the ooplasm in vicinity of rough endoplasmic reticulum (RER). From Fair *et al.* [10].

The **small tertiary follicle** up to about 1 mm in diameter presents an oocyte with a complete zona pellucida traversed by numerous granulosa cell or, when the cumulus oophorus is formed, cumulus cell projections with bulbous endings invaginating the oolemma and forming gap and intermediate junctions to the oocyte (Fig. **6**). The mitochondria are distributed throughout the ooplasm and round and elongated forms are equally present. Lipid droplets and vesicles are more numerous as are Golgi complexes in the cortical ooplasm. Clusters of cortical granules are numerous and often related to large membrane bounded vesicles and Golgi complexes (Fig. **12**). Cisternae of RER are still distributed in the ooplasm (Fig. **13**). The oocyte nucleoli are typical fibrillo-granular with numerous fibrillar centers scattered within the granular component (Figs. **3** and **9**). For unknown reasons the bovine oocyte does not present a visible dense fibrillar component. Abundant oocyte transcription is encountered after 30 min incubation with 3H-uridine (Fig. **10**).
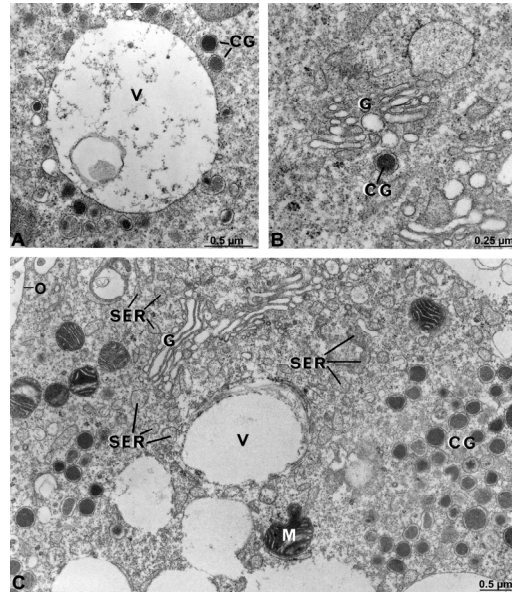
Figure 12: Electron micrographs of cortical granules in bovine oocytes. (**A**) Detail form early tertiary follicle showing cortical granules (CG) associated with a large vesicle (V) in the ooplasm. (**B**) Detail form early tertiary follicle showing a cortical granule (CG) associated with a Golgi complex (G) in the ooplasm. (**C**) Detail from a fully grown oocyte presenting vesicles (V), mitochrondria (M) and a Golgi complex (G), all closely associated with the smooth endoplasmic reticulum (SER). Note the big cluster of cortical granules (CG) located at some distance from the oolemma (O). From Fair *et al.* [10].


Figure 13: Electron micrograph from oocyte from an early tertiary follicle. Note the well-developed rough endoplasmic reticulum (RER) connected to smooth endoplasmic reticulum (SER) which associates with mitochondria (M). ZP: Zona pellucida; Mv: Microvilli; Coated pit: CP. From Fair *et al.* [10].

In the **larger tertiary follicles** the oocyte ultrastructure may be classified according to the inside zona pellucida diameter of the cell. In oocytes <100 μm the nucleus is, in general, located slightly off centre, the mitochondria are relocated to the perinuclear and deep cortical ooplasm in many cases and the microvilli remain embedded in the zona pellucida. The particular hooded mitochondria, unique to ruminants, are observed for the first time (Fig. **7**). The oocyte is transcriptionally active and its nucleus presents large fibrillo-granular nucleoli (Figs. **3**, **14** and **15**).

Oocytes from 100 to 110 μm in diameter in many cases display formation of a perivitelline space, the process of which is associated with the release of the previously embedded microvilli from the zona pellucida. The oocyte nucleus is, in general, displaced further towards the periphery as are the Golgi complexes and mitochondria amongst
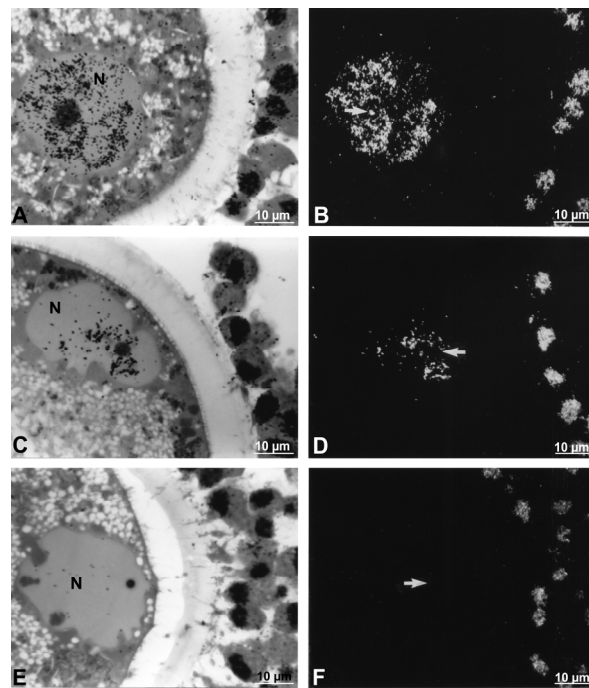

Figure 14: Autoradiograms of bovine oocytes following 30 min 3H-uridine incubation. (**A, C, D**) Bright field microscopy; autoradiographic labeling seen as small black grains. (**B, D, F**) Epipolarized light microscopy; autoradiographic labeling seen as small lucent grains. (**A, B**) Detail from oocyte <100 μm in diameter. Note the autoradiographic labeling over the nucleus (N) as well as over part of the nucleolus (arrow). (**C, D**) Detail from oocyte with a diameter between 100 and 110 μm. Note the sparse autoradiographic labeling over the nucleus (N), but not the nucleolus (arrow). (**D, E**) Detail from oocyte >120 μm in diameter. Note the lack of autoradiographic labeling over the nucleus (N) and the nucleolar remnant (arrow). From Fair *et al.* [8].

which the hooded form becomes more numerous. RER is less abundant whereas the Golgi complexes are more numerous. In some oocytes, electron-dense accumulations are established around the fibrillar centers of the nucleoli indicating changes in rRNA transcription and processing (Fig. **15**). In other oocytes, the fibrillar centers of the nucleoli have migrated towards the periphery and form lentifom structures on the surface of the spherical granular component. This process presumably represents the withdrawal of the rRNA-genes from the nucleolus and, thus, the termination of rRNA and ribosome synthesis signaling that oocyte growth is more or less completed. In such oocytes, autoradiographic examination after 30 min incubation with 3H-uridine show lack of nucleolar transcription, i.e. rRNA transcription, and a decreased rate of transcription of other portions of the genome (Fig. **14**). A majority of oocytes from 110 to 120 μm present a well developed perivitelline space, occupied by numerous microvilli that tend to form stacks, and a peripherally located nucleus. The hooded mitochondrial form is the most common and the number of Golgi complexes, lipid droplets and vesicles has increased further. The latter two components are more or less equally distributed throughout the ooplasm whereas mitochondria and Golgi complexes are peripherally located. RER is uncommon. In some oocytes, the nucleoli have completely marginalized the fibrillar centers to a single lentifom structure on the outside of the granular component (Figs. **3** and **15**). Accordingly, no oocyte transcription can, in general, be detected after 30 min 3H-uridine incubation (Fig. **14**). In a majority of oocytes, however, the process of nucleolar inactivation has proceeded and the granular component has been lost, leaving the nucleolus to consist of a remnant sphere of tightly packed fibrils with a fibrillar centre attached (Fig. **16**). Oocytes >120 μm present even more lipid droplets and have completed nucleolar inactivation in by and large all cases. The nucleus is located peripherally in the ooplasm and the perivitelline space is evident, but the cumulus cells are still connected to the oocyte through long slender projections penetrating the zona pellucida. Numerous large clusters of cortical granules are present in the cortical ooplasm together with mitochondria of the hooded type. At this point, the oocyte has completed the growth phase and achieved the ultrastructure characterizing the full developed gamete (Figs. **17**, **18** and **19**).
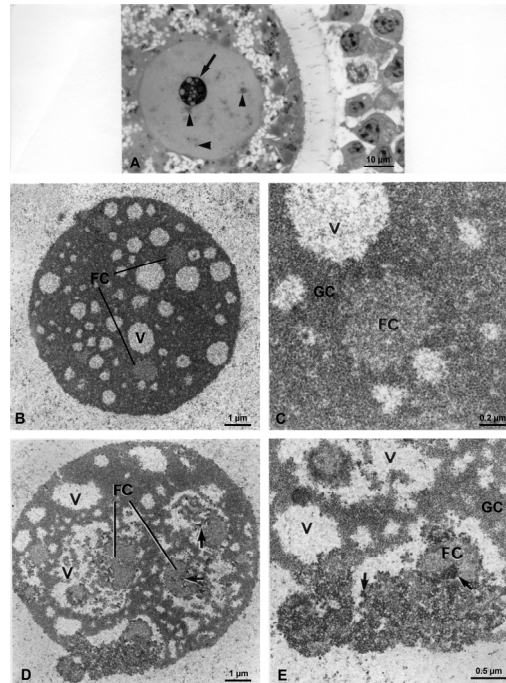
Figure 15: Light (**A**) and electron (**B, C, D, E**) micrographs of the development of the oocyte nucleolus during the late phases of bovine oocyte growth (see Fig. 16 for continuation). (**A**) Detail of bovine oocyte <100 μm in diameter. Note the nucleus with a vacuolated nucleolus (arrow) and heterochromatin (arrowheads) of which portions are connected with the nucleolus. (**B, C**) Fibrillo-granular nucleolus from an oocyte <100 μm in diameter. Note the granular component (GC), the fibrillar centers (FC) and the vacuoles (V). (**D, E**) Fibrillo-granular nucleolus from an oocyte between 100 and 110 μm in diameter. Note the granular component (GC), the fibrillar centers (FC), the vacuoles (V) as well as the electron-dense material (arrows) gathering in association with the fibrillar centers. From Fair *et al.* [8].
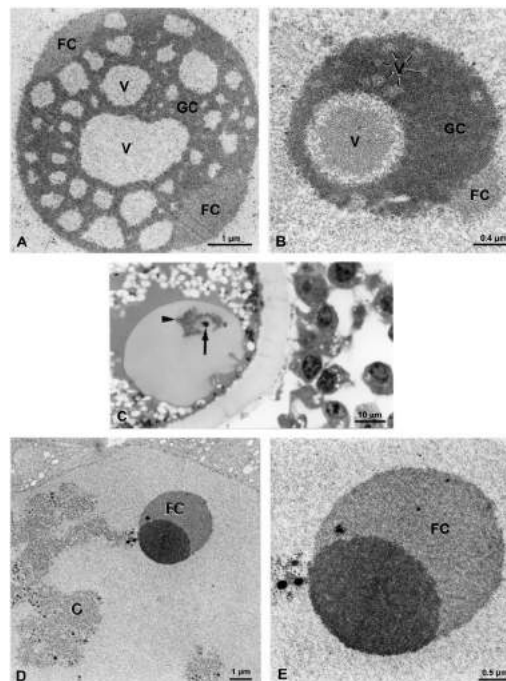

Figure 16: Electron and light micrographs of the development of the oocyte nucleolus during the late phases of bovine oocyte growth. (**A**) Electron micrograph of fibrillo-granular nucleolus from an oocyte between 100 and 110 μm in diameter. Note the marginal localization of the fibrillar centers (FC) being extruded from the granular component (GC), as well as the formation of several smaller and a central larger vacuole (V). (**B**) Electron micrograph of fibrillo-granular nucleolus from an oocyte between

100 and 110 μm in diameter. Note the complete marginalization of a single fibrillar center (FC) and the presence of several smaller and a central larger vacuole (V) in the granular component. (**C**) Light microscopical detail of an oocyte between 110 and 120 μm in diameter. Note the nucleolar remnant (arrow) surrounded by chromatin (arrowhead). (**D, E**) Electron micrographs of nucleolar remnant from an oocyte between 110 and 120 μm in diameter. Note the nucleolar remnant consisting of a fibrillar center (FC) attached to a sphere of densely packed fibrillar material. The remnant is associated with chromatin (C). From Fair *et al.* [8].
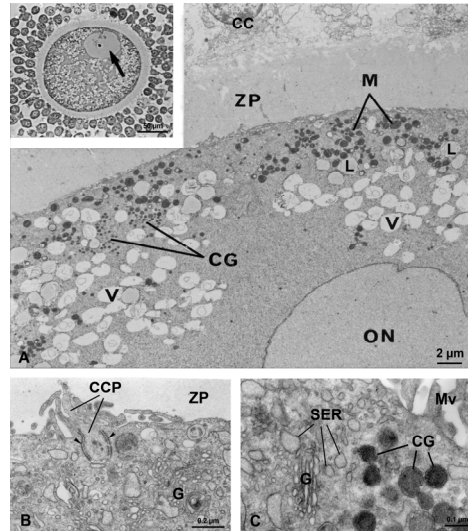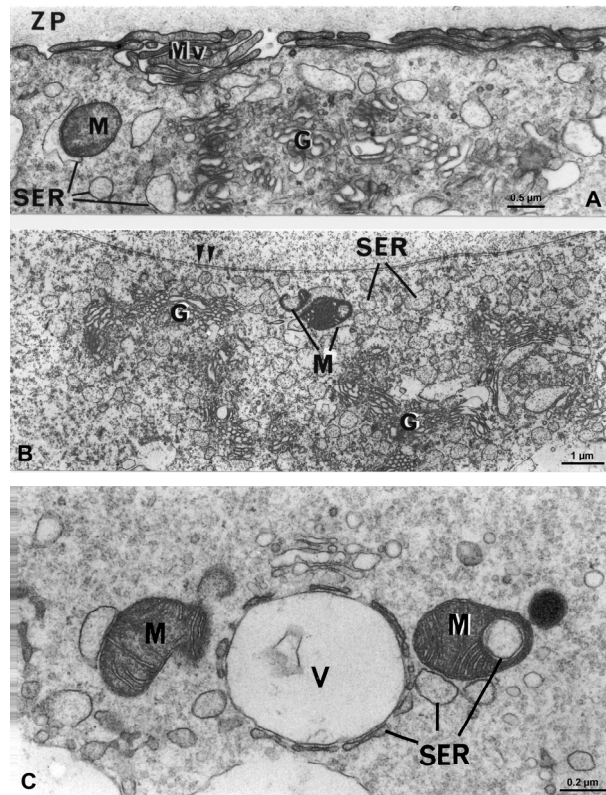

Figure 17: Characteristic electron microscopic features of the fully developed bovine oocyte (see Figs. 18 and 19 for continuation). (**A**) Detail from oocyte presenting cumulus cells (CC), zona pellucida (ZP) and peripherally located clusters of mitochondria (M) and cortical granules (CG). Also, numerous vesicles (V) and lipid droplets (L) are seen. ON: Oocyte nucleus. Insert: Light micrograph of fully developed oocyte presenting the peripherally located oocyte nucleus (arrow). (**B**) Detail from oocyte showing a cumulus cell projection (CCP) penetrating the zona pellucida (ZP) and invaginating the oolemma for establishing intermediate junctions (arrowheads) to the oocyte. In the proximity of the projection, numerous Golgi complexes (G) are seen in the cortical ooplasm. (**C**) Detail from oocyte showing microvilli (Mv) extending into the perivitelline space and a cluster of cortical granules (CG) closely associated with a Golgi complex (G) and smooth endoplasmic reticulum (SER). From Hyttel *et al.* [16].
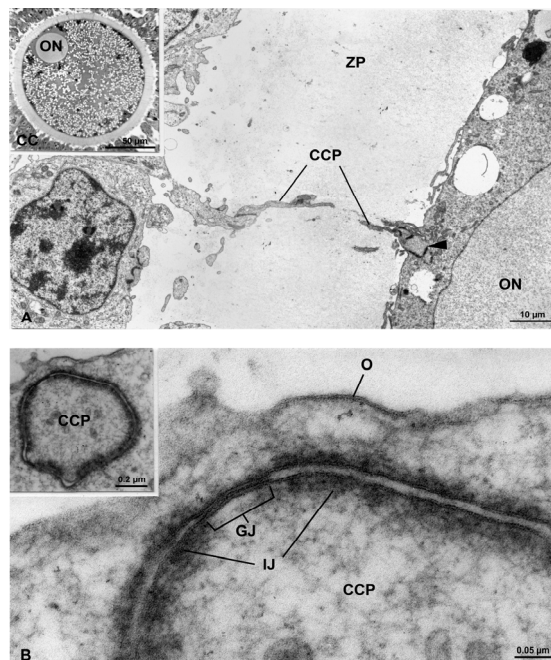
Figure 18: Characteristic electron microscopic features of the fully developed bovine oocyte. (**A**) Detail from oocyte showing a cumulus cell projection (CCP) penetrating the zona pellucida (ZP) for invaginating the oolemma (arrowhead). ON: Oocyte nucleus. Insert: Light micrograph of oocyte showing the cumulus cells (CC) and the peripherally located oocyte nucleus (ON).

(**B**) Detail from oocyte showing the ending of a cumulus cell projection (CCP) establishing contact with the oolemma in the form of intermediate junctions (IJ) and a gap junction (GJ). O: Oolemma. Insert: The cumulus cell projection (CCP) at a lower magnification. From Hyttel [21].


Figure 19: Characteristic electron microscopic features of the fully developed bovine oocyte. (**A**) Detail from the periphery of an oocyte showing the zona pellucida (ZP), stacks of microvilli (Mv) and a peripherally located mitochondrion (M) and Golgi complex (G), both closely associated with the smooth endoplasmic reticulum (SER). (**B**) Detail from oocyte showing the nuclear envelope (arrowheads), hooded mitochrondria (M) and Golgi complexes (G) closely associated with smooth endoplasmic reticulum (SER). (**C**) Detail from oocyte showing the intimate relationship between the smooth endoplasmic reticulum (SER), a vesicle (V) and mitochondria (M). From Assey *et al.* [12] and Hyttel *et al.* [16].

Interestingly, the oocyte achieves the competence to complete meiotic maturation to metaphase II *in vitro* at a diameter of about 110 μm coinciding with the de-activation of its transcriptional machinery, indicating that the necessary compartment of proteins and mRNAs has been formed at this stage of development.

Oocyte Capacitation in Cattle

With the growth of the dominant follicle, the ultrastructure of the fully grown oocyte is modified during its so-called capacitation. This process has been examined in a model, where the dominant follicle of the first follicular wave of the estrous cycle in cows was induced to ovulate by treatment with a prostaglandin F2α analogue on Day 7 after ovulation [12]. This model allows for collection of oocytes for ultrastructural analyses from the dominant follicle throughout the entire phase of dominance. The results are summarized in Table **1** and Fig. **20**. During the days approaching the regression of the corpus luteum, i.e. the final period of the luteal phase, the frequency of microvilli stacks on the oocyte surface decreases as does the size of the oocyte Golgi complexes, whereas that of the lipid droplets increases. The cortical granule clusters are dislocated to more superficial locations and some granules migrate to solitary positions along the oolemma. During the period between luteolysis and the LH-surge, i.e. the initial period of the follicular phase, individual cumulus cells exhibit elongation and some of the cumulus cell process endings are retracted to a more superficial location on the surface of the oolemma (Fig. **21**). The perivitelline space enlarges and the size of the Golgi complexes is further reduced. Moreover, the oocyte nuclear envelope becomes

undulating, especially in the regions facing the zona pellucida, and the nucleolar remnant displays vacuolization where the fibrillar center is incorporated into a shell-like structure together with the dense fibrillar material (Fig. **21**). Both of these phenomena are presumably related to the subsequent breakdown of the oocyte nucleus.
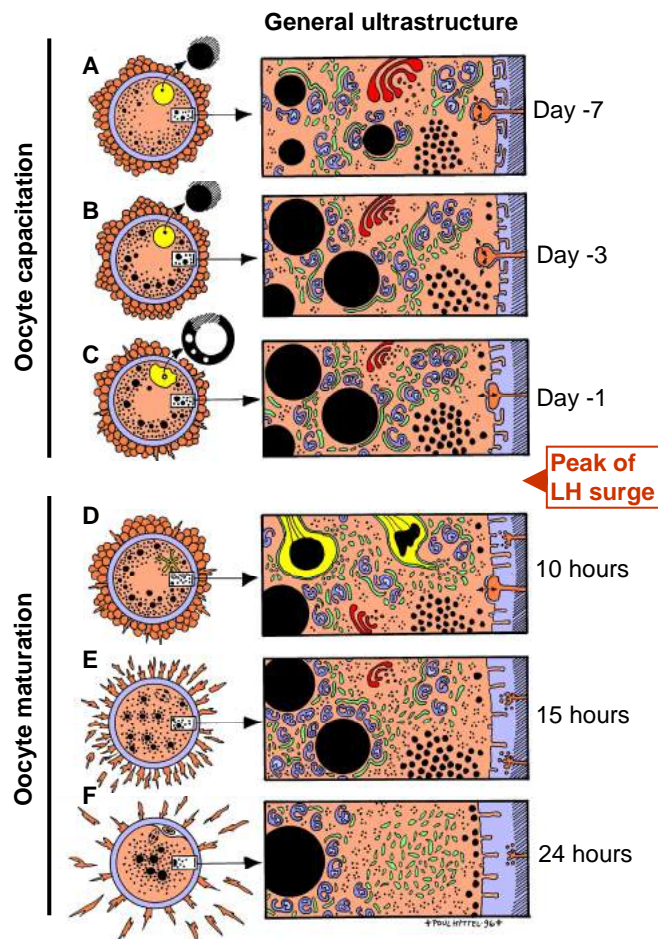
Figure 20: Schematic drawing of ultrastructural aspects of bovine oocyte capacitation in the dominant follicle up to the LH peak and final oocyte maturation after the peak. See Fig. 3 for labels. **(A)** Oocyte from a dominant follicle 6 days before the LH peak. The general ultrastructure is identical with that obtained at the end of oocyte growth (Fig. 3F). **(B)** Oocyte from a dominant follicle 3 days before the LH peak. The number of microvilli stacks have decreased as have the size of the Golgi complexes, the amount of lipid droplets has increased, and the cortical granule clusters have dislocated to a more superficial location. **(C)** Oocyte from a dominant follicle on the day before the LH peak. Some individual corona cells display elongation and the corona cell projections have been retracted to a more superficial location, the perivitelline space has enlarged, the microvilli have become more erect, and the size of the Golgi complexes has been further reduced. Moreover, the envelope of the oocyte nucleus has become undulating and the nucleolar remnant has transformed into a ring-like structure including the fibrillar centre with a central and several secondary vacuoles. **(D)** Oocyte at "germinal vesicle breakdown" from an ovulatory follicle at 9-12 h after the LH peak. The perivitelline space develops further and in the oocyte the mitochondria tend to arrange around the lipid droplets and the nuclear envelope is disolved into tubules of SER and microtubules appear adjacent to the condensing chromosomes. **(E)** Oocyte at MI from an ovulatory follicle at about 15 h after the LH peak. The number and size of the lipid doplets has increased and mitochondria have assembled around the droplets and these conglomerates have attained a more even distribution throughout the ooplasm. Numerous ribosomes have appeared especially around the chromosomes and the size of the Golgi complexes has decreased further. **(F)** Oocyte at MII from an ovulatory follicle at about 24 h after the LH peak. The bulk of the cortical granules are distributed at solitary positions along the oolemma. The lipid droplets and mitochondria have attained a more central location in the ooplasm leaving a rather organelle free peripheral zone in which the most prominent features are large clusters of SER. Golgi complexes are practically absent.

Data reported by Hendriksen *et al.* [13] indicate that the competence of the oocyte to produce blastocysts *in vitro* increases with completion of capacitation. Superovulation with exogenous gonadotropins may have an adverse effect on oocyte capacitation as indicated by a lack of at least the vacuolization of the nucleolar remnant [14].
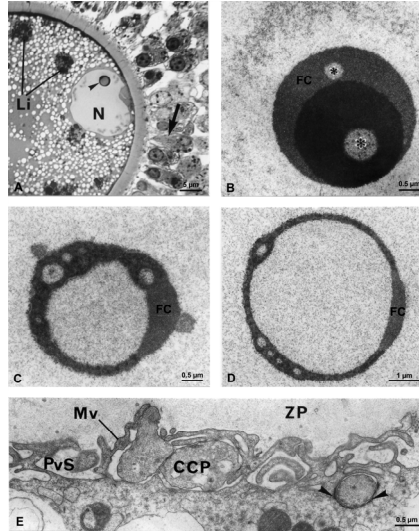
Figure 21: Light (**A**) and electron (**B, C, D, E**) micrographs of bovine oocytes during the process of capacitation. (**A**) Detail of oocyte presenting a nucleus (N) with clear undulations of the nuclear envelope and a vacuolated nucleolar remnant (arrowhead). Note the elongated cumulus cells (arrow) and the lipid-mitochrondrial clusters (Li). (**B, C, D**) Detail from oocytes showing gradual vacuolization (asterisk) of nucleolar remnants. Note that the originally peripherally located fibrillar center (FC) becomes incorporated into the shell-like nucleolar remnant. Asterisks: Vacuoles. (**E**) Detail from the periphery of an oocyte showing the zona pellucida (ZP), microvilli (Mv) extending into the perivitelline space (PvS) and a cumulus cell projection ending (CCP) retracted to a more superficial location without junctional contact to the oolemma. Note that another cumulus cell projection has retained its intermediate junctions (arrowheads) with the oolemma. From Assey *et al.* [12].

Oocyte Maturation in Cattle

The maturation of the oocyte, which in cattle occurs during the approximately 24 hour period from the LH-peak to ovulation, comprises the progression of meiosis from the diplotene stage of prophase I to metaphase II accompanied by a series of ultrastructural and molecular changes in the ooplasm. The ultrastructural changes have been described in detail in relation to the time of the LH-peak in unstimulated [15] as well as gonadotropin stimulated cattle [16]. The results are summarized in Table **1** and Fig. **20**. Other researchers have described certain ultrastructural aspects of the immature or maturing oocyte [17-19]. The breakdown of the oocyte nucleus occurs approximately 9 to 12 hours after the LH-peak and studies of oocyte maturation *in vitro* have demonstrated that first the nuclear envelope becomes extremely undulating and, second, it appears to be dissolved into SER (Figs. **22**, **23** and **24**; [20]). In
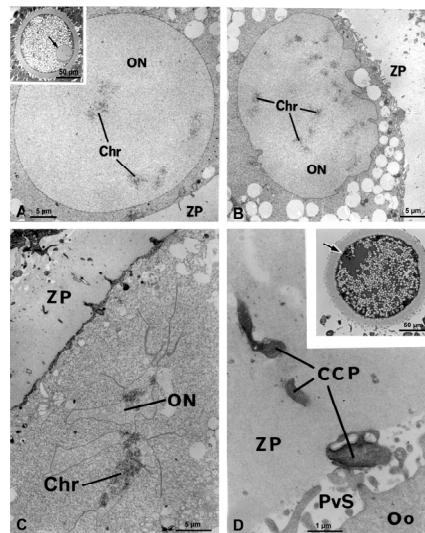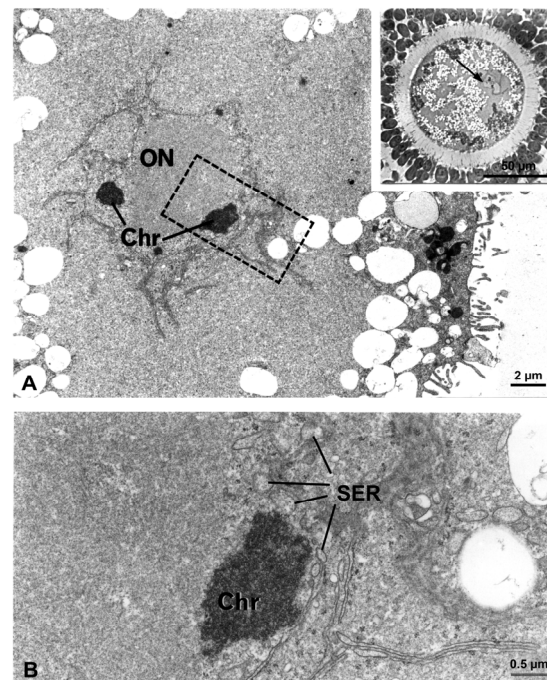

Figure 22: Electron micrographs of different phases of the breakdown of the bovine oocyte nucleus, i.e. germinal vesicle breakdown (see Figs. 23 and 24 for continuation). (**A**) Detail from oocyte before capacitation showing typical spherical oocyte

nucleus (ON) with sparse amounts of heterochromatin (Chr). ZP: Zona pellucida. Insert: Light micrograph of the same oocyte showing the peripherally located spherical oocyte nucleus (arrow). (**B**) Detail from oocyte during capacitation showing undulations of the envelope of the oocyte nucleus (ON), particularly in the portion facing the zona pellucida (ZP), and increased amounts of heterochromatin (Chr). (**C**) Detail from oocyte in which the nucleus (ON) is in the process of breakdown. Note the pronounced undulations of the nuclear envelope and the increased condensation of chromatin (Chr) to heterochromatin. (**D**) Detail from oocyte showing a decoupled cumulus cell projection (CCP) traversing the zona pellucida (ZP) ending in the perivitelline space (PvS) without any junctional contact to the oocyte (Oo). Insert: Light micrograph of the same oocyte showing the oocyte nucleus in the process of breakdown (arrow). From Hyttel *et al.* [16, 20].

parallel, the chromatin gradually condenses to form chromosomes associated with dense areas probably representing condensations of the nucleoplasmic matrix. These processes are accompanied by dissolution of the nucleolar remnant and the gradual decoupling of the cumulus cell endings from the oocyte (Fig. **22**; [21]). Moreover, the Golgi complexes are reduced in size and the mitochondria tend to accumulate around the lipid droplets organizing what are referred to as metabolic units of the oocyte [15].

At about 15 hours after the LH-peak most oocytes have reached metaphase I and the lipid-mitochondrial clusters move from the peripheral location to a more even distribution throughout the ooplasm (Fig. **25**). Concomitantly, the mitochondrial clustering around the lipid droplets is enforced and the Golgi compartment is further reduced. Interestingly, the metaphase chromosomes, as well as their associated microtubules, are positioned in a dense area probably representing the condensed nucleoplasmic matrix. At about 20 hours after the LH-peak, most oocytes have reached metaphase II and the first polar body is abstricted. Studies of *in vitro* oocyte maturation have demonstrated that immediately after abstriction of the first polar body, the chromosomes with their associated microtubules are individually localized in the cytoplasm adjacent to a dense area of presumptive condensed nucleoplasmic matrix (Fig. **25**; [20]). When the metaphase plate is subsequently arranged, the chromosomes become incorporated into the dense area, and two smaller dense areas emerge at the tentative spindle poles. Ovulation occurs at about 24 hours after the LH-peak. During the last hours of maturation, the lipid droplets and mitochondria attain a more central location in the ooplasm, the SER tends to form large aggregates in the cortical ooplasm and the cortical granules migrate to solitary positions along the oolemma (Figs. **25** and **26**). The peripheral migration of the cortical granules appears to be

Figure 23: Electron micrographs of different phases of the breakdown of the bovine oocyte nucleus. (**A**) Detail from oocyte showing the oocyte nucleus (ON) in the process of breakdown. Note the marked undulations of the nuclear envelope and the completely condensed chromatin (Chr). Insert: Light micrograph of the same oocyte showing the peripherally located oocyte nucleus (arrow). (**B**) Boxed area from "A". Note the condensed chromatin (Chr) and the undulating nuclear envelope dissolving into smooth endoplasmic reticulum (SER). Hyttel *et al.* [22].
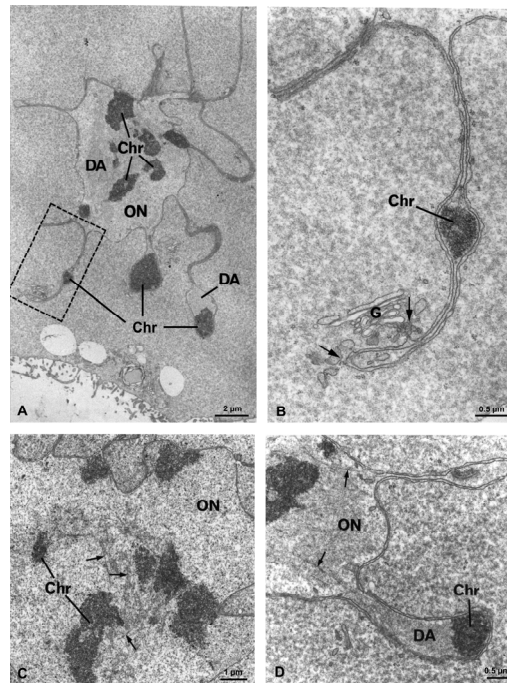
Figure 24: Electron micrographs of different phases of the breakdown of the bovine oocyte nucleus. (**A**) Detail from oocyte showing the nucleus (ON) in the process of breakdown. Note the pronounced undulations of the nuclear envelope and the blocks of condensed chromatin (Chr) frequently found in extensions of the nucleus (boxed area). Also, note the dense areas (DA) in the nucleus formed by condensation of the nucleoplasmic matrix. (**B**) Boxed area from "A". Note the condensed chromatin (Chr) enclosed in an outpocketing of the nuclear envelope and the dissolution of the nuclear envelopes into smooth endoplasmic reticulum (arrows) closely associated with a Golgi complex (G). (**C, D**) Details from oocyte showing the nucleus in the process of breakdown. Note the condensed chromatin (Chr) with dense areas (DA) of condensed nuclear matrix attached in the oocyte nucleus (ON) as well as the presence of microtubules (arrows) attaching to the chromosomes. From Hyttel *et al.* [20].
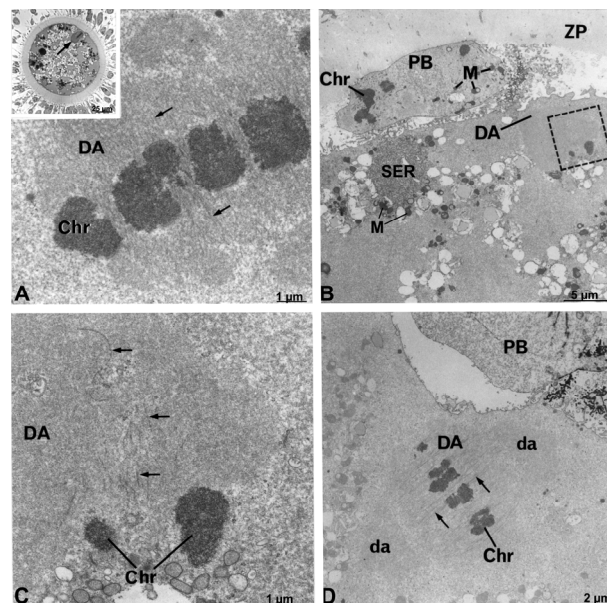
Figure 25: Electron micrographs of progression of meiosis from metaphase I to metaphase II in bovine oocytes. (**A**) Detail from oocyte showing chromosomes (Chr) at metaphase I. Note that the chromosomes are arranged in a dense area (DA) presumably representing the condensed nucleoplasmic matrix. Insert: Light micrograph showing the same oocyte with the metaphase chromosomes located in a dense area of the cytoplasm (arrow). (**B**) Detail from oocyte showing the first polar body (PB) containing chromosomes (Chr) and mitochondria (M). Note that the oocyte chromosomes (boxed area) are located adjacent to a

dense area (DA). Also, note the large cluster of smooth endoplasmic reticulum (SER) surrounded by mitochondria (M) located peripherally in the ooplasm. ZP: Zona pellucida. (**C**) Boxed area from "B". Note the chromosomes (Chr) with associated microtubules (arrows) as well as the adjacent dense area (DA). (**D**) Detail from oocyte showing the first polar body (PB) and in the oocyte, chromosomes (Chr) arranged in a metaphase plate with associated microtubules (arrows) which are located in a large dense area (DA). Note the smaller dense areas (da) at the spindle poles. From Hyttel *et al.* [20].
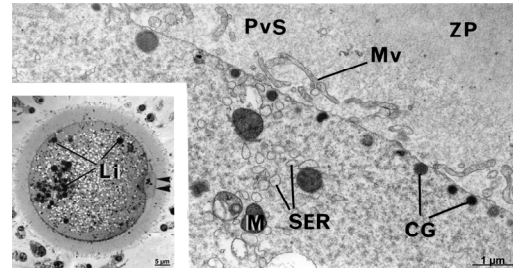

Figure 26: Electron micrograph from bovine oocyte at metaphase II showing the solitary location of cortical granules (CG) along the oolemma which forms microvilli (Mv) protruding into the perivitelline space (PvS). Note the mitochondria (M) closely associated with smooth endoplasmic reticulum (SER). Insert: Light micrograph of same oocyte showing the first polar body (arrowheads) and large clusters of lipid droplets and mitochondria (Li). From Assey *et al.* [12].

compromised to a certain degree during oocyte maturation *in vitro* rendering such oocytes more susceptible to polyspermic fertilization [22]. The large SER aggregates may play a role as intracellular deposits of Ca^{++} ions as in skeletal muscle cells allowing for the intracellular increase in the Ca^{++} concentration in conjunction with oocyte activation at fertilization. At both metaphase I and II, the chromosomes are contained within a spherical organelle-free area displaying increased electron-density of the cytoplasmic matrix, previously described as dense areas. The density of these areas is very similar to that observed in the oocyte nucleus before its breakdown. Hence, it is tempting to speculate that substances from the nucleoplasmic matrix remain in contact with the metaphase chromosomes without being delineated by a nuclear envelope. Interestingly, spherical areas of similar density are found adjacent to the sperm head during its decondensation in the ooplasm after fertilization and, moreover, the prophase chromosomes of the first mitosis, located centrally in the zygote, are also embedded in such material (see later).

The growing and dominant follicle is capable of maintaining oocyte meiosis arrested at the diplotene stage of prophase I. During the process of follicular development, numerous tertiary follicles undergo atresia. Interestingly, such atretic follicles may loose the ability to retain the oocyte in meiotic arrest. Hence, oocytes in atretic follicles may display different stages of meiotic maturation; even reaching metaphase II (Fig. **27**). Along with this aberrant resumption of meiosis, also certain aspects of cytoplasmic oocyte maturation may occur.

Through the described phases of growth, capacitation and maturation, the oocyte has now reached the stage where the ultrastructural architecture for fertilization and initial embryonic development is established.
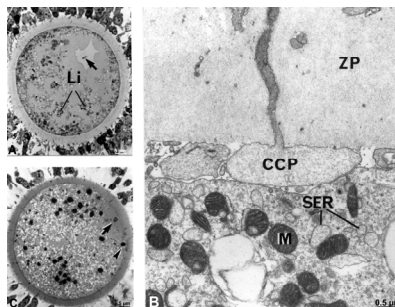

Figure 27: Details from bovine oocytes retrieved from atretic follicles. (**A**) Light micrograph of oocyte showing breakdown of the oocyte nucleus. Note the undulating nuclear envelope and the small condensed mass of chromatin (arrow) in the nucleus. Also, note the clusters of lipid droplets and mitochondria (Li). (**B**) Electron micrograph from oocyte showing a cumulus cell projection (CCP) traversing the zona pellucida (ZP) ending in the perivitelline space without junctional contact to the oocyte, in which mitochrondria (M) closely associated with smooth endoplasmic reticulum (SER) are seen. (**C**) Light micrograph of oocyte at metaphase II. Note the first polar body (arrowhead) in the perivitelline space and the metaphase chromosomes (arrow) in the oocyte. From Assey *et al.* [12].

Fertilization and Development of the Zygote in Cattle

Fertilization occurs in the poorly defined transition between the ampulla and the isthmus of the oviduct resulting in the formation of the zygote, i.e. the one-cell fertilized egg. The further journey though the isthmus of the oviduct to the uterus occurs within about 4 days in cattle. During the process of fertilization the ultrastructural components developed during oocyte growth, capacitation and maturation are re-utilized for a number of specific purposes. The ultrastructural changes associated with fertilization has precisely been described in relation to the estimated time of ovulation as determined by timing of the LH-peak in gonadotropin stimulated cows [23]. The results are summarized in Fig. **28**. Other researchers have described more specific ultrastructural features of fertilization in unstimulated [24] or stimulated cattle [25], but without relating the findings to the endocrine timeline and, thus, ovulation. Also, the ultrastructural investigations of bovine *in vitro* fertilization have added to the understanding of the process of fertilization [26-27].

The oocyte sheds the accompanying expanded cumulus cells during or within the first hours after ovulation. Hence, the spermatozoa have direct access to a major surface area of the zona pellucida (Fig. **29**). After the fertilizing spermatozoon has attached to the zona, the acrosome reaction occurs. This process is characterized by swelling of the acrosome, except at the equatorial segment, and appearance of a few vesicles in it. This feature is followed by multiple fusions between the sperm plasma membrane and the outer acrosomal membrane resulting in formation of tiny hybrid vesicles and release of the acrosomal content (Fig. **30**).
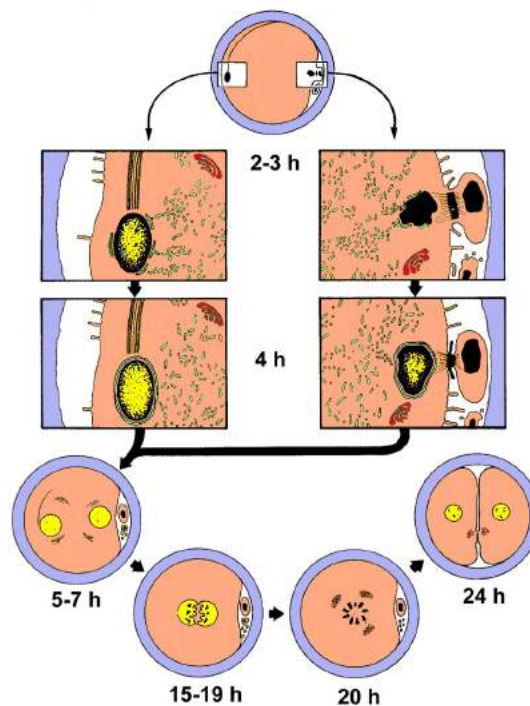
Figure 28: Schematic drawing of pronucleus development in the bovine zygote at different hours after ovulation. The left panel depicts the development of the paternal component whereas the maternal is depicted at the right. See Fig. 3 for colors and labels. At 2-3 h after ovulation, the decondensing sperm chromatin is gradually enclosed by profiles of smooth endoplasmic reticulum (SER) approaching it. The maternal chromatin is at the telophase, and the condensed chromatin block, destined to contribute to development, is likewise surrounded by SER in the oocyte. At 4 h, complete nuclear envelopes have formed around both maternal and paternal chromatin, and small pronuclei have been established. During the following hours, the pronuclei swell to their typical spherical shape. Annulate lamellae form in their proximity, and the paternal pronucleus is still recognizable by its association with the sperm tail. At 15-19 h, the pronuclei migrate to a close apposition, and the apposing regions of the nuclear envelopes display pronounced undulation. Likewise, heterochromatin is formed in the apposed regions of the pronuclei. At around 20 h, the nuclear envelopes have dissolved and the prophase chromosomes are localized in a spherical electron-dense region of the ooplasm surrounded by conspicuous Golgi complexes. At around 24 h, cleavage to the 2-cell stage occurs and prominent Golgi complexes are found at the cytoplasmic bridge containing the midbody.

Figure 29: Scanning electron micrograph of bovine oocyte at 1 h after insemination *in vitro* showing cumulus cells (CC) as well as the free area of the zona pellucida (ZP). Note the numerous spermatozoa (arrowheads) attached to the zona pellucida.
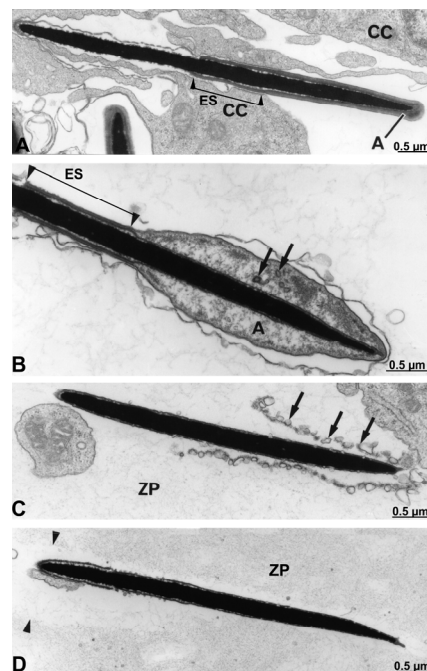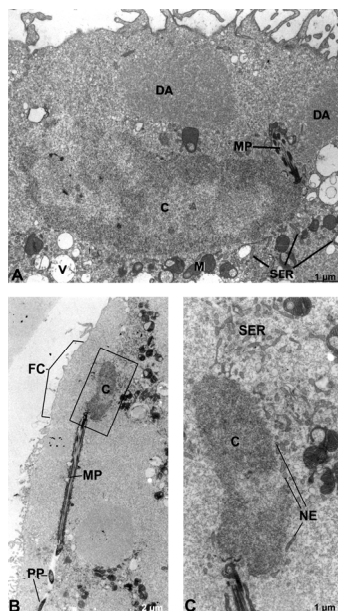

Figure 30: Electron micrographs of the acrosome reaction at *in vitro* fertilization of bovine oocytes. (**A**) Detail showing cumulus cells (CC) and a sperm head with an intact acrosome (A) located in the anterior portion. Note the equatorial segment (ES) of the sperm head. (**B**) Detail of sperm head in which the acrosome is swollen and small vesicles (arrows) have appeared in it. Note that the equatorial segment (ES) is not included in the acrosomal swelling. (**C**) Sperm head located at the surface of the zona pellucida (ZP). Note the hybrid vesicles (arrows) formed between the plasma membrane and the outer acrosomal membrane. (**D**) Sperm head in the process of penetrating the zona pellucida (ZP). The arrowheads indicate the track produced by the sperm head in the zona pellucida. Note that the hybrid vesicles have been detached from the sperm head, the anterior of which is now covered by the inner acrosomal membrane. From Hyttel *et al.* [27].

Upon penetration of the zona pellucida, the oocyte microvilli contact the equatorial segment of the sperm head where fusion between the two gametes, i.e. syngami, initially occurs. Subsequently, and probably within the first 2-3 hours after ovulation, the content of the spermatozoon including the nucleus, the proximal centriole as well as the distal centriole, prolonged into the axoneme of the sperm tail, and its surrounding coarse fibers, are expelled into the ooplasm along with the sperm plasma membrane being incorporated into the mosaic plasma membrane of the zygote. Along with the initial phases of this process, the content of the cortical granules are released into the perivitelline space by exocytosis, establishing the block against polyspermic fertilization (Fig. **31**). After this event, only few solitary cortical granules are scattered in the ooplasm. Also, within the initial 2-3 hours after fertilization, the paternal chromatin is denuded from its membrane coverings and this event is followed by decondensation of the chromatin (Fig. **32**). At the same time, the maternal chromatin is advancing through ana- and telophase II (Fig. **31**).

Spherical organelle-free areas, where the cytoplasmic matrix displays increased electron-density, are found adjacent to the decondensing sperm head (Fig. **32**). At this early stage of development, pronucleus formation is initiated as SER moves towards both the paternal and maternal chromatin where it starts to form small portions of tentative nuclear envelope (Figs. **31** and **32**). Apparently, within an hour or so, the two sets of chromatin are completely
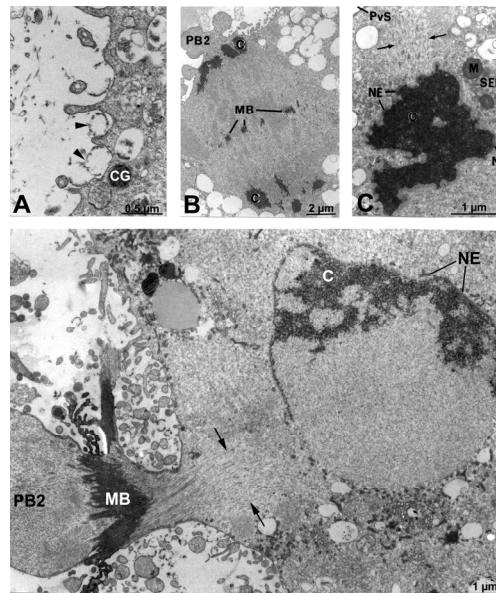

Figure 31: Electron micrographs of cortical granule release and formation of the maternal pronucleus in bovine zygotes. (**A**) Detail from the cortex of zygote showing the release (arrowheads) of cortical granules (CG) by exocytosis. (**B**) Detail from zygote showing the chromosomes (C) in anaphase II with the midbody (MB) being visible on the continuous microtubules. Note the initial protrusion of the second polar body (PB2). (**C**) Detail from zygote in telophase II showing the condensed maternal chromatin block (C) surrounded by mitochondria (M) and smooth endoplasmic reticulum (SER) in the periphery of the zygote. Note the microtubules (arrows) stretching towards the site of polar body extrusion and the small portions of nuclear envelope (NE) forming around the chromatin. PvS: Perivitelline space. (**D**) Detail from zygote at late telophase II showing the decondensing maternal chromatin (C) surrounded by an almost complete nuclear envelope (NE). Note the microtubules (arrows) stretching towards the midbody (MB) and the second polar body (PB2). From Hyttel *et al.* [23].

Figure 32: Electron micrographs of the formation of the paternal pronucleus in bovine zygotes (see Fig. 33 for continuation). (**A**) Detail of zygote showing decondensing sperm chromatin (C) with its adjacent midpiece of the sperm tail (MP) located in the

cortical ooplasm. Note that the sperm chromatin is surrounded by mitochondria (M), vesicles (V), smooth endoplasmic reticulum (SER) and dense areas (DA), the latter potentially representing the condensed nucleoplasmic matrix of the oocyte. (**B**) Detail from zygote showing the principal piece (PP) and mid piece (MP) of the sperm tail, the decondensing sperm chromatin (C) with an overlying fertilization cone (FC) protruding into the perivitelline space. (**C**) Boxed area from "B" showing the decondensing sperm chromatin (C). Note how smooth endoplasmic reticulum (SER) migrates towards the sperm chromatin and forms portions of nuclear envelope (NE). From Hyttel *et al.* [23].

surrounded by nuclear envelopes establishing two small pronuclei (Figs. **31** and **33**). Along with this process, the abstriction of the second polar body occurs accompanied by the gradual degradation of the first polar body. The midpiece of the sperm tail is still spatially associated with the paternal pronucleus. Apart from the importance of the sperm chromatin, the role of the proximal centriole of the spermatozoon should also be emphasized. The centriole is a prominent component of the cell centrosome and acts as a microtubule enucleation centre. Interestingly, the oocytes of the large domestic species lack centrioles, and the centrioles forming the microtubule asters of the first and succeeding mitoses arise from doublings of the proximal centriole of the fertilizing spermatozoon [28]. During these initial steps of fertilization, well-developed Golgi complexes re-appear in the ooplasm. Subsequently, the pronuclei swell to their characteristic spherical appearance accompanied by completion of chromatin decondensation, and about 10 hours post ovulation most zygotes exhibit spherical pronuclei (Fig. **33**; [29]). Along with this process, so-called nucleolus precursor bodies, which later act as enucleation sites for nucleolus formation (see later), are formed in the pronuclei [30]. The precursor bodies, which consist of tightly packed fibrils, are about 1 µm in diameter and they share ultrastructural similarities with the nucleolar remnants of the fully grown oocyte. With the progression of the cell cycle, they may develop a central vacuole. The precursor bodies are not active in rRNA transcription and ribosome formation. Another feature, accompanying pronucleus swelling, is the synthesis of numerous arrays of parallel cytoplasmic annulate lamellae connected to the SER. The function of these lamellae is not clear, but it has been hypothesized that they are implicated in the regulation of gene expression in cells having an extended interval between transcription and translation [31]. This context certainly applies to the zygote and early embryo, where mRNAs transcribed during oocyte development are translated with a remarkable delay during initial embryonic development.
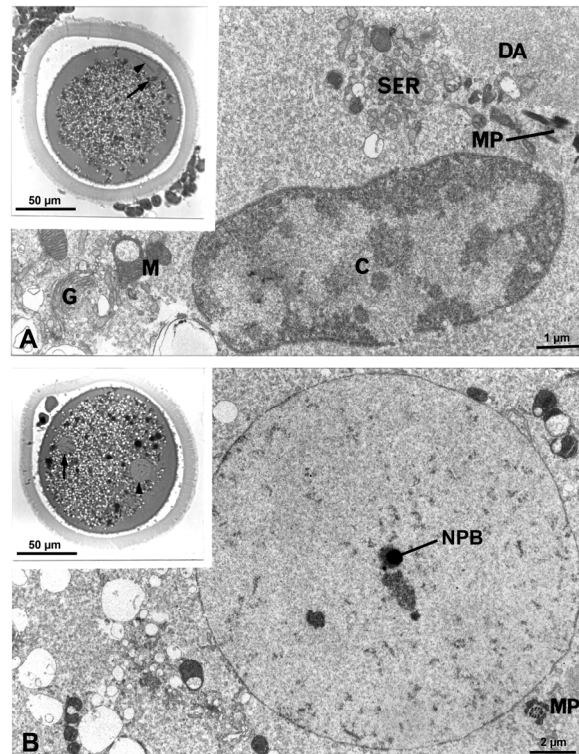
Figure 33: Electron micrographs of the formation of the paternal pronucleus in bovine zygotes. (**A**) Detail of zygote showing a paternal pronucleus where the sperm chromatin (C) is surrounded by a complete nuclear envelope. Note the midpiece (MP) of the sperm tail, a cluster of smooth endoplasmic reticulum (SER), mitochondria (M), a Golgi complex (G) and dense areas (DA), the

latter potentially representing the condensed nucleoplasmic matrix of the oocyte, adjacent to the pronucleus. Insert: Light micrograph showing a developing paternal pronucleus (arrow) adjacent to the sperm tail (arrowhead). (**B**) Detail showing a fully developed paternal pronucleus after swelling. Note the nucleolus precursor body (NPB) in the pronucleus and the midpiece (MP) of the sperm tail adjacent to the pronucleus. Insert: Light micrograph of the same oocyte showing the maternal (arrow) and paternal (arrowhead) pronucleus located at some distance in the zygote. From Hyttel *et al.* [23].

The two pronuclei migrate to a close apposition slightly off centre in the zygote, and about 14 hours after ovulation most zygotes exhibit apposed pronuclei (Fig. **34**). In cattle, the DNA-synthetic phase (the S-phase) of the first post-fertilization cell cycle is initiated at about 12 hours after ovulation and terminated at about 19 hours [29]. Along with this process, the organelles, vesicles and lipid droplets tend to accumulate centrally in the ooplasm around the pronuclei, leaving a rather organelle free cortical zone. In conjunction with pronuclear apposition, pronounced undulations of the nuclear envelopes of the pronuclei are seen in the apposed regions, probably preparing for breakdown of the envelopes which is seen at about 24 hours after ovulation. This process is often referred to as synkaryosis, but it should be emphasized that the two pronuclei do not fuse, but undergo dissolution of the nuclear envelopes similar to the one seen at the breakdown of the oocyte nucleus at resumption of oocyte meiosis. Chromatin condensation and dissolution of the nucleolus precursor bodies occur in the pronuclei already before synkaryosis, preparing for the first mitosis, and following synkaryosis the assembled maternal and paternal chromosomes are found in an organelle-free area displaying increased electron-density surrounded by numerous Golgi complexes. Immediately after synkaryosis, karyokinesis and cytokinesis proceed resulting in the formation of two daughter nuclei enclosed in each their blastomere.
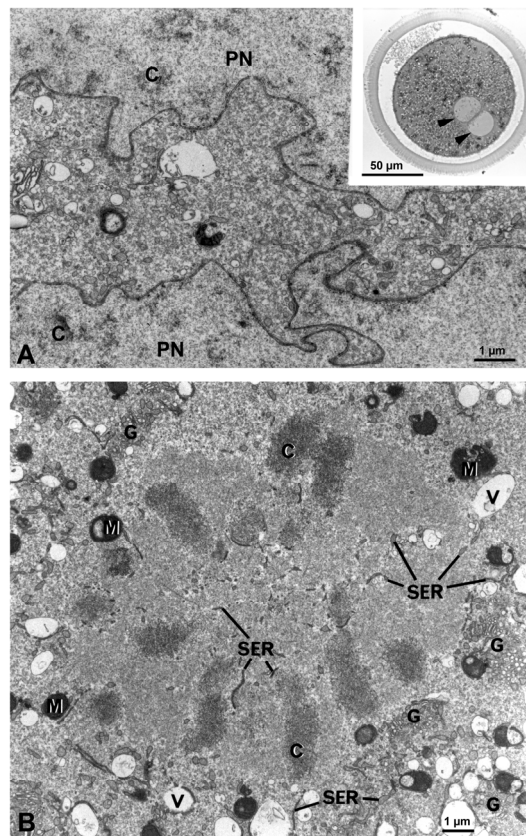
Figure 34: Electron micrographs of pronucleus apposition and synkaryosis in bovine zygotes. (**A**) Detail from zygote showing the apposed region of the two pronuclei (PN). Note the pronounced undulation of the nuclear envelope and the increasing amounts of heterochromatin (C). Insert: Light micrograph of the same zygote showing the apposed pronuclei (arrowheads). (**B**) Detail from zygote showing the chromosomes (C) arranged in the metaphase of the first mitotic division. Note that the chromosomes are localized in a particularly dense area of the cytoplasm presumably representing the condensed nucleoplasmic matrix from the pronuclei and, in turn, from the oocyte nucleus. This area is surrounded by numerous mitochondria (M), vesicles (V) and Golgi complexes (G), but the area itself is free of organelles except for smooth endoplasmic reticulum (SER). From Hyttel *et al.* [23].

Pre-Hatching Embryonic Development in Cattle

Morphologically, the first cleavages consist of repartitions of the cytoplasm of the zygote into smaller and smaller compartments without changing its overall volume [32]. Soon after the first cleavage, a certain degree of developmental asynchrony is often seen between the individual blastomeres of the embryo. Thus, 3-cell embryos are not rare. Due to this phenomenon, the ultrastructural features of a blastomere are most correctly referred to the context of the number of the post-fertilization cell cycle that specific blastomeres have reached. Hence in the following, the 2nd cell cycle refers to the 2-cell stage, the 3rd to the tentative 4-cell stage, the 4th to the tentative 8-cell stage, and the 5th to the tentative 16-cell stage. Thereafter, the terms morula, compacted morula, early blastocyst, expanded blastocyst and hatched blastocyst apply.

Along with the initial cleavages the embryonic genome is gradually activated during the so-called maternal-embryonic transition (Fig. **2**). Thus, a low rate of transcription of the embryonic genome has been detected as early as during the 1st, i.e. the zygote [33], and 2nd post-fertilization cell cycles [34-35], and during the 4th cell cycle a major transcriptional activation occurs [36]. Correspondingly, the duration of this cell cycle is about 21-30 hours, which is considerably longer than both the former and the subsequent ones [37]. The activation of the embryonic genome has a profound effect on embryo physiology and it is, therefore, not surprising that this process is reflected by dramatic changes in the ultrastructural appearance of the embryo as well.

The ultrastructure of the bovine embryo developed both *in vivo* and *in vitro* has been chronologically described in detail by Plante and King [38]. Also a number of other researchers have contributed to the understanding of the general embryonic ultrastructure based on either *in vivo* or *in vitro* developed embryos [39-40, 36, 41-47]. These data are summarized in Table **2**.

The general ultrastructure of the embryo during the 2nd cell cycle, i.e. the 2-cell stage, basically remains rather unchanged as compared with the first cycle. The blastomeres are organized with the nucleus in the centre surrounded by most organelles, vesicles and lipid droplets leaving a rather organelle-free cortical zone. The organelles include mitochondria, with the hooded form still predominating, well-developed Golgi complexes with dilated cisternae, cytoplasmic annulate lamellae and SER, the latter of which associates with mitochondria, vesicles and lipid droplets. Early during the second cell cycle, nucleolus precursor bodies resembling those described for the pronuclei are established in the nuclei (Fig. **35**). As during the first cell cycle, they undergo extended vacuolization with the progression of the cell cycle, but they are never transformed to ribosome-synthesizing fibrillo-granular nucleoli [34]. Thus, the protein synthesis of the embryo must be based on the ribosome pool inherited from the oocyte, and polyribosomes are still abundant during the 2nd cell cycle. The nuclei also present small clumps of heterochromatin, which tend to be located in the periphery of the nucleus, as well as nuclear annulate lamellae, the significance of which are unknown. The nucleolus precursor bodies are finally dissolved in conjunction with mitosis.

Apart from the reduced blastomere volume, the general ultrastructure of the embryo during the 3rd cell cycle, i.e. the tentative 4-cell stage, to a great extent resembles that of the previous cycle. Early during the cell cycle, nucleolus precursor bodies resembling those from the previous cell cycles are established. Again, the precursor bodies undergo vacuolization with the progression of the cell cycle, but fibrillo-granular nucleoli are, in general, not formed. As a consequence of the lacking ribosome-synthesis, the amount of polyribosomes decrease as do the total protein synthesis of the embryo [48]. Also, the number of microvilli on the blastomere surface decreases slightly.

During the 4th cell cycle, i.e. the tentative 8-cell stage, which in cattle is considerable longer than the previous and subsequent cycles, the first ultrastructural signs of intercellular connection between the blastomeres develop as short contact areas between the apposed plasma membranes. With the extension of these areas the involved membranes develop an electron-dense coating which is interpreted as a feature preparing for establishment of true intercellular junctions [38]. Early during the cell cycle, nucleolus precursor bodies resembling those from the previous cell cycles are re-established. Again, the precursor bodies undergo vacuolization with the progression of the cell cycle, and during the second half of the cycle, which is considerably longer than during the previous ones, the precursors develop into fibrillo-granular nucleoli according to the following mode of action: First an eccentrical primary vacuole and later several peripheral secondary vacuoles form (Fig. **35**). Subsequently, tiny fibrillar centers

surrounded by a dense fibrillar component develop in the peripheral zone of the precursor bodies. The centers are closely associated with heterochromatin. Presumably, this chromatin arrangement allows for the rRNA-genes to be localized in the centers as a prerequisite for rRNA-gene transcription. Additionally, portions of dense fibrillar component form at the inner lining of the large primary vacuoles. The dense fibrillar component and the fibrillar centers expand and a presumptive granular component, consisting of pre-ribosomal particles, emerges and occupies the remaining portion of the precursor body (Fig. **35**). Thus, through this process a spherical fibrillo-granular nucleolus emerges. Concomitant with the first observation of fibrillar centers towards the end of the 4th cell cycle, it is, for the first time in the embryo, possible to localize RNA polymerase I and other important nucleolar enzymes of significance for rRNA-gene transcription to the fibrillar centers by means of immunocytochemistry and confocal laser scanning microscopy [46]. The development of the nucleolus, allowing for ribosome synthesis, is a prerequisite for continued embryonic development. Thus, the amount of polyribosomes decreases to a minimum during the 4th cell cycle as does the level of embryonic protein synthesis [48]. At mitosis the nucleoli are dissolved, as are the precursor bodies during the previous cell cycles. Along with the activation of the rRNA-genes, sets of other genes resulting in transcription of different mRNAs are activated during the 4th cell cycle.
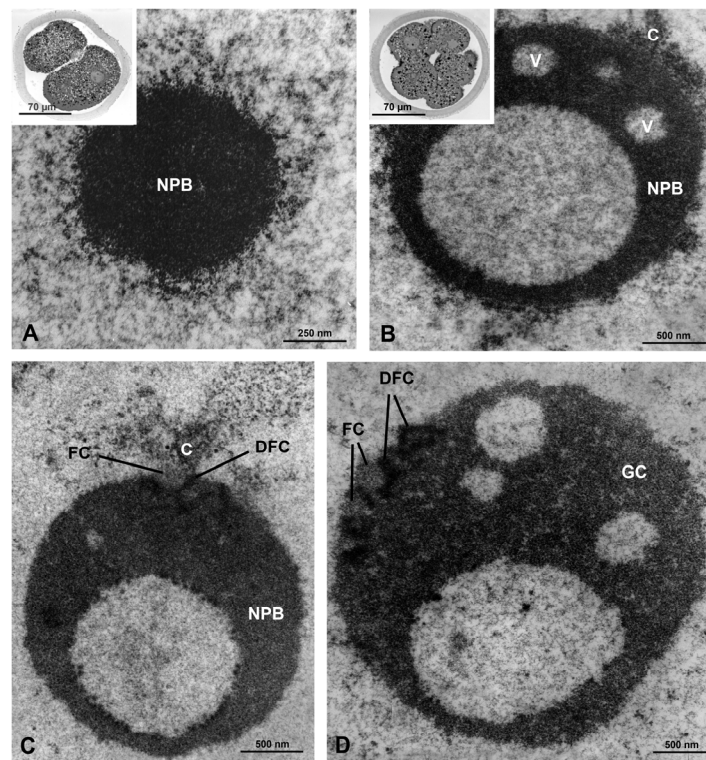
Figure 35: The transformation of the nucleolus precursor body into a functional nucleolus in bovine embryos. (**A**) Detail from 2-cell embryo showing a nucleolus precursor body (NPB). Insert: Light micrograph of 2-cell embryo. (**B**) Detail from 8-cell embryo showing a nucleolus precursor body (NPB) displaying a large central and several smaller peripheral vacuoles (V). Note the chromatin (C) associated with the NPB. Insert: Light micrograph of 8-cell embryo. (**C**) Detail from 8-cell embryo showing a vacuolated nucleolus precursor body during initial nucleolar transformation. Note the development of a fibrillar center (FC) peripherally in the NPB. The FC is surrounded by dense fibrillar component (DFC) which is also seen in the large central vacuole. Chromatin (C) is connected to the FC. (**D**) Detail from 8-cell embryo showing a young fibrillo-granular nucleolus with fibrillar centers (FC), dense fibrillar component (DFC) and granular component (GC).

During the 5th cell cycle, i.e. the tentative 16-cell stage or early morula, tight junctions represented by electron-dense areas at the latero-apical regions of neighboring external blastomeres become discernable for the first time preparing for the sealing of the subsequent trophectoderm. The density of microvilli on the blastomere surfaces has decreased and the Golgi complexes present less dilated cisternae. In the nuclei, spherical fibrillo-granular nucleoli develop already from the onset of the cell cycle. Consequently, the amount of polyribosomes increases as does the total embryonic proteins synthesis [48]. The nuclei display more clumps of scattered heterochromatin.

Initially, the individual blastomeres of the morula are clearly discernable as individual entities protruding from the embryo surface, but along with the process of compaction, the profiles of the individual blastomeres are lost and the surface of the embryo levels out. With this development, two cell populations are established: External cells that, in general, appear to have a lower electron-density of the cytoplasmic matrix, and internal cells that display a higher electron-density of the matrix. The external cells are connected by developing tight junctions while the internal cells are only connected by focal membrane contacts with electron-dense coating as described for the 4th cell cycle. The ultrastructural appearance of the mitochondria changes markedly at this stage of development. Thus, mitochrondria of the hooded form, which were established back during the development of the oocyte in the early tertiary follicle, become fewer, and elongated types with transverse cristae become more numerous. Moreover, the mitochondrial matrix appears less electron-dense; a change that apparently occurs gradually from the 2nd cell cycle and onwards. RER starts to develop again for the first time since the termination of the oocyte growth phase, signaling the synthesis of proteins for export out of the cells. Phagosomes and lysosomes become a regular finding in the cells. The action of these compartments may be related to the elimination of cells undergoing apoptosis; a phenomenon that is considered as being a normal feature of initial bovine embryonic development from the morula stage and onwards [49-50].

In the blastocyst, the fully developed trophectoderm surrounds the blastocyst cavity and the inner cell mass. Neighboring trophectoderm cells are connected by well developed tight junctions and desmosomes. In the cytoplasm, the internal face of the desmosomes is connected to a prominent meshwork of intermediate filaments. The cells of the inner cell mass remain connected with the focal electron-dense contacts as described earlier. The amount of microvilli on the surface of the trophectoderm cells increases again. Elongated mitochondria with transverse cristae and electron-lucent matrix are predominating and with the expansion of the blastocyst, the amount of lipid droplets decrease indicating that this compartment presumably constitutes an energy reserve consumed during initial embryonic development. The RER becomes more abundant whereas the SER decreases in amount.

OOCYTE, ZYGOTE AND PRE-HATCHING EMBRYONIC ULTRASTRUCTURE IN THE PIG

The following section will address oocyte maturation, fertilization and pre-hatching embryonic development in the pig. Due to the fact that the pig represents an alternative biological model of development than cattle with respect to the nucleolar re-activation during the major embryonic genome activation, the initial pre-hatching embryonic development is included in this species.

Oocyte Maturation in the Pig

The ultrastructure of porcine oocyte maturation has been analyzed at a detailed qualitative and quantitative level by Cran [51]. The immature porcine oocyte shares many features with its bovine counterpart. It is characterized by a peripherally located oocyte nucleus and an abundance of vesicles, lipid droplets and mitochrondria, all of which display an intimate relationship with the SER. The mitochondria, which present only few cristae, are concentrated in the cortical region and hooded forms, as seen in cattle, are very infrequent. Also in the cortical ooplasm, Golgi complexes are residing. Cortical granules are, in the immature oocyte as well at all later stages of maturation, confined to the cortical 4 μm of the ooplasm. They are never seen clustered as in the immature bovine oocyte. Ribosomes are evenly distributed and form free polyribosomes as well as, much more infrequently, polyribosomes attached to the RER.

The breakdown of the oocyte nucleus occurs more than 20 hours after initiation of maturation. Along with this process, the gap junctional communication between the oocyte and the cumulus cell projections is lost. During the initial maturation, when the oocyte nucleus is intact, the number of mitochondria is constant, but after the nucleus breakdown, the mitochrondrial number decreases, whereas the mitochondrial volume remains constant probably due to fusion of mitochondria. Moreover, during the same period of time, the mitochrondria migrate from the periphery towards the center of the ooplasm. Finally, a rather organelle-free zone is established peripherally in the ooplasm.

Fertilization and Development of the Zygote in the Pig

In the pig, the oviduct transport is more rapid, and the embryos reach the uterus already about 2 days after ovulation. The ultrastructure of fertilization is described in detail by Szollosi and Hunter [52] using a model where a low

degree of polyspermia is induced *in vivo*. Additional information is provided by Norberg [53], and we have extended this knowledge by an analysis of pronucleus formation in relation to the progression of the 1st cell cycle [54].

The oocyte apparently sheds the cumulus cells more or less during ovulation, and tubal oocytes only present few attached cells with their projections embedded in the zona pellucida. Some spermatozoa close to the zona pellucida may not have undergone the acrosome reaction whereas all spermatozoa observed in the substance of the zona have completed this process. Soon after penetration of the oocyte, the maternal chromatin forms a compact mass around which portions of nuclear envelope are established. Nuclear pores appear in those portions of the envelope that are closest to the chromatin. The development of intra-nuclear annulate lamellae is a common finding. The chromatin of the second polar body is, likewise, arranged in a compact mass around which fragmentary formation of nuclear envelope may be seen.

The paternal pronucleus formation is an early phenomenon, and the nuclear envelope starts forming in conjunction with the earliest stages of chromatin decondensation. Again, the development of nuclear pores and intra-nuclear annulate lamellae is a consistent finding. Another characteristic is the formation of lateral projections of the forming pronucleus. It is believed that these projections contain thin sheets of chromatin. The mitochrondria of the mid-piece of the fertilizing spermatozoon display swelling and degeneration. Swelling of the pronuclei to a spherical appearance is completed within 10 hours after ovulation and migration of the pronuclei to a close apposition within 20 hours. The S-phase of the first cell cycle is initiated at approximately 14 hours after ovulation and completed at about 20 hours [54]. Abundant cisternae of SER, prominent Golgi complexes, abundant vesicles containing flocculent material, and arrays of parallel cytoplasmic annulate lamellae are located centrally in the ooplasm around the pronuclei, and this complex of tightly packed elements is, in turn, surrounded by a zone rich in numerous large lipid droplets still leaving the most peripheral zone of the ooplasm rather organelle-free. The lipid content is higher than in cattle zygotes. Mitochondria are found scattered all around in the ooplasm and are mostly spherical or ovoid presenting only few peripheral cristae. The vesicles and to some degree the lipid droplets are surrounded by profiles of SER.

The pronuclei contain large nucleolus precursor bodies of up to about 4 μm in diameter, i.e., about 4 times as large as in cattle, appearing as spherical masses of tightly packed fibrils. With the apposition of the pronuclei, the precursors become localized together with condensed chromatin in the apposed regions of the pronuclei, where marked undulations of the nuclear envelope develop. Vacuolization of the nucleolus precursor bodies is not observed.

Pre-Hatching Embryonic Development in the Pig

In the pig, the major activation of the embryonic genome takes place already during the 3rd cell cycle [55-56]. Accordingly, this cell cycle is of a duration of about 50 hours, which is considerably longer than the previous and subsequent ones [57]. There is no knowledge of whether the major transcriptional activation is preceded by a minor activation as demonstrated in cattle. The general ultrastructure of the pre-hatching porcine embryo has been described by Norberg [58], Szöllösi and Hunter [52], Barends *et al.* [58], Stroband *et al.* [59], Stroband and van der Lende [60], and Antalíková *et al.* [61]. We have extended this knowledge by a chronological ultrastructural investigation of embryonic development *in vivo* and *in vitro* [62]. Furthermore, we have conducted a detailed chronological investigation on nuclear ultrastructure in *in vivo* developed embryos with special emphasis on elucidating the activation of the embryonic genome as visualized by the rRNA-gene activation and the resulting nucleolus formation [56]. The results are summarized in Fig. **3**. Certain ultrastructural data on the nucleolus formation were also previously available [63, 55, 54].

During the 2nd cell cycle, i.e., the 2-cell stage, the blastomeres maintain the same overall organization as observed for the zygote. The nucleus is located in the centre surrounded by a zone rich in tightly packed SER, Golgi complexes, vesicles and cytoplasmic annulate lamellae which, in turn, is surrounded by a zone occupied by abundant large lipid droplets. Mitochondria, still of the spherical or ovoid type, are distributed throughout the cytoplasm and are often located adjacent to lipid droplets. In the nuclei, large nucleolus precursor bodies are seen, but again no vacuolization occurs with the progression of the cell cycle.

At the onset of the 3rd cell cycle, i.e. the tentative 4-cell stage, the blastomeres are round and present the same internal organization as during the previuous cycles. Later during the cycle, however, they assume a more

polyhedral outline leading to increased appositions of their plasma membranes resulting in a decrease in the intercellular spaces. This phenomenon is regarded as the first phase of compaction [64]. Early during the 3rd cell cycle, nucleolus precursor bodies, resembling those from the previous cell cycles, are established again. However, from around 20 hours post cleavage to the 3rd cell cycle, the nuclei of the blastomeres display different stages of nucleolus formation, ranging from the precursor bodies to fibrillo-granular nucleoli presenting fibrillar centers, a dense fibrillar component and a granular component. Different stages of nucleolus development are observed even within the same nucleus. Apparently, nucleolus formation is initiated by the formation of the dense fibrillar component and the granular component and later the fibrillar centers on the surface of, but not in, the nucleolus precursor body (Fig. **3**). Throughout this process, semilunar fibrillo-granular nucleoli more or less encapsulate precursor bodies. Soon after the observation of fibrillar centers it is, for the first time in the porcine embryo, possible to localize at least RNA polymerase I to the fibrillar centers by means of immunocytochemistry and confocal laser scanning microscopy [56].

During the 4th cell cycle, i.e., the tentative 8-cell stage, the next phase of compaction occurs. The blastomeres tend to become polarized. The nucleus is located towards one pole adjacent to a zone rich in organelles and vesicles with a rather organelle-free zone rich in large lipid droplets towards the other pole. Moreover, the apposition of the plasma membranes of the blastomeres develops to such a degree that the intercellular spaces are no longer visible by light microscopy. Fibrillo-granular nucleoli, as described for the previous cycle, are already formed at the onset of this cell cycle. Again, the fibrillo-granular nucleoli and inactive nucleolus precursor bodies can be seen within the same nuclei.

Already during the 5th cell cycle, i.e. the tentative 16-cell or early morula stage, a peripheral layer of trophectoderm cells and a group of internal cells becomes more or less obvious. The outer cells may still posses the polarized appearance. The mitochondria are more filamentous and their inner membranes form more cristae and tubules. In the morula the outer cells have a bean-like shape and they are connected by tight junctions, thus, forming a seal to create an internal environment for the inner cells.

In the early blastocyst, the blastocyst cavity is small and the mural trophectoderm only consists of one or two cells. The inner cell mass still has a compacted organization. At the internal face of the inner cell mass and the trophectoderm cells, cytoplasmic extensions, rich in microfilaments, free ribosomes, and connected to each others by desmosomes, are formed. The flattened trophectoderm cells are covered with microvilli towards the zona pellucida and the vesicles, mitochondria and SER assume a polarized localization in the cytoplasm. These cells are connected by well developed tight junctions and desmosomes. The organelle rich cytoplasm is gradually replaced by cytoplasm devoid of SER and Golgi complexes but rich in swollen RER and free ribosomes. These cytoplasmic changes occur finally in all cells, although some of the internal cells preserve their organelle rich cytoplasm for en extended period.

OOCYTE AND ZYGOTE ULTRASTRUCTURE IN THE HORSE

In many aspects, the horse displays unique features with respect to reproductive biology. For example, unfertilized eggs are prohibited from entering the uterine cavity from the oviduct, the developing blastocyst forms a second protective layer, the capsule, underneath the zona pellucida, and the embryo remains spherical and unattached in the uterine cavity for an extended period of time. With respect to the ultrastructural aspects of oocyte maturation and fertilization, the horse resembles cattle except for a pronounced polarization of the ooplasm with respect to distribution of organelles and inclusions. The ultrastructure of oocyte maturation, fertilization and pre-hatching embryonic development has been addressed by Betteridge [65] and Groendahl *et al.* [66, 5].

Oocyte Maturation in the Horse Zygote in the Fox

In follicles less than about 20 mm in diameter, which have presumably not yet been stimulated by the LH-surge, the equine oocyte presents a centrally located spherical nucleus. In follicles between 20 and 37 mm in diameter, when the LH-surge has presumably been initiated, the oocyte nucleus has migrated to a peripheral position in the ooplasm retaining its spherical shape. In follicles between 38 and 40 mm, the oocyte nucleus first flattens and subsequently breaks down, and metaphase I is established, and in follicles from 37 to 47 mm, metaphase II oocytes can be retrieved.

The ultrastructure of oocyte meiosis is comparable with that described for cattle. Cortical granules are, however, never seen clustered as in the immature bovine oocyte. In conjunction with the peripheral migration of the oocyte nucleus, the ooplasm become very polarized with the mitochondria preferentially occupying a semilunar position in one half of the ooplasm and the lipid droplets being localized to the other half. Around the time of oocyte nucleus breakdown, the cumulus cell projections are retracted into the perivitelline space and the junctional contact between these cells and the oocyte broken. In conjunction with the oocyte nucleus breakdown and establishment of metaphase I, the number of cortical granules found along the oolemma increases, and this feature becomes even more pronounced at metaphase II.

### Fertilization and Development of the Zygote in the Horse

The equine zygote is characterized by a pronounced polarization inherited from the oocyte. Thus, more or less one half of the ooplasm is occupied by mitochrondria while the other half is occupied by large lipid droplets. The pronuclei present nucleolus precursor bodies of similar ultrastructure and diameter as described for cattle. No vacuolization of the precursor bodies is seen at any stage of development. The ultrastructural events associated with cortical granule release and pronucleus development are comparable with that described for cattle.

OOCYTE AND ZYGOTE ULTRASTRUCTURE IN THE FOX

The fox, and with it also the dog, is completely unique with respect to oocyte maturation and fertilization. The majority of oocyte maturation occurs after ovulation in the oviduct which potentially allows for fertilization to occur well before the oocyte has reached metaphase II. The processes of oocyte maturation, fertilization and pre-hatching embryonic development have been ultrastructurally addressed by Hyttel *et al.* [6] and Farstad *et al.* [67].

Oocyte Maturation in the Fox

Before the LH-peak, the oocyte is characterized by a centrally located oocyte nucleus with an active fibrillo-granular nucleolus (Fig. **36**). One to two days after the LH-peak, the nucleus has migrated to a peripheral position in the ooplasm and has assumed a flattened shape, and the nucleolus developed into a non-ribosome synthesizing remnant. At this stage of development ovulation occurs, and further oocyte maturation takes place in the oviduct where metaphase I is reached at 2 to 3 days and the metaphase II at about 5 days after the LH-peak. Hence, meiotic maturation is a prolonged phenomenon in the fox.
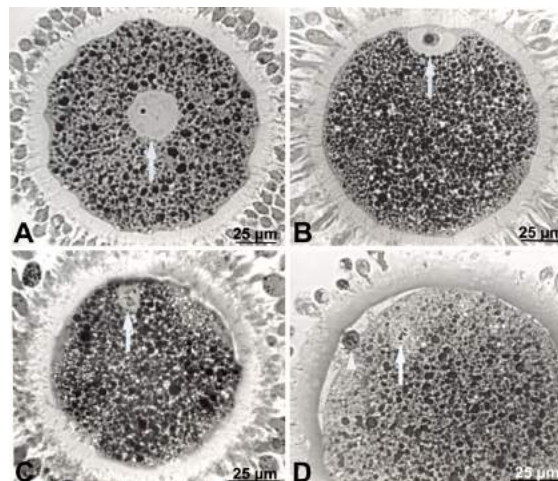

Figure 36: Light micrographs of fox oocytes at different stages of meiosis. (**A**) Oocyte at the prophase I with a centrally located spherical oocyte nucleus (arrow). (**B**) Oocyte at the prophase I with a peripherally located flattened oocyte nucleus (arrow). (**C**) Oocyte with a nucleus in the process of breakdown (arrow). (**D**) Oocyte at metaphase II with metaphase plate (arrow) and first polar body (arrowhead). From Hyttel *et al.* [6].

Fox oocytes are extremely rich in lipid droplets distributed more or less evenly throughout the ooplasm. In the immature oocytes, mitochondria are localized in the cortical ooplasm together with well-developed Golgi

complexes, mitochrondria and cortical granules, and well-developed junctional coupling between the oocyte and the cumulus cells is seen (Fig. **37**). Cortical granules are never seen clustered as in the immature bovine oocyte. Interestingly, the oocyte microvilli extend into the zona pellucida and a perivitelline space hardly exists in the fully grown oocyte. In conjunction with the oocyte nucleus breakdown, the junctional communication between the oocyte and the cumulus cells is broken down; a phenomenon thus occurring in the oviduct.

Fertilization and Development of the Zygote in the fox

Due to the fact that the majority of oocyte maturation takes place in the oviduct in the fox, the oocyte may potentially be fertilized before it has reached metaphase II. Indeed, structures appearing like decondensing sperm heads, with a more or less complete nuclear envelope formed around them, has been observed in oocytes in which the nucleus has migrated to a peripheral location, i.e. during prophase I (see above). No clear relationship has been noted between the development of the paternal pronucleus and the progression of oocyte meiosis. Hence, apparently the development of the paternal pronucleus is arrested until its maternal counterpart reaches a certain stage of development, and finally the typical morphology presenting two spherical pronuclei is reached. The pronuclei contain nucleolus precursor bodies similar to those described for cattle. Along with the initial paternal pronucleus formation, while the oocyte still presents a peripheral nucleus or chromosomes at metaphase I, exocytosis of cortical granules can be seen. Interestingly, components of the sperm tail have not been observed in fox zygotes.
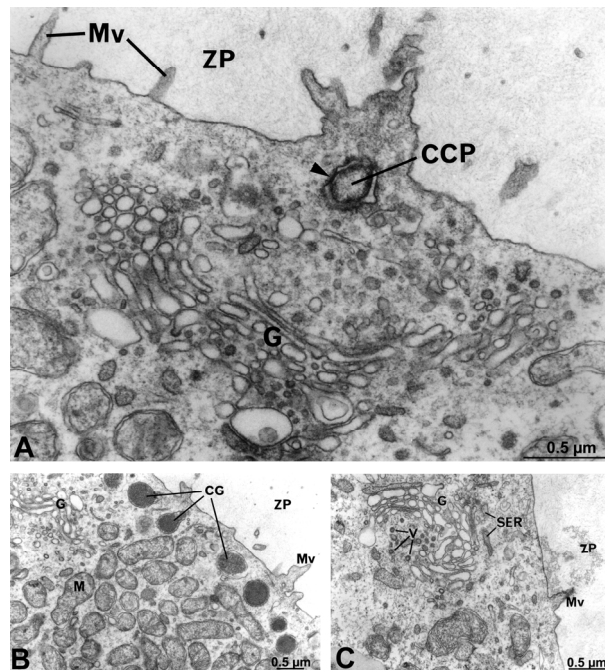
Figure 37: Electron micrographs of details from fox oocytes. (**A**) Detail from prophase I fox oocyte showing a cumulus cell projection (CCP) establishing a gap junction (arrowhead) to the oolemma. Note the well-developed Golgi complex (G) and the microvilli extending into the zona pellucida (ZP). (**B**) Detail from prophase I fox oocyte showing cortical granules (CG) along the oolemma adjacent to a Golgi complex (G) and a cluster of mitochondria (M). Note the microvilli extending into the zona pellucida (ZP). (**C**) Detail from prophase I fox oocyte showing a conspicuous Golgi complex (G) of which the immature face is associated with smooth endoplasmic reticulum (SER) and the mature face with small vesicles (V). Note the microvilli extending into the zona pellucida (ZP). From Hyttel *et al.* [6].

OOCYTE AND ZYGOTE ULTRASTRUCTURE IN THE MOUSE

In many terms, the mouse is the most well-studied model organism for mammalian biology. This also holds true with respect to oocyte maturation and fertilization. The ultrastructure of murine oocyte maturation and fertilization has been addressed in details by numerous authors including Yamada *et al.* [68], Odor and Blandeau [69], Zamboni [70-71], and Dvorak [72].

Oocyte Maturation in the Mouse

The chronological progression of meiosis in the mouse is rapid with the breakdown of the oocyte nucleus occurring within few hours [4]. At 9 hours after initiation of maturation, most oocytes have reached metaphase I, and at 15-17 hours metaphase II is predominant. Except for the chronology, the ultrastructure of meiosis is similar to what described for cattle.

The mouse oocyte is somewhat smaller than its counterparts in the larger domestic species and reaches a diameter of about 70 μm. The immature mouse oocyte contains many of the elements also found in oocytes in the larger domestic animal species, but it is much more translucent at bright field microscopy due to the presence of much smaller and fewer lipid droplets. The organelles appear to be uniformly distributed, and a well-developed RER, which is closely associated with groups of mitochondria, is seen. Moreover, numerous fibrillar arrays, each consisting of 7-15 parallel fibrils separated by a distance of about 150-200 nm, are present. It is believed that these fibrils derives from polyribosomes. At first ribosomes become arranged in a curvilinear pattern and then they fuse to give rise to a typical fibril. Golgi complexes are present in the cortical ooplasm where they are related to the synthesis of the numerous cortical granules. Cortical granules are never seen clustered as in the immature bovine oocyte. As in cattle, the junctional communication between the oocyte and the cumulus cell projections is lost in conjunction with the breakdown of the oocyte nucleus. The first polar body in the mouse is relatively large, as compared with the larger domestic animal species, and it contains the same organelles as the oocytes together with irregularly shaped chromosomes.

Along with the meiotic maturation, mouse oocytes present characteristic mitochondrial migrations [73]. As soon as maturation is initiated, mitochrondria form clusters in the cortical and perinuclear area, and in conjunction with the initial dissolution of the oocyte nucleus, the mitochondrial clusters migrate to exclusive perinuclear localization.

Fertilization and Development of the Zygote in the Mouse

As a consequence of the comparatively lesser lipid content, the murine zygote is transparent and the process of pronucleus formation is apparent at light microscopical visualization of zygotes in toto. The mouse zygote presents a well-developed fertilization cone. The nuclear envelope of the fertilizing spermatozoon displays swelling of the perinuclear cisternae and transforms into vesicles during the process of degradation. The ultrastructural events associated with cortical granule release and pronucleus development are comparable with those described for cattle. Interestingly, in the mouse it has been demonstrated that the oocyte contributes with a centrosome including microtubule organizing centers, whereas this component is paternally inherited in the other species addressed in this chapter. Large spherical nucleolus precursor bodies, comparable with those described for the pig, develop in the pronuclei.

OOCYTE AND ZYGOTE ULTRASTRUCTURE IN MAN

In spite of the difficulties in obtaining human oocytes and zygotes, unique ultrastructural studies have been published on aspects of oocyte maturation and fertilization in man by several authors including Van Blerkom and Motta [74], Sathananthan [75-76], Sundstrom and Nilsson [77] and Familiari *et al.* [78].

Oocyte Maturation in Man

The immature human oocyte is, as its murine counterpart, very translucent at bright field microscopy due to a complete absence of visible lipid droplets. The diameter of the immature human oocyte is about 100 μm, which is a bit less than in the large domestic species.

Except for a rather organelle-free cortical zone, the organelles are rather uniformly distributed in the ooplasm. A thin layer of cytoskeleton consisting of microfilaments is reported to underlie the oolemma, and internal to this, the cortical granules are located. Cortical granules are never seen clustered as in the immature bovine oocyte. In the more central ooplasm, mitochrondria, which are spherical or ovoid, are located in close association with SER. RER is very rare as are ribosomes. Again, the ultrastructure of meiosis is comparable to what has been described for cattle.

Fertilization in Man

As for the mouse, pronucleus development in man may be observed by light microscopy of zygotes *in toto*. The pronuclei contain large spherical nucleolus precursor bodies, comparable with those described for the pig, and the spatial alignment of the precursor bodies in the pronuclei may be used for prediction of zygote viability. The ultrastructural events associated with cortical granule release and pronucleus development are comparable with those described for cattle.

ACKNOWLEDGEMENTS

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Intra- and Intercellular Molecular Mechanisms in Regulation of Meiosis in Murid Rodents

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Abstract: Overviewed is the life history of murid female germ cells from their first appearance as primordial germ cells during embryogenesis, their migration, proliferation and colonization of the genital ridge, the prospective gonad; the differentiation of primordial germ cells into oocytes that embark on the protracted meiosis in the embryo; the arrest of the meiosis in the newborn; the resumption of meiosis in the sexually mature animal, and finally the release of fertilizable ovum at ovulation. Emphasized are recent advances in the molecular regulation of meiosis initiation in the embryo and its resumption in the mature follicles in response to the ovulatory stimulus by luteinizing hormone.

Advances in the understanding of the regulation of meiotic resumption are presented in their historical perspective and the development of appropriate experimental models. Within the context of somatic cell regulation of meiosis resumption, the apparent paradoxical involvement of 3'5' cyclic adenosine monophosphate in both inhibition and stimulation of meiosis are discussed; the role of somatic cell-oocyte gap junctions and the recently established obligatory role of follicular epidermal growth factor-like molecules in the mediation of the gonadotropic stimulation of meiosis and ovulation.

The involvement of steroids in mediating the gonadotropic stimulation of meiotic resumption in fishes and amphibians is well established. Nevertheless, the available data do not seem to support an obligatory role of steroids in meiotic resumption in mammals, and, particularly, not in murids. Apparently the evolution of hierarchical follicle growth and the consequent high intraovarian steroid levels resulted in the abandonment of steroids as a reliable signal for meiosis in mammals.

INTRODUCTION

Oocyte meiosis in mammals is an extended process, subject to several stop and go controls. The oocyte embarks on the first meiotic division during embryonic life or shortly after birth. This division has a protracted and uniquely complicated prophase. During this stage the chromosomes condense, pair and become linked at chiasmata, allowing exchange of paternal and maternal DNA. Meiosis is arrested, in most species, during neonatal life at the diplotene of the first meiotic prophase. In murid rodents, at this stage, referred to as diffuse diplotene or dictyate, the chromosomes decondense and become undetectable under the light microscope. The hallmark of the dictyate oocyte is the large nucleus known as "germinal vesicle" (GV). The oocyte persists in this GV stage throughout infancy and for variable periods beyond puberty. In sexually mature females, during each reproductive cycle a number of oocytes, characteristic of the species, re-enter meiosis as manifested by germinal vesicle breakdown (GVBD). These oocytes complete the first round of meiosis with extrusion of the first polar body (PBI) and without an intervening interphase progress to the second meiotic metaphase. Resumption of meiosis and its progression to the metaphase of the second meiotic division is usually referred to as "oocyte maturation". At this stage the meiotic process is arrested again (second meiotic arrest) and is completed, upon fertilization, by the extrusion of the second polar body (PBII).

Resumption of meiosis, like other ovulatory changes, such as luteinization, cumulus expansion and mucification, and finally rupture of follicle wall and release of a fertilizable ovum, is triggered by the preovulatory surge of gonadotropins. Pincus and Enzmann [1] were the first to report that copulation in rabbits (which stimulates gonadotropin release in this species) or administration of beef pituitary extract or "maturity hormone" cause resumption of meiosis. Later studies confirmed and extended these pioneering observations using purified gonadotropin preparations. Thus GVBD occurs 2-4h after hCG administration to mice [2], rats [3] and rabbits [4], whereas 18h and approximately 25h are required for reinitiation of meiosis in pigs [5] and in the human [6-7],

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respectively. While the provulatory surge of gonadotropins includes both LH and FSH, administration of an antibody to the β-chain of LH demonstrated that, at least in the rat, LH but not FSH, is the physiological trigger of oocyte maturation and ovulation [8].

As mentioned above, Pincus and Enzmann [1] have found that oocyte maturation in rabbit *in vivo* is dependent upon copulation or a pituitary hormone administration. By contrast, in the same study they observed that fully-grown oocytes, explanted from their follicles and *in vitro* cultured, matured whether the pituitary hormone was added or not. This phenomenon, later defined as "spontaneous oocyte maturation" made them assume that follicle cells keep meiosis on hold either indirectly by maintaining the oocyte "in a nutritional state wherein nuclear maturation is impossible" or directly by supplying a substance or substances which inhibit nuclear maturation. It took more than forty years to test the hypotheses of Pincus and Enzmann and another thirty five years to understand, at least in part, the regulatory pathways involved in keeping meiosis in abeyance prior the gonadotropic stimulation, the hormone-induced resumption of meiosis *in vivo* and the mechanisms responsible for spontaneous maturation of isolated oocytes *in vitro*. Here we shall overview the major advances in our understanding of follicular cells- oocyte interactions and molecular mediators keeping the meiotic process arrested or, alternatively, triggering its resumption.

THE LIFE HISTORY OF MAMMALIAN FEMALE GAMETES

The production of fertilizable ova, oogenesis, starts in mammals during embryonic development to be completed months or years later at each reproductive cycle of sexually mature females. The following description of oogenesis is based mainly on the mouse.

Primordial Germ Cells. Origin and Specification of Germ Cell Lineage

Everett [9] was the first to demonstrate the extragonadal origin of mouse germ cells. Transplanting of genital ridges of 10.5 to 14-day-old mouse embryos (10.5-14 E) beneath the kidney capsule of adult mice resulted in normal differentiation of the transplanted gonad. By contrast, transplantation of genital ridges of younger mice (9.5-10E) did not show germ cells and follicle formation. Later studies have shown that the ancestors of primordial germ cells (PGC) are derived from the proximal pluripotent epiblast (embryonic ectoderm) cells adjacent to the extraembryonic ectoderm. These cells, in addition to the germ cell line, contribute to several additional extraembryonic lineages, such as the allantois, blood islands, yolk sac mesoderm and the amnion. Using knockout models allowed the identification of members of the bone morhogenetic protein (BMP) family secreted from the extraembryonic ectoderm as inducers of PGC formation [10-13]. The pluripotent epiblast cells respond to BMP signaling by the expression of *Ifitm3* (interferon-induced transmembrane protein 3; *Fragilis*), which marks the competent cells to become PGC, even though only a small minority of them become germ cells [14]. On 6.25E about six of the *Iftm3* positive epiblast cells in contact with the extraembryonic ectoderm express the protein PRDM1 (PRDI-BF1-RIZ domain containing 1;BLIMP1) and become PGC precursors. PRDM1 is a key transcriptional regulator that apparently represses the expression of somatic genes and up-regulates PGC-specific genes [15]. A second gene that is essential for the specification of mouse germ-line is *PRDM14* [16]. Both genes regulate PGC-line specific expression of the pluripotency-associated protein SOX2 and are necessary for the establishment of the germ cell lineage in the mouse and presumably in other mammals.

PGC Migration and Proliferation

By 7.5E, when PGC specification has taken place there is a cluster of about 40 PGC in the base of the allantois that express PRDM1; they have strong alkaline phosphatase activity (AP) and express DPPA3 (developmental pluripotency-associated 3; STELLA). While AP and STELLA are markers of PGC, they are not necessary for PGC development. Nevertheless, the co-localization of PRDM1 and AP and the lineage analysis [15] confirms that AP expressing cells are PGC and gives credence to earlier studies on the migration of PGC by their PA activity [17-19]. The cluster of PGC begins to disintegrate and the PGC move through the primitive streak and invade the endoderm. By 8.5E the PGC are carried along with the invagination of the endoderm to form the hindgut. With the extension and closure of the hindgut into a tube by 9E, the PGC move around the cells of the hindgut epithelium, emerge from its dorsal side and migrate towards the genital ridges. At 10.5 PGC reach the genital ridge and by 11.5 most PGC have colonized it [20]. During their migration PGC undergo mitoses; their number increases from about 100 to

approximately 5,000 upon full colonization of the genital ridges. Thus they undergo 7-8 divisions during their four-day migration. In the rat and the mouse the PGC in the genital ridge are no longer locomotory, become rounded and are usually termed oogonia. They proliferate for two or three days reaching a peak of 75,000 in the rat embryo on day 18.5 [21-22].

The migration of PGC seems to be guided by PGC interactions with extra cellular matrix and chemoattraction. Thus expression of integrin β1/2 by PGC [23] and TGFβ signaling [24] were implicated in PGC extra cellular matrix interaction and guidance. Kit ligand is apparently a chemoattractant of PGC through the involvement of phosphatidylinositol 3-kinase (PI3K)/AKT and SRC kinase as downstream pathways in the PGC [25].

In vitro studies have implicated several growth factors and mediators in PGC proliferation. Such are the anti-apoptotic stem cell factor (SCF) and leukemia inhibitory factor (LIF) and stimulators of proliferation like adenosine 3'5'cyclic monophosphate (cAMP), pituitary adenylyl cyclase activating peptides (PACAPs) and retinoic acid (RA) [reviewed by 26].

Initiation of Meiosis

Initially the genital ridges, as well as the colonizing PGC, are bipotential or sexually undistinguishable. Thus, PGC, regardless of their genetic sex (XY or XX), are attracted to genital ridges of both genetic constitutions. Reviews of gonadal differentiation are available. The first one on their microscopic differentiation [27] and the other on molecular changes involved in the differentiation of the bipotential gonad [13].

In the genital ridge of female mouse embryos the germ cells, oogonia, enter the meiotic prophase asynchronously between 13.5E to 16.5E and proceed through leptotene, zygotene (15.5-16.5E) and pachytene (17.5E) stages. The meiotic process is arrested in the mouse and the rat at the diplotene stage around birth [reviewed by 28]. The meiotic process will be resumed normally, excluding follicles undergoing atresia or apoptosis of granulosa cells [29], shortly before ovulation. The meiotic prophase in mammalian oocytes is protracted, it may last for weeks, months or even years in some species.

The transformation of oogonia into oocytes, oocytogenesis, is the first visible demonstration of the sexual differentiation of the germ cells. Yet other, morphologically undetectable, changes occur in germ cells earlier. Among these are the epigenetic reprogramming of the inactivated X chromosome in migrating female PGC [reviewed by 30] and the mitotic arrest of XY germ cells on 12.5E. Mitoses of male germ cells are resumed only after birth.

Studies with XX-XY chimeras revealed that germ cells, being XX or XY, enter meiosis according to their somatic cell environment. In the ovary, but not in the testis, germ cells initiate meiosis [31-32]. Nevertheless, the completion of meiosis and production of fertile gametes is dependent upon the chromosome constitution of the germ cells [33-34].

Recent studies have provided new insights into the molecular regulation of germ cell sexual differentiation and these have been reviewed [13, 35-36]. In brief, it has been concluded that retinoic acid (RA) produced from the mesonephros of both sexes is the meiosis-inducing molecule [37-39]. The sex-specific regulation of cytochrome P450 26B1 (CYP26B1), which degrades RA, is responsible for the initiation of meiosis in the ovary. On 12.5E CYP26B1 is up regulated in the somatic cells of the testis, while it is down regulated in the ovary. This results in the exposure to RA of the germ cells residing in the ovary, but not in the testis, and induces them to enter meiosis. In the embryonic testes CYP26B1 is associated with Sertoli cells and some interstitial cells [37, 40]. CYPB26 enzymes are associated with local regulation of RA levels in several developmental systems, for example in the patterning of the central nervous system [41].

The RA target, *Stra8* (stimulated by retinoic acid gene 8), a bHLH (basic helix-loop-helix) transcription factor is expressed only in XX oogonia before initiation of meiosis [42]. An additional protein, NANOS2 represses the expression of *Stra8* in the XY germ cells in face of reduced CYPB26 levels in the testis after 13.5E [43].

An additional gene necessary for production of germ cells capable for undergoing meiosis and normal gametes production is *Dazl* (deleted in azoospermia-like). It is expressed in both XX and XY germ cells upon their

colonization of the genital ridge [44]. In *Dazl*-deficient ovaries condensation of germ cell chromosomes, evidencing the initiation of meiosis, fails to occur. Likewise, *Stra8* expression, required for premeiotic DNA replication and subsequent meiotic prophase [45], is markedly reduced in such *Dazl* –deficient ovaries. *Dazl* is, therefore, required upstream to *Stra8* action on meiosis initiation. Germ cells of both sexes normally express *Sycp3* (synaptonemal complex protein 3). In *Dazl* – deficient embryonic ovaries *Sycp3* expression is markedly reduced and results in failure of chromosomes to perform their meiotic function and recombination. Thus, DAZL serves upstream in the embryonic germ cells as an intrinsic factor rendering them meiosis-competent and responsive to the extrinsic RA. But, DAZL acts also downstream through SYCP3 on meiotic prophase progression in the embryonic ovary oocytes. Embryonic male germ cells, by contrast, with down-regulated *Sycp3* arrest at G_0 [16], but require *Sycp3* for meiosis progression during spermatogenesis [39].

Folliculogenesis and Follicle Growth

Incomplete cytokinesis of oogonia undergoing mitotic divisions results in clusters of synchronously dividing germ cells [46]. Entry into meiosis and its progression are accompanied by massive attrition of germ cells. Four waves of oocyte degeneration were described in the rat, reducing their number from 75,000 on 18.5E to about 27,000 by day two after birth [21]. Following meiotic entry, the oocytes remain in cysts that undergo breakdown into single oocytes, some of them becoming enclosed by flattened somatic cells, pre-granulosa- or the first granulosa cells, and form the first follicles. Follicle formation, folliculogenesis, starts in mouse embryos around day two after birth. Folliculogenesis is also associated with extensive germ cell death, leaving only one third of the oocytes surviving and becoming enclosed within primordial follicles [47-49].

A germ cell-specific bHLH (basic helix-loop-helix) transcription factor, FIGLA (factor in the germline α), is required for primordial follicle formation. Female mice lacking the *figlα* gene show absence of germ cells and primordial follicles on day-2 after birth [50-51]. Steroids were implicated in the inhibition and the NOTCH signaling pathway in the promotion of folliculogenesis [for review see 13].

The primordial follicles form the pool from which some follicles are recruited for growth throughout life, from infancy to the exhaustion of the pool. Autocrine, paracrine and juxtacrine factors, that are not fully understood, have been implicated in recruitment of primordial follicles into the growing trajectory and affecting their number and rate of growth. As the follicle grows, hormones assume a leading role in the regulation of its differentiation and demise through atresia, involving apoptosis of granulosa cells. Regulation of follicle growth has been reviewed extensively [48, 52-53] and is beyond the scope of this oocyte maturation-centered review.

Primordial follicles initially consist of a germ cell and a few granulosa cells. Growth of such follicles results in the development of several layers of granulosa cells, basement membrane, theca layers with their vascular and neural supply, marks the establishment of a physiological unit that responds to endogenous and exogenous stimuli in a coordinated manner. Such coordination is achieved, at least in part, by the extensive communication through gap junction channels that join granulosa, cumulus cells and oocytes inner to the basement membrane [54-56]. Further studies revealed that connexins, the core proteins of gap junctions, are expressed in the mammalian ovary, and at least two of them, connexin 43 (Cx43) and 37 (Cx37) are essential for normal follicle development. Ovaries from Cx43-knockout newborn mice transplanted under the kidney capsule of wild-type mice did not proceed beyond the primary follicle stage and oocyte growth was impaired [57, 58]. In Cx37 null mice follicular growth was arrested at early antral stage and the oocytes did not reach their normal size and failed to acquire full meiotic competence [59-62]. Taken together, these findings seem to suggest an indispensable role for Cx43 early in folliculogenesis whereas Cx37 seems to be essential only at later stages of follicle development. The role of gap junctions in regulation of meiosis will be discussed below (Oocyte-cumulus Cells Communication: Role of Gonadotropins).

Oocyte-somatic cell interactions are essential for follicular development as demonstrated by re-aggregation experiments [63]. Two oocyte-derived growth factors that affect follicular growth have been demonstrated thus far. Growth differentiation factor 9 (GDF9) is produced in oocytes of primary follicles throughout their growth and ovulation [64]. Follicles of *Gdf9*-deficient mice do not grow beyond primary stage, the oocytes grow faster and the granulosa cells show reduced proliferation and aberrant differentiation [65-66]. Bone morphogenetic protein 15 (BMP15), a second growth factor produced by the oocyte [67], does not seem to be required for follicle growth. Nevertheless, *BMP15*-knockout mice are subfertile, showing reduced ovulation and fertilization rate [68].

In rodents, antrum formation is a hallmark in follicle growth and development. The dependence of antrum formation on FSH [69, 13] heralds the increasing role of endocrine regulation in follicle growth, in addition to local paracrine factors.

IN VITRO MODELS FOR STUDYING RESUMPTION OF MEIOSIS

Isolated Oocytes (Spontaneous Maturation)

The first model employed for investigating meiotic maturation *in vitro*, the culture of fully-grown rabbit oocytes in rabbit serum, revealed that the explanted oocytes matured whether pituitary extract or "maturity hormone" were added to the medium or not. Such spontaneous maturation *in vitro*, in contrast to the dependence on hormonal stimulation *in vivo* was interpreted to indicate either that follicle cells produce a substance or substances that directly inhibit nuclear maturation or that maintain the oocyte in a state wherein resumption of meiosis is impossible [1]. It took the development of additional complementary *in vitro* models and more than seventy years of research to reveal some of the cellular, regulatory and molecular factors that participate in keeping the meiotic process in abeyance and stimulating resumption of meiosis upon gonadotropic stimulation of ovulation.

These pioneering observations in rabbits were confirmed and extended to other mammalian species [6, 70-73]. Furthermore, it was found that spontaneous maturation of oocytes occurs in appropriate media, whether the oocytes are cultured within their intact cumulus cell complex or after the removal of these somatic cells (denuded oocytes) [74-77].

This model and its specific adaptations proved themselves most efficient for testing inhibition of spontaneous maturation by putative physiological or metabolic inhibitors of meiotic maturation and of follicle cells, for the investigations of intra-oocyte molecular mechanisms and of bidirectional oocyte and somatic cumulus cell interactions. Some of the results of such studies will be reviewed below. An elegant modification for studying the role of the oocyte in the regulation of cumulus cell differentiation and action, the oocytectomy, i.e., surgical removal of the oocyte from the cumulus-oocyte complex (COC) in culture, was established by Eppig and his colleagues [78-81] for studying mouse oocytes.

Follicle-Enclosed Oocytes (Hormone-Dependent Maturation)

Spontaneous maturation in culture of isolated oocytes is starkly different from the maturation of oocytes *in vivo* that is dependent on gonadotropin stimulation [1, 3, 82]. It was assumed, therefore, that the investigation of the molecular mechanisms involved in the regulation of meiotic resumption requires a model system replicating the need for hormone stimulation. By explanting rat Graafian follicles on the morning of the day of proestrus, Tsafriri and colleagues [83] were able to demonstrate that resumption of meiosis was dependent upon addition of LH or FSH or other stimulators to the medium. By contrast, follicles explanted after the preovulatory surge of gonadotropins matured in a hormone-free medium [82]. The use of mature proestrous rats as a source for preovulatory follicles requiring LH/hCG stimulation for trigerring meiosis was soon replaced with immature PMSG-treated rats [84]. Follicle-enclosed oocyte models were successfully applied to other mammalian species [85, 86]. Initial use of mouse ovarian fragments [87] was replaced by follicle cultures [88, 89]. More recently a method for injecting mouse follicle-enclosed oocytes was developed [90].

Follicle-enclosed oocytes were instrumental for studying some of the regulatory mechanisms involved in the hormonal stimulation of meiotic resumption, such as the role of gonadotropins and EGF, steroids, prostaglandins, cyclic nucleotides, intercellular communication, and follicular metabolism. All these will be dealt below.

MEIOTIC AND DEVELOPMENTAL COMPETENCE

In Rodents

Dictyate oocytes acquire the capability to resume meiosis (meiotic competence) upon approaching their final stage of growth. Accordingly, growing oocytes are usually meiotically incompetent and fully-grown oocytes are competent. At birth, the ovary is populated by a homogenous population of meiotically incompetent oocytes, of which a selected population starts growing with animals' age. Thus, oocytes harvested from mice before day 15 after birth do not mature *in vitro* and the incidence of maturing oocytes increases between days 15 and 21 [91-92]. Likewise, meiotic maturation is achieved not earlier than at 23 days in hamsters [93] and between days 20 and 26 in

rats [94]. The development of meiotic competence in mouse [92] and rat oocytes is acquired gradually [94]. The ability to resume meiosis and undergo GVBD with progression to metaphase I, is followed only later with the ability to reach metaphase II. In the mouse and the rat the acquisition of meiotic competence is clearly associated with the appearance of follicular antrum [92, 94-95].

Hypophysectomy of immature rats on day 15 of life, well before the acquisition of meiotic competence significantly reduced the number of competent oocytes explanted on day 26. By contrast, hypophysectomy on day 20 did not affect meiotic competence, tested on day 26 [94]. The effect of hypophysectomy on acquisition of meiotic competence could be reversed by administration of PMSG or FSH, but not LH. The action of FSH seems to be mediated, at least partly, by ovarian estrogen production. Inhibitors of aromatase blocked the effect of FSH on meiotic competence and this inhibition was prevented by concurrent administration of estrogen [96]. These experiments and the fact that antrum formation in the rat and the mouse is FSH-dependent [69, 13] support the notion that FSH plays a role in the acquisition of meiotic competence. Nevertheless, it should be noted that some oocytes might acquire meiotic competence when cultured *in vitro* without FSH [97-99]. In addition, a small number of grown oocytes from immature hypogonadal (hpg) mice that are deficient of hypothalamic gonadotropin-releasing hormone (GnRH) having thread-like uteri and very small ovaries [100] acquire meiotic competence autonomously and resume meiosis *in vitro* [101]. Treatment of hpg mice with gonadotropin promotes the development of meiotic competence in virtually all the oocytes [101]. Therefore, it seems that under regular physiological conditions the acquisition of meiotic competence by the regular numbers of oocytes is FSH dependent.

The mechanism by which hormonal or paracrine factors bring about the transition of meiotically incompetent into competent oocytes is largely unknown. Being a known mediator of FSH action, the role of cAMP in the development of meiotic competence was tested. Indeed, treatment of mouse growing oocytes with either dbcAMP or forskolin (that directly activates the adenylate cylase) leads to acquisition of meiotic competence [102].

While the detailed molecular mechanisms involved in meiotic arrest and resumption of meiosis in competent oocytes are overviewed in Regulation of Meiosis below, here we shall mention some of the factors that possibly take part in the acquisition of meiotic competence. In fully-grown oocytes resumption of meiosis is prevented by oocyte levels of cAMP. Nevertheless, negative regulation of meiosis in growing oocytes is independent of intraoocyte concentrations of cAMP [103]. Surprisingly, inactivation of the cAMP downstream effector, cAMP-dependent protein kinase A (PKA), triggered growing oocytes to resume meiosis [104]. This apparent discrepancy could possibly be attributed to a state of constitutively active PKA in growing oocytes achieved by compartmentalization of this enzyme executed by the A kinase anchoring protein (AKAP), the expression of which in oocytes has been demonstrated [104-105]. Meiotic incompetence could also be associated with relatively low activity of the oocyte specific phosphodiesterase 3A (PDE3A). This idea is based on the report that oocytes recovered from sexually mature female mice genetically ablated of PDE3A fail to resume meiosis *in vitro*, thus resembling growing, meiotically incompetent oocytes [106]. In that sense, acquisition of meiotic competence may represent some transition in the level of expression and/or activation of PDE3A. The demonstration that meiotic competence can be achieved upon microinjection of cytoplasm from fully-grown into growing oocytes [107] may support this assumption. Accumulation of cytoplasmic factors during oocyte growth could also contribute additional regulatory components that are absolutely necessary for the completion of the meiotic cell cycle. Indeed, microinjection of Cdk1 and cyclin B1 mRNA into growing oocytes was demonstrated to stimulate the resumption of meiosis [108].

The gradual development of meiotic competence is followed by the acquisition of developmental capacity, including fertilization, cleavage, blastocyst formation, implantation and embryonic development to term. Developmental capacity and production of live offspring of mouse oocytes matured spontaneously *in vitro* was similar to these maturing *in vivo* [101].

In Other Mammals

Despite our focus on rodent oocytes, one comparative aspect of follicular growth and acquisition of oocyte competence should be considered. In species with larger follicles (and longer reproductive cycles) acquisition of meiotic competence occurs only in tertiary follicles, long after antrum formation. Thus in cattle [109], porcine [110] and goat [111] full meiotic competence is acquired at a follicle size of 3mm, corresponding to an oocyte diameter of

about 110mm. Developmental competence is achieved only upon further growth of the oocyte within dominant follicles [for reviews see 112-113]. This growth is accompanied by ultrastructural changes in the oocyte, referred to as "oocyte capacitation" [114]. It seems that, also in the human, the development of meiotic and developmental competence is acquired at later stages of oocyte and follicle growth. A higher fertilization and cleavage rates were observed in oocytes retrieved from follicles larger than 16 mm [115-117].

This brief account of the acquisition of meiotic and developmental competence by murid oocytes and of species with larger follicles underlies the difference in the relationship and synchronization between oocyte and follicle growth in these species. Furthermore, they may reflect temporal differences in the action of endocrine, paracrine and autocrine regulators of their growth and differentiation. These should be considered in the development of improved methods of *in vitro* maturation (IVM) for animal husbandry and the clinic [118, 113].

REGULATION OF MEIOSIS

The cAMP Paradox

Pioneering studies demonstrated that cAMP serves as the second messenger for the LH-induced stimulation of ovarian steroidogenesis. It was shown that LH stimulates the formation of this nucleotide by ovarian cells or homogenates [119-120], that cAMP or its cell-permeable derivative dibutyryl cAMP (dbcAMP) mimic the steroidogenic action of LH [121-122] and that methyl xanthines, that inhibit cAMP degradation, potentiate this action of LH [123-124]. In follicle-enclosed oocytes, stimulation of meiosis resumption by gonadotropins was associated with a rise in follicular cAMP production [83, 125-126]. Furthermore, injection of dbcAMP, but not cAMP, into the antrum of follicles *in vitro* stimulated GVBD, while follicle-enclosed oocytes cultured in a medium containing dbcAMP remained immature [83]. These results support the notion that gonadotropin stimulation of oocyte maturation is mediated by cAMP. By contrast, dbcAMP and/or phosphodiesterase inhibitors prevent the LH-induced meiosis resumption in follicle-enclosed oocytes [127-128] disagreeing with this conclusion. Nevertheless, these latter observations may suggest that cAMP serves as a negative regulator of meiosis.

Studies with isolated oocytes in culture revealed that addition of dbcAMP into the medium inhibits sponataneous resumption of meiosis [129, 130, 131]. All these studies showed that rodent fully-grown oocytes released from the ovarian-follicle are kept meiotically arrested *in vitro* when either provided with permeable derivatives of cAMP or upon preventing its degradation by phosphodiesterase inhibitors. Consistent with this view, maturation is blocked in oocytes incubated with an invasive adenylyl cyclase that elevates intraoocye cyclic nucleotide levels [132]. These reports, followed by observations that a decrease in intraoocyte cAMP concentrations is associated with both, spontaneous as well as LH-induced resumption of meiosis [132-134], laid the groundwork for identification of cAMP as the negative regulator of meiosis in oocytes.
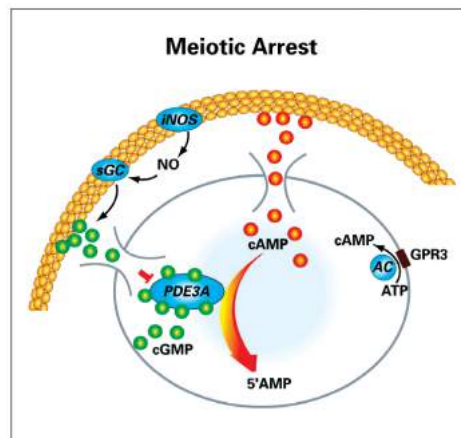
Figure 1: Major follicular events in meiotic arrest. A constitutively active adenylate cyclase in granulosa cells (yellow cells) generates basal levels of cAMP that are continuously transferred to the oocyte (large ellipse) through gap junctions. Oocyte cAMP, transmitted from the somatic compartment combined with some locally GPR3-mediated production, is maintained at the threshold levels required for meiotic arrest by the oocyte specific PDE3A, the hydrolyzing activity of which is restrained by follicular cGMP concurrently transferred to the oocyte through gap junctions. Enzymes- *italics*. Further details in the text.

Taken together, these studies present an apparent paradoxical dual action of cAMP, namely stimulation as well as inhibition of meiosis. This paradox was already apparent when intrafollicular injection of dbcAMP stimulated meiosis, while its addition to the culture medium was ineffective [83]. Attempts to resolve this puzzle and identify the molecular mechanisms involved in these apparently opposing responses included analysis of the doses required for inhibition as compared to induction of oocyte maturation [135]. The results of this study revealed that inhibition of meiosis in isolated oocytes is achieved by relatively low concentrations of cAMP, whereas higher levels of this nucleotide are required for the induction of maturation in follicle-enclosed oocytes. Other reports further showed that it is the continuous presence of cAMP that blocks LH-induced maturation in follicle-enclosed oocytes, whereas a transient exposure to elevated levels of the nucleotide will, by itself, induce meiosis resumption [125]. All in all, the results of these studies suggest that basal, sustained levels of cAMP maintain meiotic arrest (Fig. **1**), whereas transiently elevated, LH-stimulated concentrations of the nucleotide mediate the induction of oocyte maturation (Fig. **2**). This conclusion assumes that it is the tight compartmentalization and differential regulation of cAMP levels in the two major follicular compartments, the oocyte and the somatic cells that allow these apparent paradoxical responses to cAMP. In particular, only the granulosa/cumulus cells, but not the oocyte, express LH receptors [136] and respond with increased cAMP production upon exposure to this gonadotropin. Under these conditions, the rise in cAMP in the somatic compartment of the ovarian follicle induced by LH is not necessarily associated with an elevation of the inhibitory nucleotide within the oocyte. Yet, a transient increase in cAMP within the oocyte
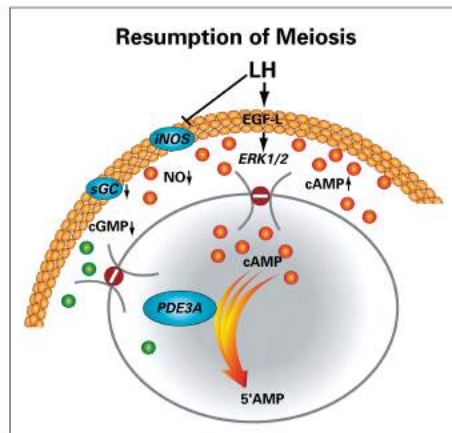
Figure 2: Follicular changes leading to resumption of meiosis. In response to the preovulatory surge of LH, accompanied with stimulation of granulosa cell cAMP production, gap junctional communication in the ovarian follicle is terminated and the transfer of both, cAMP and cGMP is stopped. The resulting decrease in the supply of cAMP, combined with the elevated hydrolyzing activity of PDE3A, due to the relief of cGMP inhibition, bring about a decrease in intraoocyte cAMP to levels that are lower than these required for meiotic arrest, thus allowing the resumption of meiosis.

that stimulates resumption of meiosis has been recently reported. This study suggests that an intraoocyte pulse of cAMP activates an AMP-activated protein kinase (PRKA) that overcomes the inhibitory action of cAMP. According to this study this rise is secondary to the LH-induced increase in cAMP in the granulosa that is, in turn, transferred to the oocytes that through gap junctions [137]. This novel idea is supported by our recent unpublished observations [Edry and Dekel, unpublished]. Additional mechanisms involved in compartmentalization and differential regulation of oocyte cAMP levels are further discussed in the following sections.

Oocyte-Cumulus Cells Communication

Role of gonadotropins. Oocytes reside in ovarian follicles where their immediate neighbors are the cumulus cells, a subpopulation of granulosa that upon antrum formation separates from the mural granulosa cells. Each follicle functions as one physiological unit due to an extensive gap-junctional communication network established between the oocyte and the cumulus cells as well as among the cells that comprise the somatic compartment [reviewed in 138]. As already mentioned, gap junctions are specialized regions in apposing membranes of neighboring cells that allow the exchange of small molecules [139, 62]. The molecules transmitted through gap junctions from the somatic cells of the ovarian follicle are essential for oocyte growth and development and play a major role in regulating its meiotic status [140, 125, 141]. Gap junctions are assembled from the proteins of the connexin (Cx) family, the most abundant in the ovary is Cx43 [142]. Ovarian Cx43 is phosphorylated in response to LH, an effect that apparently

results in disruption of cell-to-cell communication [143-144, 90]. Another member of the Cx family expressed in the ovary is Cx37. The expression of Cx37 is restricted to the oocyte and its contribution to cell-to-cell communication in the ovarian follicle is apparently limited to the cumulus cells-oocyte interface [59, 145-146].

As mentioned above, a relatively high intraoocyte concentration of cAMP keeps the meiotic process on hold. Two different sources are presently proposed to account for the origin of the intraoocyte, inhibitory cAMP. One suggested source is the somatic compartment of the ovarian follicle that exogenously supplies this inhibitor to the oocyte [147, 125]. Alternatively, local production of cAMP by the oocyte itself has been suggested [148-149].

The first suggestion is supported by the observation that oocytes released from the ovary resume meiosis spontaneously and the consequent conclusion that meiotic arrest highly depends on an inhibitor originating from other follicular compartments. This assumption was supported by early studies in rats demonstrating that the inhibitory cyclic nucleotide generated by the follicular cells enters the oocyte [125, 150] and a later report of actual transport of cAMP from granulosa cells into the oocyte through gap junctions that is subject to negative regulation by gonadotropins [151]. Along this line, LH-induced oocyte maturation has been shown to be associated with interruption of gap junctional communication between the oocyte and the cumulus [152, 125, 153, 144]. In that sense, the release of the oocyte from the ovarian follicle, which results in its spontaneous maturation, actually mimics the effect of LH in terminating the supply of the follicular inhibitory cAMP. In strong support of this view are recent studies in which resumption of meiosis in follicle-enclosed oocytes was induced by blocking follicular gap-junctions with either the non-selective gap junction blocker, carbenoxolone (CBX), or with the anti Cx37 antibody [154-155]. Importantly, CBX- induced resumption of meiosis was accompanied by a decrease in intraoocyte cAMP concentration [154].

It should be noted, that since spontaneous maturation is not only observed in cumulus-free oocytes, but rather takes place in isolated cumulus–enclosed oocytes [147, 131], it is apparent that mural granulosa cells supply the major bulk of the inhibitory cAMP. The cumuls cells serve as a conduit providing the gap junctions for the actual transfer of this inhibitor to the oocyte. This role of the cumulus is clearly evident since anti Cx37 antibodies, that exclusively block gap junctions at the oocyte-cumulus interface, effectively induced maturation in follicle-enclosed oocytes [155].

The theory of in ovo generation of cAMP relies on the presence of a ligand, continuously generated by the granulosa cells, which in turn activates an oocyte membrane-bound Gs protein that stimulates the oocyte adenylyl cyclase [148-149]. In this case, the spontaneous maturation that occurs upon the release of the oocyte from the ovarian follicle may represent the termination of its exposure to the granulosa-derived Gs-activating ligand. This mechanism is supported by the reported rise in cAMP concentration following incubation of isolated denuded mouse oocytes with forskolin, an activator of adenylate cyclase [156-157], as well as by the demonstrated expression of this cAMP generating enzyme in rodent oocytes [158]. Along this line, it has been shown that microinjection of antibodies against Gs into mice resulted in the resumption of meiosis [148]. Furthermore, in a knockout mouse model of GPR3, a G protein-coupled receptor that activates Gs, the females have mature oocytes in their antral follicles independently of LH stimulation [149]. However, this model was not oocyte specific, as GPR3 was omitted from granulosa cells as well. It is possible that the LH-independent oocyte maturation in this model represents the impaired constitutive production of cAMP by the granulosa cells that results in reduction of cAMP available for transfer into the oocyte to maintain the levels sufficient for meiotic arrest. In addition, the ligand that activates GPR3 remains unidentified and the precise regulatory mechanism employed by LH to terminate its action has not yet been determined. Finally, it was recently discovered that LH does not affect the activity of G(s) in mouse oocytes and, hence, this signaling pathway does not seem to participate in reinitiation of meiosis [155].

The question whether local cAMP production in the oocyte is sufficient to keep meiotic resumption in abeyance by itself under physiological conditions, as well as the regulation of oocyte adenylate cyclase(s) remain to be investigated.

Role and Regulation of Phosphodiesterases (PDEs)

As discussed above, low sustained levels of cAMP block meiosis while LH induction of meiosis is mediated by a rise in follicular cAMP levels, that is immediately followed by a short term-pulse in oocyte cAMP [137 and Edry

and Dekel, unpublished]. Nevertheless, termination of the supply of cAMP from the somatic compartment of the follicle that leads to the drop in oocyte cAMP level remains an absolute prerequisite for resumption meiosis [reviewed by 159]. However, a complementary mechanism that controls the intracellular concentration of cAMP is provided by its degradation, catalyzed by cyclic nucleotide phosphodiesterases (PDEs). Eleven different PDE families have been identified in mammals [160] of which members of the type III and IV PDE are expressed in the ovary. PDE3A was shown to be expressed in rat oocytes by in situ hybridization [161] and was later cloned from a mouse oocyte cDNA library [162]. Two members of the type IV PDE family, PDE4D and PDE4B, were exclusively observed in mural granulosa and in theca cells, respectively [161]. The development of type-specific PDE inhibitors allowed further confirmation of the differential expression of PDEs in the various follicular compartments by identification of specific PDE hydrolyzing activity in either of them as follows.

Cilostamide, a type III PDE inhibitor, eliminated all the PDE activity measured in denuded rat oocytes. The cilostamide-sensitive PDE activity, namely activity of PDE3, measured in cumulus-oocyte complexes was similar to that measured in oocytes alone, supporting the notion that PDE3A is the only PDE expressed and active inside the oocyte [163]. A role of this specific PDE is demonstrated by the fact that spontaneous GVBD in isolated rat oocytes was blocked by two inhibitors of type III PDE, cilostamide and milrinone, whereas treatment with a type IV PDE inhibitor, rolipram was ineffective [161]. In agreement with these *in vitro* results, injection of cilostamide or another PDE3 inhibitor, Org 9935, suppressed oocyte maturation in hormonally primed mice and rats, which consequently ovulated meiotically arrested, GV oocytes [164]. In addition, PDE3A knock out female mice are sterile and their oocytes fail to resume meiosis both *in vivo* as well as spontaneously *in vitro* [106].

Although PDE3A is clearly essential for oocye maturation [106], the regulation of its acivity in oocytes is poorly understood. Upregulation of the activity of PDE3 has been demonstrated in cumulus–enclosed oocytes isolated from hCG-stimulated follicles, but could not be detected in cumulus-free oocytes, suggesting a role for LH in activating PDE3A that is mediated by the cumulus cells [163].

Phosphorylation by different kinases has been reported to play a pivotal role in PDE3A activation [165-166]. Taking into account the consensus sequences that have been identified in PDE3A [162], the two suggested candidates for such activating kinases are PKA and PKB/Akt. Considering that PKA is instrumental in maintaining meiotic arrest, the activation state of this downstream effector of cAMP in GV-arrested oocyte should be relatively high. Nevertheless, a special cAMP signaling module has been demonstrated in which PKA and PDE4D3 are held in proximity by muscle-selective A-kinase anchoring protein (mAKAP) in heart tissue. This complex forms a negative feedback loop, in which hormone stimulation elevates cAMP that activates PKA, which in turn phosphorylates and activates the nearby PDE. The activated PDE hydrolyses cAMP in order to restore basal levels and hence, inactivates PKA and terminates the stimulus [167]. Indeed, the expression of a specific AKAP in rat [168] and mouse [169] oocytes has been demonstrated and a role for AKAP in maintaining meiotic arrest in mouse oocytes has been shown [105].

A role for PKB-Akt phosphorylation can also be considered. PKB-Akt is expressed in Xenopus oocytes and is a component of the pathway that controls resumption of meiosis in this species [170] as well as in cattle [171] and starfish [172]. Moreover, PKB/Akt is distal to PI-3 kinase that is activated by several growth factors. As discussed bellow, EGF has been shown to activate meiotic resumption in rodents [173-174]. As the PI-3 kinase PKB/Akt pathway plays an important role in EGF signaling, it is possible that EGF stimulates activation of PDE3A through PKB/Akt. Consistent with this view, an EGF-receptor has been identified in human and rat oocytes [175-176]. Indeed, phosphorylation by PKB/Akt was recently claimed to activate PDE3A in mouse oocytes as a mean to induce oocyte maturation [177]. This study showed that myristylated PKB/Akt induced resumption of meiosis in isolated hypoxanthine-arrested mouse oocytes, an outcome which required activation of PDE3A. This group of researchers also demonstrated elevated activity of oocyte PKB/Akt that peaked two hours after hCG administration and was accompanied by PDE3A activation.

The Nitric Oxide (NO) Connection

Another regulator of PDE3A in oocytes could possibly be nitric oxide (NO). NO was shown to be involved in several events in the reproductive tract including steoidogenesis, pregnancy, folliculogenesis, follicle rupture at ovulation as

well as oocyte meiotic maturation [178-179]. NO is synthesized from arginine by the catalytic action of NO synthase (NOS), for which three encoding genes have been isolated; two calcium-dependent constitutively expressed isoforms, endothelial NO synthase (eNOS) [180] and neuronal NO synthase (nNOS) [181], and a calcium-independent inducible form (iNOS) [182]. Both iNOS and the constitutive endothelial isoform eNOS, are expressed in rat ovaries; their expression as well as their pattern are affected by gonadotropins [183]. In immature untreated rats, transcripts of iNOS in granulosa cells of secondary follicles are scarce and become even lower following injection of pregnant mare serum gonadotropin (PMSG), which stimulates follicular growth. Expression of iNOS is no longer detected in ovulatory follicles or corpora lutea developed after further stimulation with LH [184-185]. In agreement, iNOS protein was hardly detectable in granulosa cells of pre-ovulatory follicles following hCG administration. On the other hand, this protein was upregulated in response to hCG in the theca cells [186, 183].

The gonadoropin-regulated expression of NOS enzymes in the ovarian follicle may suggest a role for NO in meiotic resumption. However, controversial observations were published concerning the effects of the NOS-NO system on resumption of meiosis. Specifically, some studies with mouse oocytes reported that NO has no effect [187] whereas others demonstrated full inhibition of meiosis by NO [188-189]. In agreement with these results, rat follicle-enclosed oocytes were induced to resume meiosis upon inhibition of follicular iNOS [190]. Furthermore, elevation of NO availability in follicles reversed oocyte maturation induced either by iNOS inhibition or with LH [190-191]. Likewise, injecting a NO-donor into the peri-ovarian sac (ovarian bursa) significantly decreased LH-induced ovulation *in vivo* [191]. Conversely, earlier reports showed that NOS inhibitors downregulated ovulation in rats [178, 192].

Cyclic GMP

One known effect of NO is the induction of soluble guanylate cyclase (sGC) [193] to produce cGMP that can maintain meiotic arrest [194-195]. Since cGMP serves as an inhibitor of PDE3s [162], it is quite possible that the controversial role of NO in resumption of meiosis represents an additional layer of regulation of oocyte PDE3A activity.

The role of cGMP in regulating PDE3A activity as well as its involvement in the control of meiosis have been suggested by recent studies. Firstly, it was demonstrated that an inhibitor of cGMP production effectively induces maturation of follicle-enclosed rat oocytes in the absence of LH. This effect was abolished by the addition of 8-Bromo-cGMP [191]. Another study [196] further reported that cGMP passes through gap junctions into the oocyte, where it inhibits PDE3A activity thus maintaining a high intraoocyte concentration of cAMP and blocking the resumption of meiosis. These authors also demonstrated that LH reverses the inhibitory signal by lowering cGMP levels in the somatic cells. This effect is enhanced by closure of gap junctions that results in a decrease in oocyte cGMP followed by a relief of PDE3A inhibition. The resulting drop in intraoocyte cAMP allows the resumption of meiosis. A more recent study [195] confirms and extends these findings. Specifically, this last study also demonstrates a LH-induced reduction in the levels of cGMP in both the granulosa cells and the oocyte suggesting that this decrease is one of the signals that leads to meiotic maturation.

EPIDERMAL GROWTH FACTOR-LIKE MOLECULES

In 1985 Dekel and Sherizly [173] demonstrated that epidermal growth factor (EGF) mimicks the action of LH on large antral follicles in culture and stimulates oocyte maturation. This puzzling finding was further confirmed in mice [197]. More significantly, it was shown that rat follicle-enclosed oocytes induced to mature by EGF, like those stimulated by LH, are fertilizable and developmentally competent. Therefore it was suggested that EGF or other growth factors produced in the ovary might act locally to control follicle development [198]. The essential role of EGF receptor (EGFR) activation in the mediation of LH-induced oocyte maturation was later demonstrated in mice [174, 199]. These reports also show that LH increases the transcription of the epiregulin, amphiregulin and betacellulin and that these EGF-like molecules, rather than EGF itself, activate the EGFR in ovulatory follicles. These data were extended to the rat, further reporting that in explanted follicles, metalloproteinases mediate the LH-induced activation of the EGFR, which is required for resumption of meiosis, as well as for follicle rupture *in vivo* [200]. The essential role of EGF-like growth factors in mediating LH action was confirmed in transgenic mice expressing a mutated EGFR, in which preovulatory follicles failed to respond to LH [199]. Taken together, these data established the indispensability of the EGFR pathway in the LH-induced ovulatory response.

It was proposed [174] that due to the lower density of LH receptors on the cumulus cells, as compared with mural granulosa [136], the EGF-like growth factors produced at the periphery of the follicle may serve as paracrine mediators that propagate the LH signal in a centripetal manner. However, the fact that the EGF-like molecules are similarly up-regulated in mural granulosa and the cumulus cells renders this possibility unlikely [201]. Regardless their specific site of production, it is quite obvious that it is the somatic compartment of the follicle, rather than the oocyte, that responds to EGF-like growth factors and that the activated EGFR further stimulates the ERK1/2 signaling cascade in the cumulus/granulosa cells. Supporting this idea a very recent study demonstrated that upon inhibition of the EGFR in rat ovarian follicles phosphorylation of ERK1/2 is prevented and LH fails to induce oocyte maturation [202]. This study also reports of a unique mode of sustained, rather than transient EGFR activation, which is an absolute requirement for LH-induced oocyte maturation.

The role of ERK1/2 in gonadotropin-induced oocyte maturation was first demonstrated in mouse COCs [203]. It was later shown in rat ovarian follicles that ERK1/2 mediate the effect of LH on gap junctions' closure in granulosa cells, stopping the somatic cAMP influx to the oocyte [144]. This, leads to a substantial drop in intraoocyte cAMP concentrations, allowing the resumption of meiosis [154, 90]. Most interestingly, it was recently shown that oocytes recovered from genetically manipulated mice, in which the granulosa ERK1 and ERK 2 were depleted, underwent spontaneous maturation, whereas gonadotropin administration in these mice failed to induce resumption of meiosis [204].

INTRAOOCYTE REGULATORS OF THE MEIOTIC CELL CYCLE

Maturation promoting factor (MPF) is the key regulator of meiosis reinitiation, the activity of which was initially discovered in amphibian oocytes [205]. Further studies revealed that the transition from G_2 to M-phase of mitosis involves similar principles, therefore extending the definition of MPF as mitosis or M-phase promoting factor as well. MPF was identified as a complex composed of the catalytic p34cdc2 also known as cyclin-dependent kinase 1 (Cdk1) and the regulatory cyclin B1 (Fig. **3**) [206-208]. A prerequisite for Cdk1 activation is its association with cyclin B1 [reviewed by 209-210]. Following the binding of cyclin B1, Cdk1 undergoes phosphorylation on threonine 161, mediated by Cdk-activating kinase (CAK), [209] and on threonine 14 and tyrosine 15, which are catalyzed by Wee1 and Myt1, respectively [211-212]. At this configuration the Cdk1/cyclin B1 heterodimer is inactive and defined as pre-MPF. The inactive pre-MPF in meiotically arrested rodent oocytes is gradually activated upon reinitiation of meiosis reaching full activity after 5 to 7 hours [213-214]. Activation of the pre-MPF is achieved by the dephosphorylation of Cdk1 on threonine 14 and tyrosine 15, which is catalyzed by the dual specificity Cdc25 phosphatase [215]. Both B and C types Cdc25 are expressed in rodent oocytes [216-217]. However, it is the Cdc25B that is essential for the meiotic cell cycle, as oocytes from cdc25B null mice do not activate MPF and fail to resume meiosis [216].

MPF activation at the onset of meiosis is dependent on the reduction in intraoocyte cAMP [213, 218-219]. It has been demonstrated that cAMP-mediated inhibition of MPF activation is accomplished by prevention of Cdk1 dephosphrylation [213, 220]. The long interval between the reduction in intraoocyte cAMP concentrations and the upregulation of MPF activity suggests the existence of intermediate steps linking these two events. However, it took several decades before the linkage between cAMP and the downstream regulators of the meiotic cell cycle has been unveiled.

Identification of cAMP as the negative regulator of meiosis in *Xenopus* oocytes was associated with the demonstration that the cAMP-dependent protein kinase A (PKA) acts as its down stream effector in this system [221]. Microinjection of the PKA catalytic subunit that prevented the spontaneous resumption of meiosis confirmed these findings for mouse oocytes [222]. Moreover, this last study also demonstrated that the microinjection of PKI, a specific inhibitor of PKA, induced GVBD in mouse oocytes incubated in the presence of either dibutyryl cAMP or a phsphodiesterase inhibitor. A more recent study identified Cdc25B as a direct target for PKA activity in mouse oocyte [223]. Specifically, this study demonstrated that PKA–induced Cdc25B phophorylation results in inhibition of this phosphatase. Along this line, a very recent study shows that PKA inactivation induces a rapid and progressive cdc25B translocation into the nucleus that brings about dephosphorylation and activation of the nuclear MPF [224]. The activated MPF in turn promotes the export of Wee1B to the cytoplasm, amplifying MPF activation. Cdc25B sequestration was actually suggested previously as the mechanism by which PKA inhibits the Cdc25B-induced activation of MPF [223].
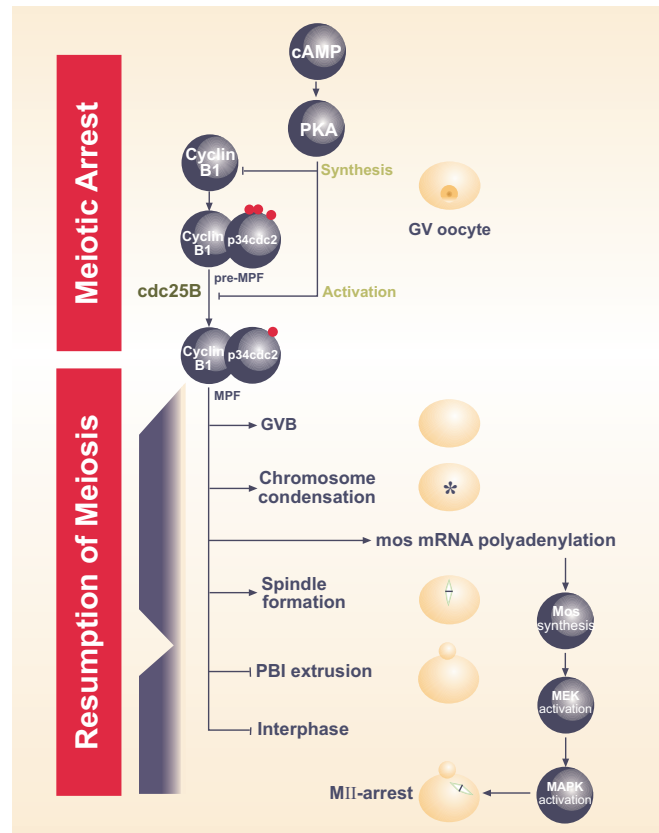
Figure 3: Intraoocyte regulators of meiosis- MPF activation and inactivation. Meiotic arrest in the oocyte is maintained by the inhibitory effect of a PKA-mediated cAMP action. The PKA-mediated cAMP inhibition is conferred at two levels: the prevention of pre-MPF activation due to sustained phosphorylation on p34cdc2 and the repression of de-novo synthesis of cyclin B1. In response to the preovulatory LH surge (or following the release of the oocyte from the ovarian follicle), intraoocyte cAMP concentration drops and MPF activation is catalyzed by the B isoform of the cdc25 phosphatase. The active MPF induces resumption of meiosis, namely GVB, chromosome condensation and spindle formation. MPF activity also stimulates the polyadenylation of mos mRNA, leading to MOS synthesis and to the activation of MAPK. Inactivation of MPF at MI is necessary for the completion of the first meiotic division and the extrusion of PBI, whereas it reactivation suppresses interphase thus ensuring the transition into MII. The MII arrest of the oocyte is exerted through the action of MAPK until fertilization (From Dekel [225], with permission).

MPF activation is necessary for reinitiation of meiosis and its progress to the first metaphase [reviewed by 225]. However, the completion of the first round of meiosis, manifested by the formation of PBI, is absolutely dependent on MPF inactivation. Down regulation of MPF activity is achieved upon proteasomal degradation of cyclin B1 [226]. Inactivation of MPF is transient and its reactivation at this stage of meiosis in rodent oocytes has a role in preventing the interphase to occur between the two rounds of meiosis [214]. An active MPF is also required for upregulation of the activity of ERK1/2 members of the MAP kinase (MAPK) family [227]. The immediate regulator of MAPK is MEK, which in oocytes is activated by Mos [228], a distinct MEK kinase that is expressed exclusively in germ cells [229-230]. The activity of the MAPK signaling pathway at this stage of meiosis is absolutely necessary for the arrest of rodent oocytes in second metaphase since genetic disruption of mos leads to parthenogenetic development of unfertilized mouse eggs [231-232]. Unlike Xenopus, the Mos/MAPK signaling cascade in rodent oocytes does not participate in early events during meiosis reinitiation since both GVBD and PBI formation are unaffected in the above mentioned mos null mice.

OTHER FACTORS IMPLICATED IN REGULATION OF MEIOSIS RESUMPTION

Steroids

In fishes and amphibian, gonadotropins induce resumption of meiosis through the mediation of steroids produced by follicle cells [233-236]. Similar regulation of meiosis, involving a peptide of neural origin (gonad-stimulating

substance – GSS) and acting on the gonadal somatic cells to produce a maturation-inducing substance (1-methyl adenine) has been described in echinoderms [237-238].

In mammals, too, the preovulatory surge of gonadotropins that triggers ovulation, including resumption of meiosis, stimulates also a rise in follicular progesterone production and a transitory rise in estrogen [239-240]. Thus, steroids like progesterone or estrogen could serve as mediators of LH in stimulating meiotic resumption in the rat and the mouse. Studies with spontaneously maturing murine oocytes resulted in ambiguous results. In some cases steroids like testosterone attenuated the spontaneous maturation and in other studies estradiol, testosterone or progesterone potentiated the inhibitory action of dibutyryl cAMP or forskolin [241, 133, 57, 242-243].

Treating rat preovulatory follicles, explanted on the morning of the day of proestrus before the endogenous surge of gonadotropins, with LH/hCG demonstrated its role in meiotic resumption. Nevertheless, addition of progesterone, dihydrorogesterone, and estradiol to the medium was completely ineffective in simulating the action of gonadotropins on meiosis [83]. Furthermore, suppression of follicular steroidogenesis by inhibitors blocked LH/hCG stimulation of progesterone, estrogen and androgen did not impair the ability of LH to induce resumption of meiosis [244-245]. All these studies led to the conclusion that steroids, though playing important roles in the regulation of reproduction, do not serve in mammals as an obligatory mediator in the stimulation of meiotic resumption [127, 243, 246].

Recently a series of studies from the laboratory of Hammes presented results implicating steroids in the mediation of the meiosis-inducing signal in the rat and the mouse [reviewed by 236]. Thus, using denuded oocytes isolated from mice that were not primed with eCG and cultured in the presence of 3-isobutyl-1-methylxanthine (IBMX), that blocks spontaneous maturation, Gill *et al.* [247] have reported stimulation of meiotic resumption by testosterone. They have concluded that androgen produced by preovulatory follicles induces oocyte maturation. In a subsequent study, Jamnongjit *et al.* [248] reported that EGF as well as testosterone or progesterone induced meiotic resumption in granulosa-oocyte cultures and in isolated oocytes cultured with IBMX. Furthermore, they found that promegestone (a progesterone agonist), estradiol and dihydrotestosterone induced the resumption of meiosis, each acting through its own receptor, and respective receptor antagonists could block the effect of each one of these steroids. In the same study, mouse follicle-enclosed oocytes responded to LH, testosterone or progesterone with resumption of meiosis. They have suggested, "steroids appear to promote similar events in mammalian oocytes, implying a conserved mechanism of maturation in vertebrates" [236]. The most recent report that estradiol could induce the maturation in follicle-enclosed oocytes explanted from PR-null mice and the implied inference that both of these steroids may serve as mediators of LH action of meiosis in the same species and cell system [249] has to be confirmed in other systems exhibiting such a broad permissibility and responsiveness to steroids with specific and distinct actions. The suggestion that steroids of three distinct groups have a similar, meiosis-stimulating effect, in the same species appears to be unprecedented in other vertebrate species.

Other studies did not support this suggestion. In mouse or rat cumulus enclosed oocytes promegestone, estradiol and DHT could not trigger the resumption of meiosis in the presence of hypoxanthine, a mild inhibitor of meiosis [250]. Furthermore, the progesterone receptor antagonists mifepristone and Org 31710, or faslodex (of estrogen) or flutamide (of testosterone) did not affect the spontaneous maturation of isolated murine oocytes [251].

Using rat or mouse follicle-enclosed oocytes, it was demonstrated that promegestone, estradiol and dihydrotestosterone did not stimulate oocyte maturation, while LH was effective. Furthermore, the PR antagonists, mifepristone or Org 31710 and the ER antagonist faslodex did not alter LH-triggered resumption of meiosis in such follicle-enclosed oocytes in the rat or the mouse.

Only addition of the androgen receptor antagonist, flutamide, to rat follicles *in vitro* resulted in a dose-dependent inhibition of LH-induced resumption of meiosis. Nevertheless, since preincubation of follicles with DHT did not alter this action of flutamide it seems that its inhibitory effect is not mediated through the androgen receptor, therefore, unrelated to its androgen receptor antagonistic activity [251].

Additional considerations argue against a mediatory role of androgens in the stimulation of meiotic resumption in murids. The immediate steroidogenic response to LH/hCG stimulation of ovulation in the rat (and most likely also in

the mouse) is suppression of androgen production [252] and CYP17A activity [253]. Finally, the fertility of two models of androgen receptor-deficient mice, albeit reduced and leading to premature ovarian failure due to its regulatory role in several genes regulating follicle development [254-255], argues against a critical role of androgen in resumption of meiosis. Taken together these findings make follicular androgen a most unlikely mediator of LH action on oocyte maturation.

In rat follicles LH/hCG stimulation induces a transient increase in progesterone and estrogen at a critical time prior to meiosis [239-240], thus affording their participation in the stimulation of meiosis resumption in murid species. Nevertheless, the bulk of studies overviewed here do not support the participation of steroids in the mediation of gonadotropic stimulation of meiotic resumption in the rat or the mouse. This conclusion is borne out by studies with transgenic mice showing that meiotic maturation is not dependent on any one of the three steroid groups, progestogens, estrogens and androgens [reviewed by 256]. Ovarian physiology in mammals is characterized by hierarchical growth of follicles and the release of one or few ova during each reproductive cycle. Consequently oocytes might be exposed during their protracted meiosis to high intaovarian steroid levels released from the larger follicles. We speculate that this difference between mammals and lower vertebrates with batches of synchronously growing follicles led to the replacement of steroids as a signal for meiosis resumption in mammals by follicular paracrine factors.

Nevertheless, in several mammalian species, with larger follicles and longer reproductive cycles, resumption of meiosis and its progression seem to be dependent on a sequence of signals [see for review 114, 257, 113], including steroids. Inhibition of steroidogenesis in ovine follicles impaired the progression of meiosis to metaphase II, but did not prevent GVBD [258]. Inversely, blocking steroidogenesis in porcine [259-260] and bovine [261] cumulus-enclosed oocytes *in vitro* prevented GVBD, which could be restored by the addition of progesterone in porcine, but not bovine oocytes. Furthermore, in these species steroids were implicated in the acquisition of developmental competence [86, 258, 262-264]. In these species steroids seem to be involved in some aspects of oocyte maturation and in the development and the acquisition of developmental competence [265]. Thus in species in which acquisition of meiotic competence is deferred to later stages of follicle development and having a longer time-interval between LH stimulation of ovulation and GVBD an appropriate steroid milieu is required for follicle development and fertilizable ova production.

Meiosis Activating Sterol (MAS)

Human follicular fluid was found to stimulate the resumption of mouse oocytes cultured in the presence of hypoxanthine. This activity was identified as a sterol (MAS; 4,4-dimethyl-5-α-cholesta-8,14,24-triene-3β-ol) [266]. MAS is the product of lanosterol 14α-demethylase and an intermediate in cholesterol biosynthesis [267]. The ability of MAS, purified or synthetic, to cause resumption of meiosis was confirmed by several laboratories and in several mammalian species, including mice and rats [266-271].

Use of inhibitors of cholesterol and sterol synthesis was reported to block spontaneous or gonadotropin-stimulated resumption of meiosis [272], while in other studies they were ineffective [270, 273-274].

The detected rise in endogenous MAS in mice and rats relatively to the timing of GVBD appears too late to be a mediator of gonadotropin-induced resumption of meiosis in these species [274-275]. Likewise, stimulation of resumption of meiosis by exogenous or endogenous MAS resulted in delay in maturation when compared to stimulation with gonadotropin [269-270, 274].

Nevertheless, the rise in follicular MAS levels by gonadotropins may be related to its beneficial effects on the follicle/oocyte increasing the rate of developmentally competent ova [269, 276-278]. For detailed reviews on MAS see [272, 279].

CONCLUDING REMARKS

The production of a fertilizable egg is an essential prelude for the creation of new life. This review presents our current understanding of the high level of complexity and the tight orchestration of the events occurring in various

follicular compartments involved in the production of such eggs. The development of highly sophisticated experimental tools, including oocyte and follicle cultures and genetic models amenable for manipulation *in vivo* and *in vitro* contributed to our understanding of the regulation of meiosis. The studies in murid rodents reviewed here provide the most accessible mammalian model systems need to be complemented by studies in other mammalian species. As already discussed, the different dynamics of follicle development and the time-interval between the stimulation of ovulation and GVBD in murids, as compared to farm animals and the human, make IVM and developmentally competent ova much more difficult to obtain in these latter species.

Intraoocyte molecular regulation of meiosis in mammals, directed largely towards MPF activation and inactivation, seems to be well-preserved and similar to lower vertebrates. Yet, follicular control of meiosis in mammals diverged. The most notable change is the abandoning of steroids as the primary somatic-cell signal for resumption of meiosis.

In spite of the vast amount of data accrued on the regulation of meiosis there are many details that need further clarification. Here, we have presented, consistent with the recent advances and commonly accepted ideas in this area, cAMP as the major somatic cell-derived regulator of meiotic arrest and resumption. Are any other factors exerting one or both of these activities? For example, an oocyte maturation inhibitory activity (OMI), derived from granulosa cells and found in follicular fluid, that was identified as a peptide [reviewed by 246], may still be involved? After the submission of the manuscript, an elegant study from the laboratory of Eppig [280] provided conclusive evidence for this concept. They have demonstrated that mural granulosa cells express natriuretic peptide precursor type C (*Nppc*) mRNA, while cumulus cells express mRNA of its receptor, NPR2 that is a guanylyl cyclase. NPPC-22 (the 22 amino acid form, [Eppig, personal communication]) increased cGMP levels in cumulus cells and oocytes and inhibited spontaneous oocyte maturation *in vitro*. Furthermore, in many oocytes in Graafian follicles of *Nppc* and *Npr2* mutant mice meiosis was resumed spontaneously. Hence it was concluded that the granulosa cell ligand NPPC and its receptor NPR2 in cumulus cells maintain meiotic arrest by suppressing oocyte PDE3A activity and keeping intraoocyte cAMP concentration at levels that prevent precocious oocyte maturation and keep resumption of meiosis and ovulation synchronized. While identification of OMI activity with NPPC remains to be established, there are remarkable common features between these two, such as a low molecular weight peptide, produced by mural granulosa cells that acts through cumulus cells but not directly on the oocyte. The stimulation of meiosis resumption by LH is mediated by closure of gap junctions leading to an abrupt decrease in oocyte cGMP levels, followed by a relief of PDE3A inhibition as previously suggested [191, 195, 281].

The recent advances in revealing the roles of paracrine EGF-like molecules, NPPC and its receptor NPR2, oocyte-specific PDE3A and cGMP in the regulation of meiosis call for further studies to unravel additional hidden surprises of the ovarian follicle.

ACKNOWLEDGEMENTS

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CHAPTER 3

The Enhancers of Oocyte Competence

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Abstract: During oocyte maturation, nuclear maturation i.e. the condensation of chromosomes and formation of the meiotic apparatus is generally considered as the most significant physiological process. However, disruption of the contact between granulosa cells and the oocyte leads to spontaneous nuclear maturation of oocytes which, however, have poor or nil developmental competence after fertilisation. Acquisition of cytoplasmic competence, i.e., the ability to sustain early development of embryos with high developmental potential, is the result of concomitant synergic actions of gonadotrophins and growth factors. The transforming growth factor beta superfamiliy, the epidermal growth factor network, insulin growth factors and growth hormone together with Leukaemia inhibiting factor are partners in the mechanism of acquisition of developmental competence in oocytes. These interactions allow the (quantitatively and qualitatively) correct storage of mRNAs and proteins necessary for the early embryonic divisions prior to genomic activation. However, the quality of the endogenous pool of metabolic intermediates such as (sulphur) amino acids is a mandatory prerequisite for oocyte activation, sperm decondensation and further on early embryo divisions. A correct timing of translation of the mRNAs stored during oocyte maturation is mandatory for the successful passage of the maternal to zygotic transition, usually considered as the critical step in early embryonic developmental arrest.

INTRODUCTION

Oocyte nuclear maturation has been considered to be the most significant parameter that influences the success of early *in vitro* fertilization experiments. The mammalian oocyte remains in a stage of quiescence for periods of several months, and meiosis is eventually resumed under hormonal control through the interactions of cumulus and granulosa cells. Completion of meiosis with the formation of the second meiotic spindle occurs in two circumstances: following extraction of the oocyte from the antral follicle, or during follicular atresia. Disruption of the contact between granulosa cells and oocyte does lead to spontaneous nuclear maturation, but in this situation the oocyte appears to be degenerated rather than mature. This "spontaneously matured" oocyte has poor, if any further developmental competence, although the timing of nuclear events is similar to that of a healthy mature oocyte. Fertilization may occur more or less normally, but the nucleus may be very poorly decondensed [1].

The efficiency of oocyte maturation in human IVF procedures is still suboptimal: during ART cycles, only approximately 5% of fresh oocytes produce a baby [2]. In fact, cytoplasmic competence in oocytes, i.e. the ability to produce embryos with high developmental potential, is poorly defined biochemically. Activation of the transforming growth factor-beta [TGFβ] superfamily, the epidermal growth factor (EGF) network, insulin like growth factor 1 (IGF1) and growth hormone (GH) also stimulate oocyte maturation and competence. All of these effectors interact with transmembrane receptors that are associated with kinase activity, and they are involved in the paracrine/autocrine loops that allow the oocyte to regulate its own environment through secretion of oocyte-secreted factors. Recent advances have clearly shown that communication between the oocyte and the follicular cells and *vice versa* is of major importance [3-4]. On the one hand, cumulus cells contribute to oocyte cytoplasmic quality after growth factor stimulation, and on the other hand, the oocyte secretes factors that improve oocyte cytoplasmic competence through the stimulation of cumulus cells. After the first phase of protein and mRNA storage, transcription is regulated during the very early stages of development, via acetylation of histones in the female nucleus. Preparation for translation of the mRNA through polyadenylation also regulates the proper timing of embryo development.

In this chapter we will focus our attention on the growth factors involved in stimulation of oocyte competence. Some of our data obtained for mRNA storage using microarrays in human GV oocytes [5], will be added in proof.

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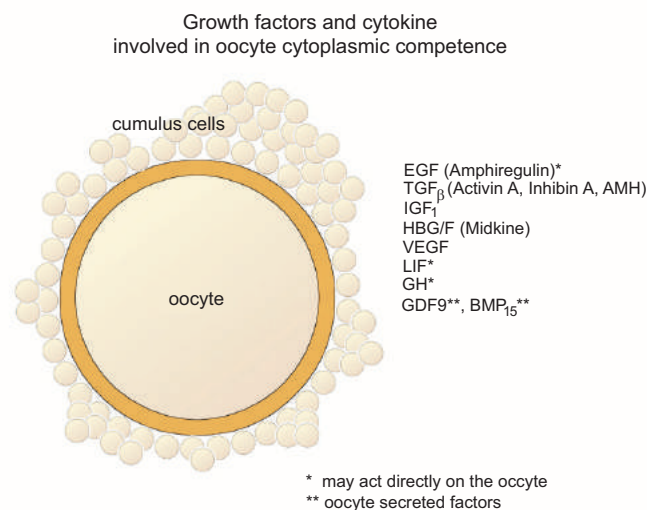## HORMONES AND OOCYTE MATURATION

Gonadotropins: FSH and LH

Gonadotropins exert their effects during the initial steps of final maturation, and induce secondary regulation through a network of growth factors. Both FSH and LH induce maturation via binding to receptors on cumulus or granulosa cells. The FSH receptor is a transmembrane receptor coupled to a G protein receptor (GPCR); its action increases cAMP levels and activates cAMP-dependent protein kinase. Gap junctions are critical at this point, despite the fact that a direct action through an oocyte receptor has been evoked. LH stimulation also involves GPRC receptor and cAMP signalling: however, downstream of this signalling, the cascades for FSH and LH are distinct, with FSH and LH playing a synergistic role during oocyte maturation. [6]. MAP Kinase is activated downstream of both FSH and LH signalling pathways, and the transcription factor involved is mainly cAMP response element-binding (CREB).

Activation of the growth factor networks is more precise: LH signalling involves the EGF receptor pathway, and EGF has a synergistic effect on FSH activity, also acting through activation of MAP kinase.

GROWTH FACTORS, CYTOKINES AND OOCYTE COMPETENCE

These effectors act by increasing transcription, either directly in the growing oocyte, or via the cumulus cells. We will here describe the most important enhancers and their signalling pathways (Fig. **1**)

Growth factors and cytokine
involved in oocyte cytoplasmic competence

cumulus cells

oocyte

EGF (Amphiregulin)*
TGF_β (Activin A, Inhibin A, AMH)
IGF_1
HBG/F (Midkine)
VEGF
LIF*
GH*
GDF9**, BMP_{15}**

* may act directly on the occyte
** oocyte secreted factors

Figure 1: Growth factors and cytokine involved in oocyte cytoplasmic competence

Epithelial Growth Factor (EGF)

Is a key member of the EGF-family of proteins, all of which have highly similar structural and functional characteristics. Apart from EGF itself, other members include heparin-binding EGF-like growth factor (HB-EGF), transforming growth factor-α (TGF-α), Amphiregulin (AREG), Epiregulin (EREG). Betacellulin (BTC) and the Neuregulins (NRGs). *In vitro* experiments have shown that the presence of cumulus cells might be necessary for their activity, but a direct action on denuded oocytes is not excluded [7-8]. This is not surprising, as EGF-R mRNA is highly expressed in the human oocyte. EREG mRNA expression is induced by FSH in granulosa cells and by LH in the mural cells that activate the cumulus cells (autocrine juxtacrine effect). AREG, EREG and BTC are efficient inducers of oocyte maturation, acting even more rapidly than LH. In primates Specific AREG, EREG and BTC appear to be much more efficient than EGF alone [9]. EGF must be present in culture in order to promote maturation and increase FSH activity: it improves oocyte competence in cattle [8-10] and mouse [11]. EGF induces cumulus expansion independently of its effect on meiosis. In human follicular fluid (IVF patients), amphiregulin is much more abundantly expressed than transforming growth factor-α and epidermal growth factor [12]. We found a very high expression of NRG1 mRNA (100x background) in the human GV oocyte [5].

Heparin Binding Growth/Differentiation Factor (HBGDF)

Midkine is a basic heparin-binding growth factor of low molecular weight, and its effect is mediated via cumulus/granulosa cells. Midkine binds to a high affinity signalling receptor associated with Janus tyrosine kinases (JAK). The association of chondroitin-sulphate or hyaluronic acid with midkine increases blastocyst formation rates in bovine IVF by 50% [13]. Hyaluronic acid is synthesized and released by granulosa and cumulus cells under the influence of transforming growth factor beta and oocyte secreted factor(s) [14]. Midkine also has an antiapoptotic role [15].

Insulin Growth Factor (IGF)

The IGF1 receptor is a transmembrane /tyrosine kinase receptor, which has the specific role of increasing the activity of FSH which in turn increases the bioavailability of IGF1. FSH and IGF1 activate the 6-phospho-inositol-3 kinase/Akt, which is involved in survival and development of granulosa cells. It enhances bovine and mouse embryonic development through a mitogenic action, increasing cell number as well as the proportion of embryos developing to blastocyst stage. Its effect on oocyte competence is mediated through cumulus cells; however, mRNAs coding for the IGFs and their receptors are present in human and bovine embryos [16-17]. IGF1 seems to be able to induce developmental capacity of oocytes from prepubertal calves [18]. The mechanism of action is not clear, but after treatment with IGF1, calf embryos appear to be transcriptionally active at the time of genomic activation, similar to the situation that is observed in embryos originating from adult animals; in particular, mRNAs for translation initiation factor and for transport of glucose are increased.

Growth Hormone (GH)

The growth hormone receptor (GHR) belongs to a superfamily of transmembrane proteins. Activation of the GHR signal transduction pathway begins with stimulation of tyrosine kinase JAK2. In the follicle, activation proceeds through MAP kinase activation, but may also occur via G-protein coupled pathways. The stimulatory effect of GH on oocyte maturation and cumulus expansion is mediated by the cAMP signal transduction pathway, and not by the tryrosine kinase pathway [19]. The stimulatory effect of growth hormone is not mediated by IGF-I [20-21]. The addition of anti-IGF-I was found to eliminate the effects of IGF-I on cell number, but did not alter GH effects. In experiments conducted by Izadyar *et al.* [19], although both GH and FSH used cAMP as second messenger, their effect on nuclear maturation differed: GH accelerated nuclear maturation, while FSH had an inhibitory effect. In humans, the GH receptor is present on the oocyte and on cumulus cells [22]. There is no doubt that GH increases oocyte cytoplasmic competence in bovine [21] and in human systems [23-24], at least for poor responders. A direct effect on the naked oocyte has also been observed, as we were able to mature and fertilize naked GV oocytes, with subsequent blastocyst development. After freeze-thawing, these blastocysts resulted in the delivery of a normal female baby [25].

Vascular Endothelial Growth Factor (vEGF)

vEGF also has a signalling mechanism that acts through phosphoInositol 3 kinase. When bovine cumulus-enclosed oocytes are incubated with vEGF *in vitro*, oocyte maturation (10%), cleavage rates and blastocyst formation in bovine are improved [26]. There is no information for other species, including human, regarding a positive effect of vEGF, although it is common knowledge that increased vascularisation and oxygenation of follicles improves oocyte quality.

Leukemia Inhibiting Factor (LIF)

LIF is a cytokine that complexes with gp130 for signal transduction, a receptor shared in common with IL6. It is phosphorylated on tyrosine residues after complexing with other proteins. Phosphorylation leads to association with JAK. However, signalling also includes a MAP kinase pathway. In human oocytes, we found expression of mRNA coding for gp130 and LIF receptor beta. *In vivo*, the action of LIF has been associated with implantation; however, *in vivo* there is also a peak in serum LIF levels immediately before ovulation. *In vitro,* LIF improves oocyte competence in the sheep, rabbit, bovine and human [27, 28]. Granulosa and *Vero* cells, which improve human oocyte maturation and increase blastocyst formation *in vitro*, secrete LIF in large quantities. When added to culture medium, LIF also increases the freezing tolerance of bovine blastocysts [29]. This is in any case a general feature: addition of efficient growth factors/interleukins increases the general tolerance of blastocysts to freeze/thawing.

Activin A, inhibin A, Anti Mullerian Hormone (AMH)

These factors belong to the TGF-β protein superfamily, and also have transmembrane receptor with serine/threonine kinase activity; they interact through SMAD, and may increase transcription. In bovine, they exert a positive effect during maturation, enhancing post cleavage development up to the blastocyst stage *in vitro,* although ultimately the percentage of matured oocytes is not increased [30]. However, a similar effect *in vivo* has not been clearly detected in the human [31].

Melatonin

Melatonin plays a complex role, even not clearly defined in reproductive physiology. A positive effect on oocyte competence has been described in goat, pigs and buffalo [32, 33]. The expression of melatonin receptor mRNAs has been found in cumulus and granulosa cells in porcine follicle [34]. In human, *in vivo* intake of melatonin has been proposed to improve IVF controlled ovarian hyperstimulation. However we were not able to detect any expression for melatonin receptors one (MT1) A and B, neither in the oocyte nor in the cumulus cells [5]. However, this does not necessary preclude a positive role, but apparently not directly, on oocyte competence.

Thyroid Hormones

The thyroid hormones, thyroxine (T_4) and triiodothyronine (T_3), are tyrosine-based hormones, responsible for regulation of metabolism. They have a small molecular weight and lipophilic substances that are able to traverse cell membranes even in a passive manner. Their impact on oocyte competence is a question mark. In fact too high or too low levels of these hormones are deleterious for oocyte competence

OOCYTE SECRETED FACTORS (OSFS)

This is probably the most important finding in relation to oocyte competence [3, 35-36] (Fig. **2**). Significant oocyte secreted factors include Bone morphogenic protein 6 and 15 (BMP 6 and 15) and growth differentiation factor 9 (GDF 9). These factors are members of the transforming growth factor beta family; their receptors in granulosa and cumulus cells are serine/threonine kinases, activating SMAD signalling. According to Sasseville *et al.* [36] EGF-R/MAPK signalling is required in the action of granulosa/cumulus cells. These compounds are secreted in the follicular fluid and they then regulate cumulus cell function in a loop way (Fig. **2**).The oocyte regulates its own environment at least in *in vitro* studies. In fact these experiments have clearly demonstrated that these factors increase the capacity of bovine embryos to reach blastocyst stage by 50% [3, 35]. In our hands, microarray experiments showed that human GV oocytes have a very high expression of mRNAs for GDF9 and a little less for BMP15 and BMP6. Based on these observations, it seems that this mechanism is present in human oocytes and probably in most mammalian species. GDF9 induces hyaluronan synthase 2, a key factor involved in synthesis of hyaluronic acid in cumulus cells [37].

Oocytes secreted factors (OSFs)

cumulus cells

* OSFs are secreted in the enviroment of the COC

** They activates the CC and increase oocyte competence

BMP15
BMP6
GDF9
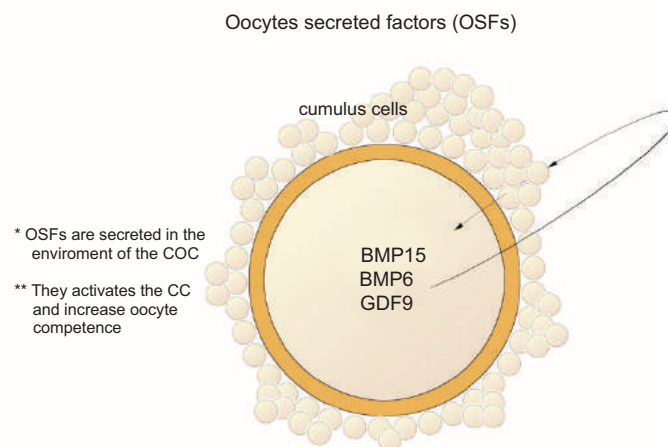
Figure 2: Oocytes secreted factors (OSFs).

RELEVANT METABOLIC PARAMETERS

We should bear in mind that oocyte competence is basically oriented towards the formation of embryos. Further basic prerequisites have to be fulfilled very early during oocyte activation, at the time of fertilization, and immediately after. All the potential of the oocyte can be lost at this point. The tertiary structure of the female nucleus maintains a lower level of transcription through hypoacetylation, compared with the male nucleus. The immediate increase and mobilisation of glutathione is achieved through two ATP-dependent steps: gamma-glutamylcysteine synthetase and glutathione synthetase, both associated with cysteine availability. The impact of glutathione mobilisation on further embryonic development is immediate: increased blastocyst formation rate and increased cell number per blastocyst formed [38]. This mobilisation is mandatory for sperm head swelling, necessary for the formation of a fully developed male pronucleus. It is also necessary for upregulation of glucose metabolism, and increased activity of the pentose phosphate pathway (PPP) then influences the initiation of the first S-phase, both in the male and the female pronuclei, as well as during embryo development up to the blastocyst stage [39-40]. The PPP, apart from the synthesis of C5 sugars, also allows the regeneration of NADP(H). This aspect is particularly important for the recycling of homocysteine, necessary for correct imprinting processes, and for synthesis of thymidine via methylenetetrahydrofolate reductase (MTHFR). This is particularly important in human oocytes [41], where recycling of homocysteine cannot be performed via the cystathionine-beta synthase (CBS) pathway, and cysteine cannot thus be made available via this pathway. The sulphur aminoacids, including methionine, are of major importance at this point, when mRNAs stored during maturation must be activated for translation via polyA tail extension. Beta oxidation and, in general, lipid metabolites are of major importance for a correct oocyte competence: this means also that the quality of the mitochondria, again usually decreasing with age, has also to be emphasized [42-43]. With a poor baseline metabolic capacity, there is no chance to increase the oocyte competence.

The cytoplasmic polyadenylation element (CPE) is necessary for this process. Microarray experiments showed that the poly (A) binding protein is highly expressed in human oocytes [44], at 300x above the background signal. The precise selection and regulation of the mRNAs to be translated and the quality of their kinetic and chronological regulation is crucial to correct development.

The Zygote Arrest (Zar) gene is a further unknown parameter. We found it to be highly expressed in the oocyte as the most intensive signal, at up to 1500x above background. Its functional significance is unclear, but mice lacking Zar are infertile – their embryos undergo cleavage arrest after the first division.

CONCLUSIONS

It must be emphasized that cytoplasmic competence is the key for firstly, correct oocyte activation, and then for fully harmonious embryo development. Acquisition of competence is obtained through multiple complementary but apparently fully redundant pathways. The EGF growth factor family is certainly one of the master elements, but in the human system GH and LIF are also potential tools for co-stimulation with FSH, both *in vitro* and *in vivo*. However, the extensive superposition and interaction of positive factors is not yet understood. These factors increase transcriptional activity in the follicle, and subsequently the mRNA content of the oocyte. However, these mRNAS must then of course be translated with a correct timing, and in precise equilibration with the metabolic pathways necessary for cellular "housekeeping". An additional question might be: do they improve the regulation of translation, and if so, how?

Do these factors intervene for DNA repair capacity? This aspect is of major importance in human assisted reproduction technology in particular. Similarly, do these factors potentially counteract the effect of female age? The answer to this point in humans is probably yes, if we consider the results obtained with GH in poor responder patients.

Although we are able to define the mode of action of signalling and describe some factors that increase the developmental capacity of the oocyte, clearly the mechanisms of action are yet to be defined. An understanding of this aspect is crucial to increasing the yields of biotechnologies related to reproduction and ART technology. Moreover, all the benefits of the factors involved can be compromised if cysteine and glutathione are not adequately

mobilised for their role in sperm head decondensation, and in general, if the pool of endogenous metabolites is suboptimal or borderline. This will result in poor oocyte activation, due to poor activity of the enzyme responsible for PI cycle mobilization, calcium release and subsequent perturbation of calcium oscillations: either an excess or a reduction may sooner or later affect embryo development [45]. Finally, an inadequate capacity for the elongation of poly (A) mRNA tails and its regulation will lead to asynchrony in translation and loss of stored mRNAs, one of the major causes of embryo developmental arrest at the time of genomic activation or immediately after.

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Genomic Regulation through RNA in Oocyte Maturation of Large Mammals

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Abstract: The oocyte is a unique cell amongst the 212 cell types that makes an individual. This cell does not divide until it resumes meiosis and remains in a special chromatin status during the dictiate stage of the prophase from oocyte formation in the gonad in the female fetus until it dies through apoptosis or proceeds to ovulation. This status requires unique features to allow transcription to be active during oocyte growth and an automatic pilot system to drive the transition from tetraploidy to haploidy during maturation and fertilization and back to diploidy, all this in a few days in large mammals. Therefore, the regulatory program for all the transformations required for chromosome separation, cell cycle progression, response to sperm entry, and embryonic genome activation must be stored in the oocyte prior to ovarian release or even prior to final chromatin condensation as it inhibits further transcription. The data related to gene regulation during this period is limited for two main reasons: limited amount of material to study in mammals and differences with somatic tissues where gene pathways are much better characterized. Nevertheless, using the genomic amplification approaches and the increasing amount of information in somatic tissues and in oocytes from lower species, it is becoming possible to study this automatic pilot system that drives the mammalian oocyte through maturation-fertilization and embryonic genome activation. This chapter will focus on the progression of our understanding of the oocyte using proteomic and transcriptomic tools.

INTRODUCTION

The oocyte is quite a unique cell. Phenotypically, it is the largest cell of mammalian body, although it is rather small compared to eggs from lower vertebrates or birds. But this cell is not only unique in the way it looks, it is also unique in the way it works. It is not clear if the oocyte's storage capacity is a remnant of evolution to ensure that there is enough nutrients until the embryo can find a way to extract its food from the uterus through the trophectoderm or eventually the placenta, or whether it serves other purposes. The meiotic maturation requires that the chromosomes condense, creating a temporary transcriptional inhibition as in normal mitosis but there is also a requirement to wait for the sperm entry to resume any possible function. Cell division and the associated chromatin condensation normally last a few minutes to a few hours in somatic cells while in the oocyte, the process may take several days. In that context, the storage capacity of eggs may ensure the proper chromatin processing through this period rather than simply providing energy reserves.

In Xenopus, the oocyte's constituents are sufficient to bring the early embryo to the mid-blastula transition several hours post-fertilization and through hundreds of cell cycles, while in the mouse, embryonic activation also occurs a few hours post-fertilization, at the 2-cell stage [1]. In larger mammals such as the cow or humans, embryonic activation occurs at the third or fourth cell cycle, several days post-fertilization, which leaves the early embryo under the control of stored proteins and RNA. Another major difference in larger mammals is the fact that oocytes are not fully competent at the end of the growth period in contrast to the mouse. In pigs, sheep, cows, horses, and humans, oocytes from smaller follicles, although fully grown, may lack the competence for forming a viable embryo, but most full-size oocytes can resume meiosis when cultured *in vitro*.

It is postulated that the oocyte must acquire specific developmental competence through the accumulation of either proteins or RNA to ensure future development [2]. This has been demonstrated phenotypically and in culture experiments, but there are actually no defined mechanism that can explain the increase in developmental competence of oocytes from larger follicles.

One rationale that could explain why oocytes are not competent is that they might be dangerous for the ovary.

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Indeed, if the oocyte acquired full totipotency and remained stuck in the follicle where the process of luteinisation permits rapid cell and tissue growth for the formation of the corpus luteum, the possibility of achieving the right conditions for cancer-type consequences is real. The best evidence of this is the mouse KO of the *cMOS* gene, that enables oocytes to activate without the presence of sperm and results in the early death of females from ovarian cancer [3]. Logically, the only way that the ovary can protect itself from an oocyte is through ovulation and, in the same logic, the appearance of LH receptor is associated with increased competence/dominance in follicles of larger mammals [4].

Using the bovine model, we have explored over the past decade, the use of proteomic and genomic tools to address the potential differences in stored proteins or RNA associated with developmental competence [5, 6]. Although this is a work in progress, collectively these experiments are allowing the formulation of a potential hypothesis linked to the control of the cell cycle machinery. The competent oocyte has accumulated enough cell cycle products such as cyclins to reach the embryonic transition while the incompetent oocyte has not.

In the next few pages, we will review the information in relation to the accumulated proteins and RNA in the bovine model to support the above hypothesis.

OOCYTE MATURATION: PROTEIN CONTRIBUTION

Protein synthesis is the major outcome of gene expression and is directly linked to the phenotype that is observed, which is not always the case with RNA. In light of this statement, it becomes clear that protein analysis should be the preferred endpoint of all physiological analysis. For this reason, our laboratory invested time and efforts in the past decade to improve the data on protein content and function in maturing bovine oocytes as well as early embryos [5, 7].

The first limitation of proteomic analysis is the requirement for large amounts of material. Using conventional gel electrophoresis analysis, 2,000 to 4,000 oocytes are required per gel depending of the stain used; silver nitrate or colloidal blue. With these stains, some 500 proteins can be visualized as single spots; the newer dyes (Cy-3, Cy-5) achieve close to 1,000. Although this number may seem interesting, it has to be put in the perspective with the theoretical number of proteins expected in a given cell, estimated to be over 100,000. In a recent deep sequencing experiment done with more than 1,000,000 reads of bovine oocytes and early embryos, a total of 14,422 genes were found out of a total bovine genome of 24,000 RNA-producing genes (results not shown). If the cow is similar to the human in the number of isoforms per gene, we should expect at least an average of 3.2 proteins per gene, for a total of about 50,000 different RNAs, and consequently, at least the same number of different proteins excluding post-translational modifications [8]. Therefore, looking at 500 or even 1,000 proteins on a gel is clearly a very small portion of the proteome.

Another approach that allows the monitoring of changes and reduces the amount of material required is radio-labelling. Using ^{35}S-methionine, our laboratory has monitored the appearance of new proteins every 4 hours during the *in vitro* maturation of bovine oocytes [7]. With this experiment, we were interested in the kinetics rather than the identification of all these newly synthesized proteins. The first observation was that the image of the newly synthesized proteins was completely different than the gels made with the native oocyte proteins. This means that the stockpile of protein in the oocyte is important and, if we compared to matured oocytes (Metaphase II) or early embryos (pre-MET), the image was truly similar, indicating that the most abundant forms do not change much during these early steps. In the Massicotte study [5], we used a gel of 3,000 metaphase II oocytes to match the embryonic gels and to allow the microdissection of the spots for further identification, re-enforcing the fact that the image of constituent proteins does not change much until the embryonic transition in the cow.

The production of new proteins therefore represents a minimal portion of the stockpile. Looking at the protein synthesis pattern in the first 4 hours, we then calculated the percentage of remaining spots on the total averaged gel; the values obtained were 100%, 65%, 60%, 47%, 38%, 42%, and 34% for time intervals of 0–4, 4–8, 8–12, 12–16, 16–20, 20–24, 24–28 hrs, respectively [7]. As with the total number of proteins detected per gel per time point, it went from around 600 in the first 4 hours to less than 400 at the end of maturation (24 hrs). These two data sets indicate that more than half of the proteins made at the beginning of maturation are not made anymore at the end of maturation. It can also be noticed that the absolute number of new proteins is decreasing during maturation.

More recently, a vast mass spectrometry analysis was done on bovine oocytes with the cumulus cells. This study revealed that 4,395 proteins were expressed in cumulus cells while 1,092 proteins were expressed in oocytes. Further, 858 proteins were common to both the cumulus and the oocytes [9]. This new powerful approach which more than doubles the number of targets to analyse is suitable for a one-time measurement but rather difficult/expensive to use for multi-comparison analysis or kinetics evaluation of protein amounts or functions. One other limitation of the proteomic approach is the limited information related to the protein database for cow or other large mammals. It is clear that the oocyte contains an overwhelming proportion of unknown proteins and RNA-related genes compared to other tissues [10-12]. The human proteome is much more complete but the quantity of material is obviously limited with human oocytes.

Therefore, it would be adequate to conclude that proteomics is still a very limited source of information for understanding mammalian oocytes, although the final understanding of the physiological process requires protein confirmation. This is very well illustrated by a recent paper by Siemer *et al.* [13] where the complexity of the phenotype depends on several proteins of the elongation factor 4 family but also on phosphorylation cascades, to name only one of the post-translational modifications. It is clear that protein analysis, either through identification (Mass Spectrometry or Wetern Blot analysis) or phenotyping (immunohistochemistry, gel shift), remains the final validation of all genomics approaches. It is surprising to see how many authors are still interpreting the phenotype based on the amount of RNA without protein confirmation despite several demonstrations that an inverse relation can often be observed between mRNA levels and protein levels [14]. In one specific case, where we compared RNA levels to protein levels for MSY2 and HMGN2, the same RNA pattern was observed but a completely opposite protein pattern was noted in addition to the cytoplasmic-to-nuclear shift of HMGN2 in bovine 8-cell embryos, potentially indicating a functional role of this transcription factor at the time when the RNA level was minimal.

OOCYTE MATURATION: RNA CONTRIBUTION

Difference in RNA Processing in Oocytes vs. Other Tissues

The oocyte is a very unique cell in the way it processes RNA. Normally, RNA is an intermediate step between a nuclear instruction and the production of a specific protein in response. Therefore, in somatic cells, the level of messenger RNA (mRNA) is a relatively good indicator of the presence of a given protein. The final activity of the protein may require other modifications, such as phosphorylation or acetylation, to be active and consequently, the outcome may be different than the measurable RNA fluctuations. But in oocyte, something special occurs: the requirement for chromatin condensation important for the two successive meiotic divisions impairs transcription and rapidly shuts down the production of any new RNA. This special transcriptional status will last until embryonic genome activation, which occurs rapidly in mouse but much more slowly in larger species such as human or cow (4- and 8-cell stages respectively), although some minor transcriptional activity has been reported in cow [15]. Our recent analysis indicates that several transcripts may be activated by poly-adenylation in the transition phase between the oocyte and the embryonic genome [16].

Uniqueness of Oocyte Genes Compared to Other Tissues

Our laboratory has been interested in the identification of oocyte genes that are highly conserved throughout evolution. For this purpose, we have initially made three different subtractive libraries of oocytes and five somatic tissues in Xenopus, mouse and cow [10]. Not only we did compare the sequences of these libraries, but we used micro-array hybridization to assess the largest number of genes that could be considered as oocyte-specific and conserved [11]. We have confirmed several known genes such as *GDF9*, *BMP15*, *ZP1-2-3*, *MATER*, *Fig1a*, *NPM2*, and *ZAR1*, but revealed close to 100 more genes, most unknown, that now need to be explored. It is obvious that several regulatory elements such as MSY2 [16] have been conserved and would likely play a similar regulatory role from amphibians to mammals. This piece of information supports the importance of comparative biology in our quest to understand the functioning of the oocyte. Although gamete changes are associated with the process of speciation [17], it seems that many important pathways have not been modified for millions of years, like the CDC2 gene in yeast that works in mammals [18] or the LH receptor that is already present in oysters and responds to human placental gonadotropins [19].

Accumulation of RNA in Mammalian Oocytes During the Growth Phase

The transcriptional activity within the oocyte starts to increase significantly when the follicles reach the secondary phase of development. Before that stage, there is little ^{3}H-uridine incorporation but at the secondary stage, the fibrillo-granular nucleoli are getting labelled as well as the nucleoplasm, indicating transcriptional activity [20-22].

With the same type of incorporation approaches, it was shown that significant transcriptional activity increases in the oocyte at a diameter of 80-100 μm, but then decreases at 110 μm to an almost complete arrest when the oocyte is fully grown around 120 μm. [20, 23]. The nucleolus also changes its morphology from fibrillo-granular with loosely compacted chromatin to dense fibrillar and compacted chromatin as the oocyte gets closer to ovulation or final atresia [23].

Our laboratory and others have shown that during maturation, there is a small period of transcriptional activity which decreases rapidly just before GVBD and is virtually absent when the first metaphase is reached [24, 25]. During this period, it has been shown that the cumulus cells produce some essential transcripts for the meiotic resumption as the use of transcription inhibitors does not allow GVBD [26].

The transcriptional repressive state is maintained until the embryonic genome gets activated, although some minor activity has been detected in bovine embryos before the 8-cell stage [25, 27]. At that time, protein synthesis increases rapidly and the nucleolus becomes fibrillo-granular again [20, 28]. The maternal-to-embryonic transition (MET) is characterised by a decrease in mRNA of maternal origin and an increase in mRNA of embryonic origin. The inhibition of transcription during that period results in developmental arrest at the 8-cell stage in cow [29]. As stated above, this transition occurs at different times in mouse (2-cell), pig (4-cell), and human (4- to 8-cell) [30].

In cow as well as in other mammals, the mechanism of transcriptional inhibition is not very well understood. In Xenopus, it seems that the transcriptional machinery is rather deficient [31, 32]. In addition, the chromatin structure or status may have a direct impact on polymerase access [33, 34]. Hypoacetylation of histones has been mentioned as a possible cause [35]. More recently, the hypothesis that repressive elements must be either destroyed or diluted has been presented in mouse [33, 36, 37]. This transition allows the destruction of maternal transcripts and the replacement by new embryonic mRNA [38].

In response to specific UTR (untranslated region) embedded instructions, cytoplasmic adenylation is a known mechanism that controls gene expression without the requirement for the transcriptional machinery that could/would be disturbed by the rapid changes that occurs at the chromosome level during maturation and fertilization. The system allows the translation of specific mRNAs according to a combination of cytoplasmic codes acting on RNA-associated proteins often through the 3' UTR of the stored RNAs. Translation permissiveness seems to be associated with a longer polyA tail (from 80-150 and more) while shorter tails (around 20 As) are repressed from translation [39]. Although pre-RNA made in the nucleus receives a long tail, it is rapidly shortened during downstream processing or degradation [40, 41]. The resulting RNAs are stored or eventually re-adenylated or degraded [42, 43].

As mentioned above, maternal RNAs are not all used upon transcription but some are stored with a short polyA tail. This storage is associated with ribonucleoproteins (mRNP) which repress translation. In Xenopus, there is more information about the different proteins involved in translation repression, such as maskin. This protein associates with cytoplasmic polyadenylation element binding protein (CPEB) located in the 3' UTR region of mRNAs that contain the cytoplasmic polyadenylation element (CPE). This association represses translation through the inhibitory action of the maskin EIF4E located at the 5' end of the RNA [44]. According to Richter, translation begins with CPEB phosphorylation which dissociates eIF4E from maskin and opens the way for polyadenylation and recruitment of the poly (A) binding proteins (PABP) and eIF4G to initiate the translation process. A very recent paper by Siemer *et al* [13] indicates that the machinery involved in the initial mRNA processing, namely the EIF4 elongation factor family and binding proteins, although present in bovine oocytes, are tightly modulated by the changing phosphorylation status of the different members, supporting a more complex cytoplasmic control than previously thought. These new results bring more support to a detailed translation plan embedded in the 3' and 5' UTRs of oocyte transcripts.

A recent paper by Piqué [45] summarizes the process of translation inhibition. The maternal RNA must have at least two CPEs in in 3 'UTR. These sites must be separated by 10-12 nucleotides and if there is a Pumilio-binding element (PBE) upstream of CPE, the repression is even greater. The same author proposes that the maskin protein is recruited by CPEB dimer, which would explain the importance of the distance between the 2 CPEs.

When oocyte maturation is triggered, some stored RNAs are released and polyadenylated while others are kept for later use. Indeed, mRNA for the protein cyclin B1 is required on several occasions during the maturation and early embryonic development process and therefore requires multiple activation since the protein is destructed at each cell cycle [46]. In several animal species, the repression of translation is correlated with a shortening of the poly A tail [47]. It is the best known mechanism for the regulation of maternal mRNA [48].

When released, the mRNA is either degraded or polyadenylated, particularly if there is a CPE present in the 3'UTR. The distance between the hexanucleotide signal of polyadenylation (HEX) and the CPE determines the efficiency of translation. Indeed, translation is maximal when the distance is 25 nucleotides and decreases when the distance reaches more than 100 nucleotides (87).

In frogs, the maturation trigger progesterone activates the phosphorylation of CPEB by Aurora A kinases, stimulating the recruitment o*f cleavage and polyadenylation specificity factor (*CPSF) on HEX to induce polyadenylation. It is not confirmed if the same cascade is present in mammals, although orthologs of the principal proteins have been identified [44, 49, 50].

Other proteins involved in mRNA storage in frogs are the Y-Box family. In *Xenopus*, FRGY2 is quite abundant and well characterized while in mammals, the MSY2 protein is believed to be a functional ortholog. MSY2 has been described in the mouse [51-53] and more recently in the cow [14]. In the male gamete where MSY2 is active, it has been suggested that it stabilizes the mRNA through a specific sequence [54]. Females deficient in MSY2 are sterile and several abnormalities are present both in oocytes and follicles [55]. In mouse [56] and cow [14], the presence of this protein decreases rapidly following the embryonic genome activation and the knock-down oocytes accumulate less mRNA [57].

Importance of Specific Maternal RNAs in Embryonic Development

Several maternal genes are clearly essential for further embryonic development. The use of KO mice for some of them has resulted in embryonic arrest [58-61]. Although their role may not yet be completely understood, they are believed to be essential elements of the embryonic genome activation. It is easier to assess the importance of a given gene when the knock-out creates a specific phenotype. When the absence of a gene results in the blockage of embryonic development as with *mater* or *stella*, it is clear that the role played by the associated protein is crucial but it does not mean that this is the mechanism by which the ovary controls oocyte competence. The absolute mRNA level of these genes does not seem to be associated with lower developmental potential in immature oocytes, thus forcing the quest in other directions. Over the past several years, the analysis of mRNA levels in different conditions where oocytes are compared according to their further capacity to produce an embryo, has led to the identification of several genes as potential key players in competence acquisition [62, 63]. Surprisingly, many of the genes identified are related to the cell cycle and chromosome segregation: Cyclin B1, Cyclin B2, PTTG1 (pituitary transforming growth factor or securin). Collectively, these results point to a new hypothesis in developmental competence regulation: some oocytes have enough ammunition (cyclins) to go through several cell cycles while some oocytes do not. This hypothesis looks very simple but it might explain why development arrests at 1, 2, 3, 4, 5 or 6 cells and not always at the MET stage (8 cells in cow). This is the reasoning that forced our analysis of the 3'UTR of cyclin B1 in cow and the discovery of new UTR isoforms associated with a potential shift in translation during and after maturation in cow [46].

To confirm the importance of cyclin B1 amount in oocyte, we explored the use of RNA interference to lower the cyclin amount in bovine oocytes. Although the phenotype was supportive of our assumptions, *i.e.* treated oocytes would block and self-activate in the presence of 6-DMAP and low cyclins, it is not possible to conclude that the resulting arrest in embryonic development is the mechanism that the ovary uses to restrict quality. Because cyclins are degraded at each cell cycle, the fine analysis of the consequences of having less of a given isoform awaits further experiments.

In addition, the presence of microRNAs in oocytes [58] offers a new possibility of mRNA abundance control. Several studies have explored the role of microRNA in embryonic development either through the gene KO induction with Dicer, the enzyme that makes the final functional form or micro RNAs or the Argonautes proteins [64-67]. Among the known microRNAs, the majority are believed to act through the 3' UTR of mRNAs and control the rate of translation/degradation resulting in altered functions [68]. More recently, it was shown that microRNAs can not only destroy a gene expression cascade but promote the translation of some element which may have ARE (AU-Rich Elements). These AREs are, for example, present in the 3' UTR and may increase translation of tumor necrosis factor α (TNFα) [69].

Given our actual knowledge of the modulation of mRNA levels, it is likely that the optimal cascade of events leading to a normal embryo is the result of a well balanced equation as illustrated in (Fig. **1**), where the essential mRNA has to be present in sufficient quantity and protected from degradation. Other inhibitory RNAs may have to be destroyed or their product diluted with the increasing amount of DNA at each cycle to permit embryonic activation (Svoboda) and a large portion of the maternal RNA must be degraded for the proper functioning of the embryo. These regulatory mechanisms would all be cytoplasmic (until it is shown that microRNAs are produced before the MET) and their cascade would be triggered by ovulation and the second wave by fertilization.
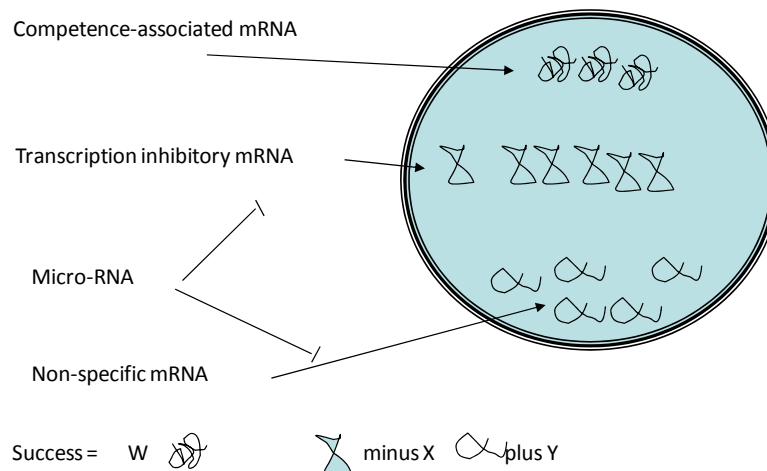
Figure 1: Influences of the accumulation and processing of important mRNA involved in the ability to achieve developmental competence.

ACKNOWLEDGEMENTS

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Meiotic Regulation by Maturation Promoting Factor and Cytostatic Factor in the Oocyte

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Abstract: In most of vertebrates, mature oocytes arrest at the metaphase of the II meiotic division, while some invertebrates arrest at metaphase-I, others at prophase-I. Fertilization induces completion of meiosis and entry into the first mitotic division. Several experimental models have been considered from both vertebrates and invertebrates in order to shed light on the peculiar aspects of meiotic division, such as the regulation of the cytostatic factor (CSF) and the maturation promoting factor (MPF) in metaphase I or II. Here, we reviewed the role of CSF and MPF and their biochemical pathways in regulating meiosis completion. Differences and similarities existing within several model systems of invertebrates, such as ascidians, cnidarians, mollusks, starfish, will be analyzed and compared to meiotic regulation in *Xenopus*. Data will be analyzed at the light of the phylogenetic conservation of MPF and CSF functions, accordingly to the position of these organisms in the evolutionary tree.

INTRODUCTION

The development from immature oocyte to a fertilizable gamete is a process called meiotic maturation [1-2]. Meiosis is the specialized nuclear division of germ cells. After a single event of DNA replication, in meiosis two nuclear divisions are necessary to produce four haploid gametes from each diploid cell, whereas in mitosis each diploid cell produces two diploid daughter cells. Several differences also exist between meiosis and mitosis in terms of checkpoints controls, DNA replication, dependency on external stimuli, and regulation of cell cycle control proteins [3-4]. One of the main difference existing between the mitotic and meiotic cell cycle, is the ability in the latter to block the oocyte at precise phases of the cell cycle until a specific stimulus (*e.g.* hormone, or sperm) removes the block. In somatic cells, a state of physiological quiescence, or cell cycle block is also described as the G0 phase of the cell cycle. However, G0 differs with respect to the meiotic blocks in terms of cell cycle regulation and the activity of the key kinases that maintain the arrest. Most oocytes block in meiosis twice (reviewed in refs [1, 2, 4-6]). Generally, the first block is at prophase I (P-I); at this stage, immature oocyte is characterized by a large nucleus called germinal vesicle (GV) and the first sign that marks a maturing oocyte is the GVBD (germinal vesicle breakdown), or rupture of the nuclear membrane. In most vertebrates, mature oocytes are blocked in metaphase of the second meiotic division (M-II), whereas in many invertebrates a block occurs in metaphase I (M-I), in others, at the pronuclear stage (G1). Fertilization induces meiotic completion. In some species like the mollusc *Spisula solidissima* and echiuroids *Urechis caupo*, maturation is directly promoted by fertilization [7], and oocytes complete both meiotic divisions without interruption. On the opposite, when maturation is induced by other stimuli, such as 1-methyladenine in starfish, serotonin in molluscs, progesterone in frogs, gonadotropins in humans (see text below), three main features can be observed related to meiotic arrest: (i) block in M-I, as occurs in insects, tunicates and other invertebrates; (ii) block in M-II, as in most of vertebrates; (iii) block in G1 of the first mitotic division as occurs in sea urchins and jellyfish [4, 7] (Fig. **1**). In all species so far investigated, the maintenance of the metaphase block and the transition from prophase to the next phase is controlled by two interacting biochemical complexes: MPF (maturation promoting factor) and CSF (cytostatic factor). MPF is active in the cytoplasm of arrested frog oocytes, and is able to induce maturation when injected into a quiescent (P-I) oocytes. Now, it is well known that MPF consists of two main components, a catalytic subunit, the serine/threonine kinase Cdc2/Cdk1, and a regulatory subunit cyclin B [8]. MPF activity increases after GVBD and is maximal during M-I and M-II, while decreases in the lag period between M-I and M-II, and at the exit from M-II. The majority of data concerning MPF regulation during fertilization is from vertebrate models (frogs, mice). However, the same pattern of activation has been

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described in all vertebrates and invertebrates so far investigated [2, 4]. As for MPF, CSF was firstly evidenced in a bioassay where cytoplasm, taken from an unfertilized egg, and injected into one blastomere of a two-cell embryo, caused arrest of the cell division in metaphase [7, 9]. Unlike MPF, the biochemical characterization of CSF has been extensively pursued, but, it remains elusive.
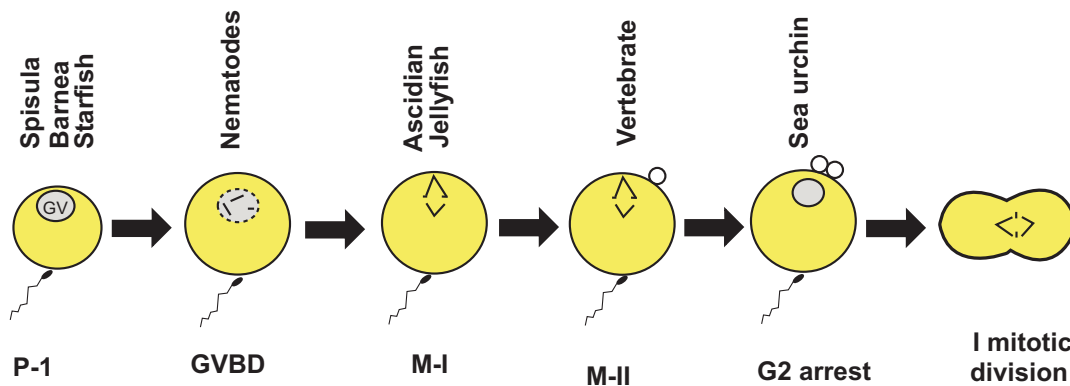
Figure 1: Meiotic arrest in oocytes of different species (see text for details).

Oocytes and embryos of African clawed frog *Xenopus laevis* have been the most widely used model systems in studying cell cycle regulation and meiotic maturation in the early phases of development in vertebrates [10]. Starfish [11] and molluscs [12] have been selected models to study maturation and meiosis in invertebrates over the last thirty years. More recently, hydrozoan jellyfish, *Clytia hemisphaerica* [13] and ascidian *Ciona intestinalis* [14-16] have been proposed as model to study meiotic regulation. *C. intestinalis* has been also largely used as model for developmental biology since the last century [17-19] and the recent publication of a draft copy of its genome provided new insights into origin and evolution of chordates [20-21].

Here, we describe the molecular mechanism regulating oocyte maturation and meiosis completion in invertebrate compared to lower vertebrate, such as *Xenopus* and fishes.

REGULATION AND CHARACTERIZATION OF MATURATION PROMOTING FACTOR (MPF)

The convergence of multidisciplinary experimental approaches implemented in different models such as yeast (genetic approach), sea urchin and *Xenopus* (embryological approach) allowed the identification of molecules and mechanisms regulating cell cycle progression [22- 23]. In 1971 the cytoplasm transfection experiments performed by Masui and Markert during meiotic maturation of amphibian showed the existence of a factor able to promote entry into meiosis, which they defined MPF [9]. In the late eighties, MPF activity was measured into dividing somatic cells from yeasts to mammals and was renamed M-phase-promoting factor to indicate its involvement in inducing metaphase in all eukaryotic cells [24]. The molecular characterization of MPF was shown through genetic screening in yeasts *Schizosaccharomyces pombe* and *Saccharomyces cerevisiae*, where *cdc2* and *CDC28* genes, coding for homologous proteins of 34 kDa, respectively, were identified as regulators of the M and S phases [25-26]. At the same time, Evans *et al.* identified in mollusc bivalves and sea urchin a class of proteins named cyclins, which were synthesized in the early stages of embryonic division and whose expression levels oscillated during the cell division showing peaks at each metaphase [27]. With the convergence of these studies, the molecular nature of MPF was defined as a complex of the serine/threonine kinase Cdk1 (also called Cdc2, as homologous to the protein encoded by the *cdc2* gene in *S. pombe*) and a regulatory subunit, cyclin B [28-31]. The binding of Cdk1 to cyclin B is necessary but not sufficient for the kinase activity of the complex. During the cell division, MPF activity is regulated through mechanisms of phosphorylation/dephosphorylation of its catalytic subunit [30]. The phosphorylated activators Cdk1 can be phosphorylated on a residue of threonine, Thr-161 (position refers to the human sequence of the protein), which promotes stability and activation of the complex [32]. The protein complex Cyclin-dependent kinase Activating Kinase responsible for this event is known as CAK and it is composed of a catalytic subunit (Cdk7 and MO15) and the regulatory cyclin H [33]. Dephosphorylation of Thr-161 is essential for the inactivation of Cdk1 at the exit of M phase [34-35]. Several proteins are involved directly or indirectly in this process: a type 2A phosphatase, named INH [36], phosphatase 2C [37], the protein Kap [38] and a phosphatase type

1 [35]. During S phase, newly synthesized cyclin B binds to Cdk1 to form the Cdk1/cyclin B complex, known as pre-MPF, where the MPF kinase activity is inhibited by inhibitory phosphorylation on residues Thr-14 and Tyr-15 in the ATP binding domain of Cdk1. Phosphorylation of these residues is carried out by Wee1 and Myt1 kinases [39, 40]. Wee 1 kinase phosphorylates Tyr-15, while Myt1 is able to phosphorylate both residues [41]. These two kinases are activated by dephosphorylation and inactivated by phosphorylation [42-43]. Several kinases have been identified as responsible for this phosphorylation: Akt [44], p90[Rsk] [10, 41], nim1 [41, 45] and Plk, a Polo family kinase [10, 46]. These kinases inhibit Myt1 and Wee1 restoring the dynamic equilibrium between MPF inhibitory kinases (Myt1 and Wee1) and Cdc25, the phosphatase which activates MPF (Fig. **2**). The Cdc25 family of phosphatases (Cdc25A, Cdc25B and Cdc25C in humans) are able to remove inhibitory phosphorylation from Tyr-15 and Thr-14 residues [47-48]. Cdc25A is classically described as an active regulator in the transition G1/S, whereas Cdc25B and C are active in G2/M. In *Xenopus* oocytes Cdc25C activity is regulated by proteolysis and phosphorylation of a conserved serine, Ser-287 (Ser-216 in the human protein) [49-51]. Phosphorylation of this residue causes binding of 14-3-3 proteins, a class of factor which specifically recognize phosphorylation residues in cell cycle regulatory proteins [50, 52]. Several kinases are involved in the phosphorylation of Ser-287. The first two to be identified have been Chk1 and Chk2 kinases [51, 53], both responsible for cycle arrest at the checkpoint active in G2. However, the essential kinase phosphorylating Ser-287 and maintaining the G2 block has been identified in *Xenopus* oocytes and corresponds to PKA (cAMP-dependent protein kinase A) [54]. Other kinase able to phosphorylate the 14-3-3 binding site of Cdc25 include C-TAK, identified in human cells [53] and the calmodulin dependent kinase (CaMKII), responsible for the calcium release after fertilization [55]. During G2/M transition, Cdc25 is activated by two independent, but functionally related events: dephosphorylation of Ser-287 and phosphorylation of Ser-193/Ser205. In *Xenopus*, release of 14-3-3 frees phosphorylated Ser-287 which can be potentially dephosphorylation by two phosphatases, PP1 and PP2A [55]. Recent studies, indicate that PP1 is likely responsible for Ser-287 dephosphorylation *in vivo* [56]. Phosphorylation of a different residue, Thr-138, probably due to Cdk2, could precede the release of 14-3-3 proteins, promoting Ser-287 dephosphorylation. At GVBD stage, dephosphorylated Cdc25 becomes phosphorylated by both Xp38, a member of the *Xenopus* MAPK family, on Ser-205, and by Plx1 on Ser-193. The order of these events has not been clearly established, but it is supposed that Ser-205 phosphorylation precedes Plx1 activity on Ser-193. Once Cdc25 has been phosphorylated by these two kinases, activates pre-MPF, which, in turns, phosphorylates Cdc25 on Thr-48 and Thr-67 triggering a mechanism of MPF self-amplification which promotes the passage through M-I and M-II [55] (Fig. **2**).
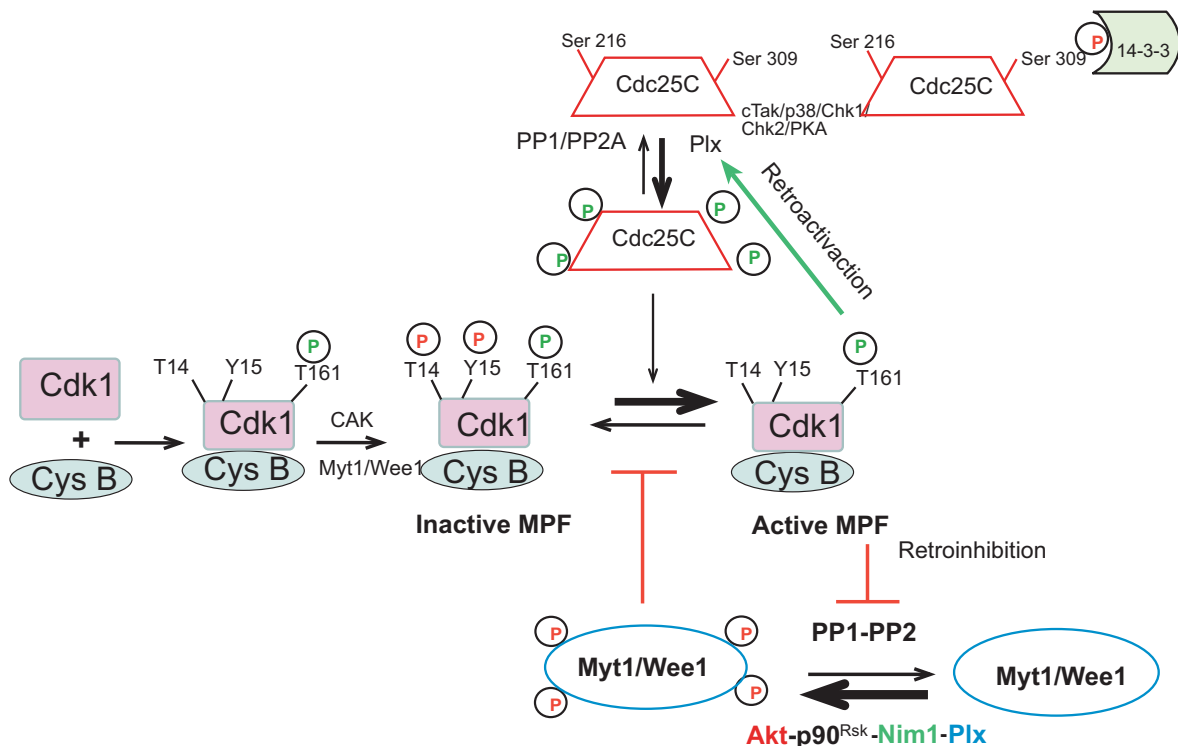
Figure 2: Regulation of MPF during maturation (see text for description)

REGULATION AND CHARACTERIZATION OF CYTOSTATIC FACTOR (CSF)

In the same work describing for the first time the existence of MPF [9], Masui and Markert also reported that injection of cytoplasm taken from *Xenopus* oocytes arrested in M-II in a blastomere at two-cell stage, blocked division of the injected blastomere; therefore, they supposed the existence of a cytoplasmic factor, present in mature oocytes, able to inhibit cell division [9]. Functional tests of CSF activity have been described from decades [9, 57] and they have been confirmed in model systems of vertebrates (*Xenopus, Rana*) [58-60]. Unlike MPF, the biochemical characterization of CSF has been intensively pursued, but remained elusive. According to recent reviews, the three main features necessary to define CSF activity from a functional point of view are: (i) CSF must appear during oocyte maturation; (ii) CSF must be present during M-II arrest (at least in vertebrates); (iii) CSF must be degraded at the time of fertilization [7, 61-62]. From a molecular point of view, CSF includes Mos kinase, a 39-kDa protein encoded by c-*mos* gene, a cellular proto-oncogene [63]. Mos is a Ser/Thr kinase originally identified as cell homologue of the *v*-mos viral gene product, which is responsible for Moloney's murine sarcoma [64] and is expressed specifically in the ovary and in the testicle [65]. In 1989, it was discovered that Mos was required for the maintenance of CSF activity, as the degradation of the c-*mos* mRNA coincided with the loss of CSF activity [63]. Subsequently, the essential function of Mos has been confirmed in all vertebrate oocytes investigated, where it is responsible for the M-II arrest, operating as an essential component of CSF [5, 63, 66-69]. In the same years, it was demonstrated that Mos was responsible for activation of p42 MAPK during oocyte maturation [70-72]. This suggested that MAPK might be a different modulator of Mos activity, as confirmed later in mammals. In fact, in mos$^{-/-}$ mice, MAPK was not activated in oocytes [73]. Previously, MEK1 dual kinase from the same pathway was identified upstream of MAPK and able to phosphorylate and activate both tyrosine (Tyr-185) and threonine (Thr-183) residues in the regulative loop of MAPK [74-76; 77-78]. In fact, MEK1 injection in immature *Xenopus* oocytes caused the activation of MAPK, while inhibition of MEK1 prevented oocyte maturation induced by progesterone [71]. The phosphorylation cascade is initiated by Mos which activates MEK1 by phosphorylating Ser-218 and Ser-222 residues [71, 79]. Later it was noticed that MAPK phosphorylated ribosomal S6 kinase, p90^{Rsk} [80]. In mammals, three different isoforms p90^{Rsk} have been identified, named Rsk1, Rsk2, Rsk3, while in *Xenopus*, only two, Rsk1 and Rsk2, are present, both active during oocytes maturation [81]. The molecular feature of CSF seems to be defined by the pathway Mos/MEK1/MAPK/p90^{Rsk} (Fig. **3**). During oocytes maturation in *Xenopus*, each component of this pathway is synthesized or activated after progesterone-induced maturation. Furthermore, an active form of each factor is sufficient to induce oocyte maturation [61]. At fertilization, owing to the increase in the cytoplasmic concentration of calcium ions (Ca^{2+}), Mos is degraded and its mRNA is deadenilated, while other components of the CSF pathway are inactivated by dephosphorylation, suggesting that in *Xenopus*, Mos is the only MAPKKK (MAPK kinase kinase) active during oocyte maturation [61]. The regulative pathway activated by Mos, and therefore responsible for the CSF-mediated M-II arrest, operates by inhibiting the activity of Cdc20/p55^{CDC}/Fizzy, an activator of the anaphase-promoting complex or cyclosome APC/C, which degrades cyclin B and inactivates MPF at fertilization [61-62]. Most studies concerning the regulation of Mos, have been focused on the translation of its mRNA and the stability of the protein. In *Xenopus* immature oocytes, Mos mRNA is not actively translated and the protein is present in small amount [63]. The translational recruitment of c-*mos* and several other mRNAs is regulated by cytoplasmic polyadenylation, a process that requires two 3' untranslated regions, the cytoplasmic polyadenylation element (CPE) and the polyadenylation hexanucleotide AAUAAA. Although the signalling events which trigger c-*mos* mRNA polyadenylation and translation are unclear, they probably involve the activation of CPEB, the CPE binding factor. In response to progesterone, the Eg2/Aurora kinase A phosphorylates CPEB, which regulates c-*mos* mRNA translation [82-83]. This allows an increase in the synthesis of Mos [84]. Some authors believe that the stability of Mos protein is due to phosphorylation of the residue Ser-3 and among the kinases responsible for this phosphorylation event, potential candidates are Cdk1 [85], p42 MAPK [86] and Mos itself [87]. Chen and Cooper [88] have shown that phosphorylation of Ser-3 facilitates interaction of Mos with MEK1, but also promotes its activation [89]. This suggests that Mos is regulated at the level of expression and by post-translational mechanisms, although the Ser/Ala mutation in position 3 does not prevent oocyte maturation or maintenance of CSF [90]. Chen *et al.* also showed that CK2β, the regulatory subunit of CK2 protein kinase, can act as a negative modulator of the Mos activity [91-92]. In 2004, Lieberman and Ruderman demonstrated the interaction between Mos and CK2β identifying the CK2β docking site on Mos sequence [93]. It remains to be defined if the inhibition of Mos by CK2β is constitutive or regulated and how this interaction regulated Mos function during oocyte maturation.
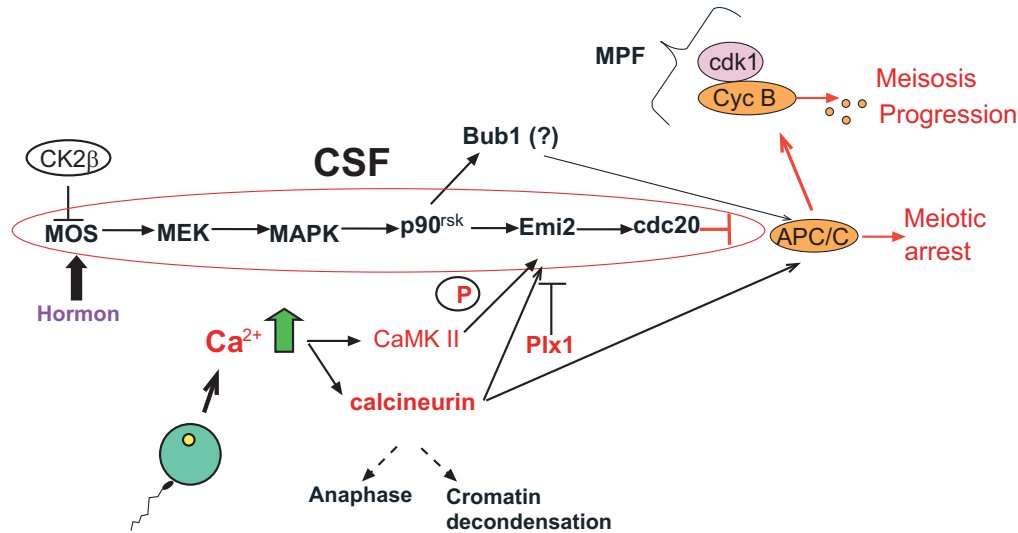
Figure 3: Regulation of CSF during maturation and at fertilization (see text for description)

More recently, the activity of CSF has been linked to the control of the spindle assembly, since p90[Rsk] can phosphorylate Bub1, a Ser/Thr kinase which inhibits APC/C activating components of the spindle checkpoint, such as Mad1, Mad2, BubR1 [61-62, 94]. However, this hypothesis remains largely circumstantial and partially elusive. In fact, the demonstration of a CSF arrest independent of the Mos/MEK1/MAPK/p90[Rsk] [95] indicated the existence of other activities, independent of the Mos/MAPK pathway. An alternative candidate, has been identified in the Cdk2/Cyclin E [96-98]. More recently, a great importance has been attributed to the APC/C inhibition by Emi/Erp factors. Initially it was thought that the protein Emi1 (*Xenopus* early mitotic inhibitor) was a component of CSF because it is necessary and sufficient to induce CSF activity [99]. However, the belonging of Emi1 to CSF has been questioned, since it has not been detected in CSF-arrested oocytes, nor its timing of degradation is consisted with its potential role in CSF activity [62, 100]. In the same context, more interesting appears the role of Erp1/Emi2, an inhibitor of the APC/C, in the maintenance of CSF [101-102]. Both in *Xenopus* and in mouse [102, 103] Erp1/Emi2 is destroyed at fertilization similarly to Mos [104]. Erp1/Emi2 is a zinc finger protein with an F-box domain in the C-terminal region. F-box proteins are part of the complex SCF (Skp1/cullin/F-box) ubiquitin ligase that, similarly to APC/C, mediates the degradation of cell cycle regulators [62]. In mammals, phylogenetic analysis of protein containing the F-box domain revealed a close relationship between Emi1 and Erp1/Emi2 [105]. Schmidt and colleagues demonstrated that Emi2 is essential for the maintenance of CSF and it is rapidly destroyed at fertilization [102], where the calcium-dependent degradation of Erp1/Emi2 occurs. However, the activity of Erp1/Emi2 required phosphorylation by Plx1 (Polo-like kinase) following Ca^{2+} increase at fertilization and CaMKII (Ca^{2+}/calmodulin-dependent protein kinase II) activation [62] which facilitates the phosphorylation-dependent Emi2 recognition by the proteasome machinary [106]. The final evidence linking Mos to Erp1/Emi2 emerged from two parallel papers showing that, in *Xenopus*, p90[Rsk] directly phosphorylates Erp1/Emi2 on Ser-335/Thr-336 both *in vivo* and *in vitro* upregulating Erp1/Emi2 stability and activity [107-108].

OOCYTE MATURATION IN LOWER VERTEBRATES

In *Xenopus*, progesterone induces completion of the first meiotic division and maturation in P-I arrested oocytes. Briefly, after GVBD oocytes complete meiosis I emitting the first polar body, enter M-II without resting in interphase and remain blocked in M-II until fertilization [10]. MPF activity oscillates during meiotic maturation: it is high in M-I, decreases during M-I/M-II transition, and increases again in M-II, where remains stable and enzymatically active due to the activity of CSF, which inhibits APC/C-mediated cyclin B degradation and other events leading to M-II exit [22]. In immature oocytes blocked in P-I, MPF is present as pre-MPF, where Cdk1 is associated with cyclin B, but enzymatically inactive since phosphorylated on Tyr-15 and Thr-14 by Myt1/Wee1 kinases [40]. As described above, Cdc25 phosphatase removes these inhibitory phosphorylation and fully activates Cdk1/cyclin B, which in turn phosphorylates Cdc25, causing a rapid amplification of MPF [2, 109]. The activation of Cdc25 is catalyzed by a complex signalling (see above for details details), which includes xPlk1 (*Xenopus* polo-

like kinase 1) and its upstream activator xPlkk1 (polo-like kinase kinase 1) or homologous kinases in mammals [110-113]. It has been shown that MPF activation induced by progesterone depends upon two mechanisms related to PKA: i. inhibition of PKA activity [114]; and ii. synthesis of new proteins which induce a decrease in the levels of cAMP resulting in decreased activity of PKA. This latter kinase is a potent inhibitor of meiotic maturation and its inhibition is sufficient to induce maturation of *Xenopus* oocytes [115]. A similar response was also described in mice, rats and fish. Inversely, oocytes from pigs, sheep and rabbits exhibit a transient increase, instead of a decrease in cAMP levels [116]; finally, even in the jellyfish, the increase in cAMP can induce oocyte maturation [116]. The link between decrease in cAMP levels and MPF activation loop seems to be the dual PKA-dependent phosphorylation of Wee1 kinase and Cdc25 phosphatase. These events lead to an increase in Cdk1/cyclin B kinase activity and, consequently, oocytes undergo GVBD [41].

Several studies reported the existence of a parallel pathway in MPF activation that involves the synthesis of Mos kinase which indirectly activates p90^{Rsk} (according to a mechanism described above), which in turns phosphorylates and inhibits Myt1 and simultaneously promotes the activation of Cdc25 [2, 114, 117]. Briefly, in addition to the pathway xPlkkl/Pxl/Cdc25C, progesterone may activate a second, independent pathway, the Mos/MEK/MAPK/p90^{Rsk} phosphorylation cascade. p90^{Rsk} phosphorylation of Myt1 may facilitate Cdc25-mediated activation of MPF and entry into meiosis I [40]. Other evidence suggest that Mos can directly phosphorylate Myt1 [118]. The exit from M-I is associated with cyclin B degradation mediated by APC/C complex. Synthesis of new cyclin B will occur before entry into M-II and its stability is maintained by the CSF [95]. At fertilization, the calcium increase induces entry into anaphase and promotes the degradation of Emi2 which causes activation of APC/C and the cyclin B degradation, as described above.

In fishes (*Carassius auratus*) it has been initially reported that the MPF is not present as pre-MPF, but Cdk1 appears as a monomer dissociated by cyclin B which is synthesized only after hormonal stimulation. The complex formed is only activated by phosphorylation of residue Thr-161, as inhibitory phosphorylations on Thr-14 and Tyr-15 residues are lacking by a mechanism which does not require the intervention of the phosphatase Cdc25 [119]. These results have been questioned by a study showing that in teleost fish *Anabas testudineus* a pre-MPF exists and that the transition G2/M induced by Cdk1/cyclin B complex dependents upon Cdc25 activation [120]. In fish oocyte, maturation occurs in three phases which are regulated by specific factors: gonadotropin (LH), maturation-inducing hormone (MIH) and MPF [119]. LH acting on the follicle induces the production of MIH (17α, 20β-dihydroxy-4-pregnen-3-one, 17α, 20β-DP); in the majority of teleosts MIH leads to new synthesis of cyclin B by translational activation of masked mRNA after a signalling which involves cAMP/PKA pathway, while Cdk1/cyclin B is activated by phosphorylation on Thr-161 [119]. After egg activation, MPF is inactivated by degradation of cyclin B by the 26S proteasome through the first cut in its NH$_2$ terminus at Lys-57 [119].

OOCYTE MATURATION IN INVERTEBRATES

In invertebrates the transition from P-I to GVBD is induced by different stimuli depending on the species. In sea urchin, oocytes complete meiosis before fertilization and arrest at the pronucleus stage; in starfish, maturation is induced by 1-methyladenine and fertilization occurs at the P-I stage [121]. In molluscs, two different situations have been described: i. oocytes, such as those of bivalves *Spisula* and *Barnea*, which are fertilized at the P-I stage, the maturation is induced by unknown molecules and proceeds with meiosis completion followed by the fusion of male and female haploid pronuclei [12]; ii. oocytes, such as those of the gastropod *Patella* and bivalves *Ruditapes* and *Mytilus*, which are also arrested in P-I, undergo meiosis after hormonal or other stimuli and secondarily arrest in M-I until fertilization occurs with the release of M-I arrest and meiosis completion [12]. Serotonin induces oocytes maturation in molluscs *Crassostrea* and *Ruditapes* (Moreau *et al*., 1996), whereas in *Spisula* fertilization causes meiotic completion [12]. In the nematode *C. elegans,* a cytoskeletal protein involved in sperm motility (major sperm protein) causes oocyte maturation and ovulation [122]. Starfish oocytes are arrested at the G2/M-phase border of meiosis I. Exposure to their natural mitogen, 1-methyladenine, leads to the activation of MPF and MAP kinase, resumption of the meiotic cell cycle, and fertilization competency [123]. In addition, in starfish, unlike the well-studied case in *Xenopus* oocytes, where MAP kinase is an essential component of the MPF activation pathway, MAP kinase is not required for either MPF activation or subsequent oocyte maturation. Instead, its major role appears to be suppression of DNA synthesis in unfertilized eggs [123]. Sea urchins are members of a limited group

of animals in which meiotic maturation of oocytes is completed prior to fertilization. Sea urchin oocytes accumulate significant amounts of cyclin B mRNA and protein during oogenesis. Cyclin B synthesis is not necessary for the entry of G2-arrested oocytes into meiosis; however, it is required for the proper progression through meiotic divisions. Mature sea urchin eggs contain significant cyclin B protein following meiosis that serves as a maternal store for early cleavage divisions [124]. In ascidians, the signals that trigger oocytes maturation are unknown. It has been suggested that follicle cells surrounding oocytes secrete a meiosis-inducing substance in several species [125].

In general, mechanisms regulating maturation and meiotic completion in invertebrates appear heterogeneous and are also difficult to reconstitute from an evolutionistic point of view. Probably, the unique conserved feature common to all species is the presence of MPF and its oscillation during maturation. As an example, in *Patella vulgata* [126] and *Mitilus edulis* [127], the metaphase block is guaranteed by the stability of M-I cyclin B which is ensured by the continuous synthesis of short lived proteins, not yet characterized. This hypothesis has been formulated based on the observation that exit from M-I was induced by treatment of oocytes with protein synthesis inhibitors [12]. In starfish, the pathway that leads to meiotic maturation does not require protein synthesis and the MPF, present as pre-MPF, is immediately activated by autoamplification mechanisms [1]. MPF promotes oocytes maturation in ascidians [4, 15]; however, this process is different in other invertebrates because the maintenance of M-I block seems to be independent from the presence of newly synthesized proteins [4, 128-129]. Similarly to *Xenopus* oocytes [130], but differently than other invertebrates where inhibition of protein synthesis causes the release of M-I block and chromosomes decondensation [1].

The role of calcium in meiosis completion has been extensively investigated in vertebrates and has been recently analyzed in excellent reviews in this book [131] and elsewhere [132-134]. In ascidians, we firstly correlated calcium oscillation at fertilization with changes in MPF [135] and sperm factor [136]: Cdk1/Cyclin B activity is maximal at M-I and M-II, and decreases at exit from meiosis I and II. A series of calcium oscillations occur simultaneously with the decrease in Cdk1 activity at M-I exit, while a second group of intracellular Ca^{2+} transients precedes the Cdk1 increase at M-II [135]. This calcium signaling system however does not appear to be the central cell cycle control mechanism during meiosis I, since inactivation of Cdk1/Cyclin B was Ca^{2+}-independent at this stage. However, oocytes did not extrude a polar body after fertilisation in the presence of calcium chelators, suggesting that calcium is involved in the completion of meiosis I. Later, others confirmed our finding [137- 138], although a major controversy regarded the role of Ca^{2+} oscillation before the M-II peak of MPF activity. We reported that inhibiting the second phase of sperm-triggered Ca^{2+} oscillations in *C. intestinalis* oocytes with calcium chelator, the reactivation of MPF that accompanies formation of the MII spindle was inhibited [135]. These data imply that the second phase of Ca^{2+} oscillations was required to reactivate MPF prior to extrusion of the second polar body. On the opposite, others showed that MPF activity initially decreases then increases even in the absence of further Ca^{2+} signals. In fact, a monotonic Ca^{2+} signal leads first to the inactivation then the reactivation of MPF activity [138-139]. These data may be revised and reconciled considering the concentration of Ca^{2+} chelator applied. In addition, differences among independent studies might be attributed to the employment of different ascidian species. More recently, our original observations on the functional link between the second wave of calcium oscillation at fertilization and MPF activity has been partially reevaluated. In fact, McDougall's group demonstrated that Cdk1/Cyclin B activity is both necessary and sufficient for the generation of second phase Ca2+ oscillations. The new and important observation regards the correlation between in Cdk1/Cyclin B activity sperm factor triggered calcium release. In fact, their data suggest that Cdk1/Cyclin B activity promotes IP_3 production in the presence of the sperm factor, rather than sensitizing the Ca^{2+} releasing machinery to IP_3 [140]. The hypothesis formulated is that, following fertilization, the ascidian sperm factor is delivered into the egg and triggers Ca^{2+} release, resulting in the first phase of Ca^{2+} oscillations. The sperm factor is then inactivated by an unknown egg-derived mechanism pausing Ca^{2+} oscillations; it is the rise in Cdk1/Cyclin B activity following the metaphase-anaphase transition of meiosis I that restores the Ca^{2+}-releasing activity of the sperm factor, resulting in the second phase Ca^{2+} oscillations. The decrease in Cdk1/Cyclin B activity at meiotic exit results in the inactivation of the sperm factor and in the block of Ca^{2+} oscillations. The Authors hypothesized that Cdk1 modulates sperm factor activity directly by phosphorylation [140].

Regarding the role of CSF in meiotic maturation in invertebrates, to date Mos kinase has been identified in starfish, where it seems to be essential for the maintenance of meiotic block [69] and a homolog of Mos has also been cloned in *Drosophila* [141], where the deletion of the gene does not prevent meiotic completion, suggesting the existence of

a redundant pathway in meiotic regulation by CSF in *Drosophila* [141]. More recently, a phylogenetic survey has revealed that c-*mos* genes are conserved in cnidarians and ctenophores, but not found outside the metazoa or in sponges [142]. Mos orthologs from *Pleurobrachia* (ctenophores) and *Clytia* (cnidarians) activated MAPK and blocked by classical RNA injection assay into *Xenopus* blastomeres. Unusually, cnidarians were found to possess multiple Mos paralogs. In *Clytia*, one of two maternally expressed paralogs accounted for the majority MAPK activation during maturation, whereas the other may be subject to differential translational regulation and have additional roles [142]. Very recently, the genome of *C. intestinalis* has been used as an essential tool to reconstitute, from a phylogenetic point of view, CSF pathway in ascidians. In this study, the existence of an active CSF in ascidians has been experimentally proved by microinjection of cytoplasmic extracts from *C. intestinalis* oocytes blocked in M-I into embryos at the two-cell stage [129]. The Authors also suggest that the cascade reaction leading to CSF arrest is common to vertebrate species for the presence in *C. intestinalis* genome of the main protein kinases involved in the pathway, from Mos to p90^{Rsk}. The rapid decrease of Cdc2/Cdk1 and MAPK enzymatic activities following fertilization can be interpreted as the expected inactivation of MPF and CSF observed in meiotic arrested oocytes. In *C. intestinalis*, continuous protein synthesis appears not required to maintain an active CSF and MPF as in lower vertebrates (*Xenopus*, fishes) and differently than other invertebrates. On the opposite, protein synthesis is essential to permit M-II entry, probably for the strong requirement of newly synthesized cyclin B [129]. This study suggests that, in ascidians, CSF is essential to maintain high MPF and M-I block, but it is not essential to remove the metaphase arrest and complete meiosis, since this event occurs in the presence of high MAPK activity. This apparent contradiction is explained evoking the presence of alternative pathways. In fact, redundant pathways exist in *Xenopus* independent from Mos to ensure CSF arrest and APC/C inhibition, *e.g.* Cdk2/Cyclin E [61]. In addition, in invertebrates such as *Drosophila*, the presence of Mos did not affect meiosis completion [141]. Furthermore, the presence in *C. intestinalis* Mos of an N-terminal domain not present in vertebrate homologs, suggests extra functions for this kinase to be explored. Perhaps, in *C. intestinalis* the Mos/MEK1/MAPK/p90^{Rsk} is necessary to establish CSF arrest, but not to maintain it. Future studies are necessary to address this aspect. The other main difference emerging in CSF regulation between ascidian and *Xenopus* derive from the *in silico* analysis of Mos pathway. Russo and colleagues [129] reported that the cascade of phosphorylation Mos/MEK/MAPK/p90^{Rsk}/Bub1/Cdc20 could be active in *C. intestinalis*, differently than the parallel pathway Mos/MEK/MAPK/p^{90Rsk}/Emi2/Cdc20 established in *Xenopus*. In fact, neither Emi1, nor Erp1/Emi2 has been found in *C. intestinalis* genome; therefore, it is possible that the Mos/Emi-Erp pathway controlling APC/C inhibition and M-II arrest in vertebrates [62] is not active in Urochordata. On the opposite, the presence of Bub1 downstream to Mos might represent the only way to ensure CSF stability in invertebrates, while in vertebrate, although still present [94], this alternative pathway could have become redundant during evolution [129]. A further convergence between ascidian and *Xenopus*, not yet investigated in other invertebrates, regards the role of CK2 in regulating CSF. As depicted in Fig. **3** and reported above, CK2β seems to negatively regulate CSF function acting directly on Mos. Our group recently characterized the independent regulation of the catalytic, CK2α, and regulatory, CK2β, subunits of CK2 during meiosis completion in *C. intestinalis* at fertilization [16]. An interesting observation emerged from the alignment of the carboxyl-terminal region of CK2β in invertebrates versus all vertebrates. Briefly, we observed that Ser-209 of CK2β is absent in all invertebrates having a *bona fide* meiotic block in metaphase of the first meiotic division, such as insects (*Drosophila*, *Anopheles* and *Spadoptera*) and worms (Platyhelminthes *Schistosoma*). Ser-209 is embedded in a perfect consensus site for Cdk1/cyclin B phosphorylation and it is conserved among vertebrates, all having mature oocytes arrested in M-II. As an example, *H. sapiens* and *C. intestinalis* CK2β are highly homologous, except for few residues, including Ser-209. In all invertebrates sequences we analyzed, including *C. intestinalis*, Ser-209 is substituted by other residues although the remaining part of the consensus site for Cdk1/cyclin B phosphorylation is conserved. Since many invertebrates present a meiotic block in P-I/M-I, these data support the hypothesis that Ser-209 might be an important requirement to ensure a M-II arrest in vertebrates, or, more in general, be involved in regulating CSF in oocytes [16]. This hypothesis remains to be supported by experimental data.

CONCLUSION

One aspect of biology that fascinated and still fascinates biologists is undoubtedly fertilization and meiosis. Thanks to the investigation of excellent experimental models, including *Xenopus*, it has been possible to outline most of the mechanisms regulating these fundamental biological processes. On one hand, it has been possible to understand, from a physiological and molecular point of view, how regulatory mechanisms of fertilization and meiotic completion have been conserved during evolution; on the other hand, many questions remain unsolved.

The present review underlined the heterogeneity and redundancy existing in the mechanisms regulating maturation and meiosis completion in invertebrates. Although the "universal" role of MPF is confirmed, more complex appears the function of CSF in these organisms. Neglected until few years ago, the presence of Mos and its function as CSF component in invertebrates has been demonstrated by recent studies. However, "alternative" functions of Mos in oocytes are emerging, as well as its different cross-talks with other CSF components. In this view, it must be kept in mind that even if MPF and CSF have been identified together and have been often considered "twin" complexes from a physiological point of view, they strongly differ from a biochemical point of view, being MPF an enzyme constitutes by two structurally different subunits, while CSF represents a complex pathway of signal transduction which includes several proteins with multiple functions. This difference accounts for the enormous difficulty in characterizing CSF in oocytes from different species. Although recent data suggests that Mos kinase appeared early during animal evolution in regulating meiosis in oocytes, the function of CSF seems to change among invertebrate species. As an example, CSF machinery in *C. intestinalis* oocytes is closer to *Xenopus* and other vertebrates, than to invertebrates, possibly reflecting the position of ascidians in the evolution of Chordata.

The introduction of new experimental models supported by the availability of tools and sequences from complete genomes will certainly help in the next future to trace phylogenetic patterns and clarify unknown aspects of meiosis and fertilization.

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Gamete Binding and Fusion

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Abstract: Fertilization is a stepwise process that starts before the actual gamete binding and fusion. Spermatozoa travel through the female reproductive system and respond to chemotaxis and thermotaxis to reach the oocyte. In most mammals, zona pellucida (ZP) glycoprotein ZPC is the primary sperm receptor that mediates sperm-ZP binding and acrosomal exocytosis (AE). During AE, the outer acrosomal membrane, already primed for AE during capacitation, fuses with the plasma membrane and undergoes vesiculation. The acrosomal matrix (AM) is exposed and dispersed in a step-wise manner. Sperm-ZP penetration is supported by sperm motility and by enzymatic activity of the hypothetical egg coat "lysin", an acrosomal protease that digests the fertilization slit. Acrosin has been considered as a zona lysin candidate. However, male mice lacking the *Acr* gene are fertile. Recently, researchers have been focusing on the 26S proteasome as a mammalian and non-mammalian egg coat lysin. Following zona penetration, spermatozoa reach the perivitelline space and adhere to and fuse with the oolemma. Tetraspanin superfamily members CD9 and CD81 appear to act as sperm receptors on the oolemma, possibly supported by integrins and other elements within the cortical tetraspanin web. IZUMO, a member of immunoglobulin superfamily is a sperm ligand candidate for oolemma tetraspanins. Both CD9 and IZUMO are essential for gamete adhesion and fertility in the mouse. However, there is no evidence yet supporting the involvement of IZUMO and CD9 in sperm-egg plasma membrane fusion. After sperm-oolemma fusion, the fertilizing spermatozoon is incorporated into the ooplasm, a process aided by oocyte cortex microfilaments.

EVENTS IMMEDIATELY PRECEDING GAMETE BINDING

Sperm Migration/Transport in the Female Reproductive System

Depending on the species, mammalian spermatozoa are deposited into the female vagina, cervix or uterus. In vaginal depositors such as primates and ungulates, spermatozoa first contact cervical mucus which is thought to be able to filter out spermatozoa with poor morphology and motility. Only good (morphologically normal and motile) spermatozoa pass through the cervix and uterus. Finally, a few thousand spermatozoa reach the oviducts where they are stored transiently for several hours or even days in a segment referred to as the sperm reservoir. Bound by their heads to the apical surfaces of the oviductal epithelial cells, these spermatozoa are maintained in a fertile state until ovulation. In response to ovulation, spermatozoa become capacitated and hyperactivated, which releases them from the sperm reservoir and allows them to approach the fertilization site. At this stage, spermatozoa move toward the oocyte by a combination of chemotaxis and thermotaxis [1].

Sperm Chemotaxis

Chemotaxis, defined as a reaction to chemoattractant or chemorepellent, is the migration of a cell or organism to/from a chemical source [2]. Sperm chemotaxis has mainly been studied in marine species because females release eggs into seawater, and the released spermatozoa need guidance to find them [3]. In mammals, high numbers of spermatozoa released during ejaculation enter the female reproductive tract and move spontaneously while aided by peristaltic action. However, a low number of spermatozoa reach the fertilization site, e.g., in humans, only about 251 (range 79–1386) spermatozoa are found in both fallopian tubes [4]. Although their number decreases dramatically during sperm migration, multiple spermatozoa still must contact the egg to complete fertilization. Chemotaxis of spermatozoa in mammals is directed toward follicular fluid in mice and oviductal fluid in humans, as well as toward medium conditioned by eggs and cumulus cells [5]. In humans and rabbits, minute concentrations of progesterone seem to attract spermatozoa. The low concentration gradient of progesterone (1-100 pmol/L) stimulated human

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spermatozoa chemotactically [6]. Follicular fluid contains progesterone; however, only a small amount of follicular fluid was released at ovulation [7]. After ovulation, cumulus cells continue synthesis and secretion of the sperm chemoattractant progesterone [8-11] and its carrier protein [12], which maintains progesterone in soluble form. In the rabbit, cumulus oophorus also seems to secrete progesterone as the chemotactic response was inhibited by anti-progesterone antibody *in vitro* [13]. Progesterone secreted by cumulus cells also appears to be a sperm chemoattractant in humans [14]. At present, progesterone is a strong candidate for mammalian/human sperm chemoattractant, while other mechanisms may work in synergy with it.

Sperm Thermotaxis

Capacitated spermatozoa respond to a temperature gradient, a reaction called thermotaxis that is also found in other cell types [15]. Thermotaxis in mammalian spermatozoa plays an important role for guiding spermatozoa from the cooler sperm reservoir site toward the warmer fertilization site, a difference of approximately 2°C. Thermotaxis and chemotaxis are thought to act in synergy in guiding spermatozoa toward the fertilization site near the oviductal isthmus. Thermotaxis is a long-range guidance mechanism; by contrast, short-range guidance is by chemotaxis [13-15]. The difference in temperature between the sperm reservoir and the fertilization site is observed at ovulation, mediated by a temperature rise from cooler sperm reservoir toward warmer fertilization site. In rabbits, approx. 2°C difference has been observed between the isthmus and the isthmic ampullary junction [16], and in pigs, 0.7°C difference has been measured between the isthmus and the ampulla [17]. Both rabbit and human spermatozoa respond to even small temperature differences (0.5°C) [15]. Several mechanisms have been postulated to produce a temperature drop at the isthmus [18]. First, Luck *et al.* [19] suggested endothermic hydration, a process by which the acidic mucus glycoprotein in the isthmus is hydrated by circulating steroid hormones [20] and causes a temperature drop during ovulation. Secondly, cold blood from the ovary may decrease the blood temperature of the vasculature surrounding the sperm reservoir [17].

Sperm-Cumulus Oophorus Interactions

During oocyte growth, cumulus oophorus cells play an important role in maintaining the oocyte in meiotic arrest. Following the preovulatory LH-surge, cumulus cells convey the signal for meiotic resumption and support nuclear and cytoplasmic maturation of the oocyte [21]. The cumulus oophorus cells are embedded in an extracellular matrix (ECM) rich in water-binding glycan, hyaluronan. Mammalian spermatozoa carry a hyaluronan-receptor and bind to hyaluronan-rich ECM [22]. Spermatozoa circle a cumulus enclosed oocyte before fertilization [23]. Stock *et al.* [24] suggested that a high rate of acrosomal exocytosis (AE) can be induced by sperm co-incubation with cumulus cells, and the human cumulus cell-conditioned medium also induced acrosomal activity [25]. Cumulus cells secrete progesterone, a sperm attractant discussed above [26], and hyaluronic acid present in cumulus ECM can increase the intracellular calcium concentration of spermatozoa [27]. It is reasonable to conclude that cumulus oophorus is involved in fertilization [28].

SPERM-ZONA PELLUCIDA INTERACTIONS

Sperm-ZP binding

Following penetration through cumulus ECM, spermatozoa bind to another type of highly organized ECM, the mammalian egg coat, zona pellucida (ZP). The ZP is composed of three (most mammals) or four (murids, primates) ZP-glycoproteins sharing the conserved, immunoglobulin-like ZP domain [29]. Based on sequence similarity, mouse (m) and human (h) ZP proteins are designated mmZP1 (hZPB), mZP2 (hZPA), mZP3 (hZPC) and ZP4 (hZPB2) [30-32]. The general mouse model of zona assembly suggests that ZP2-ZP3 (ZPA-ZPC) heterodimer fibers are cross-linked with ZP1 (ZPB) molecules, and ZPC serves as the primary sperm receptor on the egg coat [33]. In pigs, a heterodimeric complex of ZPB/ZP3alpha (homologue of hZPB2/ZP4) and ZPC seems to act together as a sperm receptor [34]. Such a synergy may also exist between ZP3 and ZP4 in humans [35]. The sperm receptor has a dual function of sperm-ZP binding and induction of AE. The latter may start even before sperm-ZP binding, but it seems to be completed during that process. This dual function is somewhat, but not universally, species-specific [36]. Evidence suggests that the branched triantennary and tetraantennary N-glycans and O-glycans decorating the sperm receptor, rather than its protein backbone, mediate sperm-ZP binding [37]. Consequently, transgenic mouse ova carrying human sperm receptor ("humanized" mouse zona) still bind only mouse spermatozoa [38-39], most likely because the human ZPC protein secreted by these murine ova carries mouse-like ZPC-associated glycans.

Furthermore, recombinant human ZPC protein will only bind to capacitated human spermatozoa and induce AE if it carries glycans consistent with those naturally occurring on the human ZP surface [40]. It is important to note that the soluble zona proteins are capable of inducing AE, a well known fact that is sometimes ignored when alternative models of sperm-ZP interactions are proposed.

Both spermatozoa with an intact acrosome and those progressing through AE can bind to ZP [41], at least in some species. A number of proteins on the acrosomal surface were proposed as ZP-binding ZPC-receptors, but the evidence for any candidate remains inconclusive. These include SP56/ZP3-receptor [42], β1,4-galactosyl transferase (GALT) [43], zonadhesin (ZAN) [44], several spermadhesins [45], arylsulfatase A (ASA) [46-47], P43H [47], proacrosin [48], SP38/IAM38/ZPBP1&2 [49-50] and PH20/SPAM1 [51].
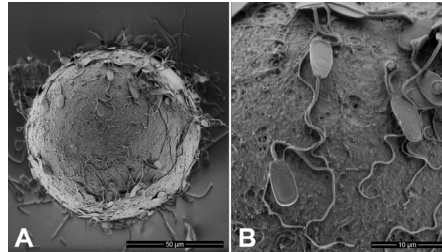
Figure 1: A low magnification (A) and a detailed view (B) of sperm-zona pellucida binding in the pig, visualized by scanning electron microscopy (SEM). Magnification is indicated by scale bars.

Acrosome Reaction Versus Acrosomal Exocytosis

The process by which spermatozoa respond to sperm receptors on the ZP has historically been referred to as acrosome reaction (AR), while it is now being reinterpreted based on new research and perhaps more appropriately called acrosomal exocytosis (AE) [52]. Exocytosis in general is a process by which cargo-carrying vesicles dock to the inner face of the plasma membrane, fuse with it and release their cargo in the extracellular space. This is typically a continuous process that can be turned on/off as needed. The AE/AR is unique in this context as it is an irreversible, one time event by which the sperm acrosome is altered and part of it is lost. While vesicle fusion during AE may employ pathways similar to those observed in somatic cells [e.g. SNARE hypothesis proteins of synaptic vesicle fusion [53-54], the purpose of AE is to expose the acrosomal matrix rather than to deliver a cargo concealed inside the vesicles to the extracellular space. The vesicle fusion during AE is heterologous, thought to arise from the fusion of inner acrosomal membrane with plasma membrane, forming what is called "hybrid vesicles" of the acrosomal shroud [41,55].

The acrosome is thought to originate primarily from the Golgi during spermiogenesis, while the perinuclear theca components that eventually form a capsule around the sperm nucleus are also thought to contribute, and lysosome-like enzymes are found in the acrosomal matrix [56-58]. During AE, the outer acrosomal membrane (OAM) fuses with the acrosome-covering plasma membrane, giving rise to said vesiculation event. The acrosomal matrix (AM) is exposed and partially dispersed, while the inner acrosomal membrane (IAM) with some adhering acrosomal matrix components remains intact throughout the process of sperm-ZP binding and sperm-ZP penetration [41].

Gerton [52] summed up the difference between AE and AR definitions as follows: AR is a binary (1/0 or ON/OFF) event (acrosome is either intact/ON, or reacted/OFF) that has no intermediate steps and starts with sperm-ZP binding. The AE is an "analog" event, a progression of distinct steps that starts with the priming of the outer acrosomal membrane and plasma membrane already occurring during sperm capacitation. Then the AE progresses through sperm-ZP binding, fusion of the PM with OAM, formation of the acrosomal shroud and a stepwise exposure/dispersion of AM layer-after-layer. Contrary to most exocytotic events observed in somatic cells that are continual and can be turned off and resumed, AE is an irreversible, one-time event. Thus, caution is in order when the mechanism of AE is extrapolated from exocytosis studied in other cell types.

Signaling during the AE is mediated by second-messenger molecules such as IP3, cAMP and Ca^{2+} ions. Two distinct pathways have been proposed by Breitbart to be activated during AE [59]. In both cases, a central event is

the increase of Ca^{2+} ion content inside the acrosome. In one scenario, the influx of external Ca^{2+} ions in the acrosomal interior is controlled by gating of Ca^{2+}-channels via activation of the cAMP-dependent kinases (PKA), which is a consequence of ZPC binding to a Gi-coupled receptor on the sperm plasma membrane. An alternative pathway that could cause cytosolic calcium increase inside the acrosome is through activation of a phospholipases enzyme (PLCgamma) by a tyrosine kinase associated with the sperm receptor for ZPC. PLCgamma hydrolyses phosphatidyl inositol di-phosphate (PIP2) into inositol 3-phosphate (IP3) that can trigger intra-acrosomal Ca^{2+}-release, and diacyl-glycerol that activates protein kinase C (PKC), causing the opening of Ca^{2+}-channels. In both scenarios, the influx of Ca^{2+}-ions triggers microfilament depolymerization in the acrosome to promote fusion and vesiculation of OAM.

The pH of the acrosome is acidic [60-61] and increased during capacitation [61]. The contents of sperm acrosome are alkalinized by calcium influx, and sperm begin acrosomal exocytosis [62]. Kim and Gerton [63] classified the progression of mouse sperm AE based on epifluorescence microscopy of GFP-acrosin expressing transgenic spermatozoa and anti-sp56 coated beads binding to the acrosome. At stage 1, the acrosome is acidic and maintains intact OAM and plasma membrane (PM). Next, the pH around the acrosome begins to increase and OAM and PM start to vesiculate. In the third stage, the acrosomal components disperse due to vesiculation of OAM and PM. Finally, at stage 4, the OAM and PM vesiculated completely, and acrosomal components are released from acrosomal matrix [63]. During acrosomal exocytosis, some acrosomal enzymes/components undergo proteolysis and post-transitional modifications, exemplified by proteolytic cleavage of pro-acrosin into enzymatically active acrosin [64-65].

Sperm-Zona Penetration

To what extent does the sperm-zona penetration rely on flagellar motility and proteolysis, respectively? Two opposing but not necessarily irreconcilable hypotheses address this question: the mechanical and the enzymatic hypotheses of sperm-zona penetration. Only sperm motility is required for sperm-ZP interaction in the "mechanical" scenario, while in the enzymatic paradigm, the acrosomal enzymes are of primary importance and sperm motility is secondary. In general, spermatozoa will only penetrate the zona once they have undergone the acrosome reaction. This morphological and biochemical change activates and exposes the proteolytic enzymes present in the acrosomal matrix. Possibly the most studied acrosomal protease, acrosin, has properties similar to trypsin, and both trypsin and crude acrosin extracts remove the zona from oocytes [66]. Because of this, acrosin has for many years been regarded as the principal contender for the role of zona "lysin", a fertilization slit digesting acrosome borne enzyme, the existence of which was proposed as early as 1958 [67]. If the fertilizing spermatozoa make the penetration slit by proteolysis rather than by mechanical disruption or cutting movement, as proposed by Bedford [68], the egg coat "lysin" would be expected to remain on the IAM after AE. Guinea pig spermatozoa indeed retain some acrosin on the IAM after the acrosome reaction [69].

Acrosin has been studied extensively as a candidate for mammalian zona lysin [70-71], but acrosin gene ablation studies demonstrated that it is not essential for mouse fertility. Male mice homozygous for the targeted mutation in the *Acr* gene were mated with homozygous F2 female mice and produced normal litters. In addition, mouse oocytes were fertilized with acrosin-lacking spermatozoa *in vitro* and showed normal sperm penetration and fertilization rates (98% sperm penetration and 79% fertilization), similar to wild type mice (99% sperm penetration and 76% fertilization). The only difference observed was a slight delay (approx. 30 min) in sperm penetration and fertilization, observed in homozygous mutant mice [72]. A similar result was later reported by another group: Homozygous *Acr*-/- mice produced offspring, but showed a somewhat delayed fertilization [73]. Therefore, acrosin, while perhaps facilitating acrosomal remodeling during AE, is not an essential factor in sperm ZP-penetration. This conclusion led to re-examination of the mechanical hypothesis of fertilization, while results of *Acr* gene deletion led to renewed interest in other acrosomal enzymes.

The enzymatic hypothesis has been revived recently by Honda *et al.* [74]. Besides acrosin, several acrosome-borne trypsin-like enzymes have been implicated in sperm-ZP penetration. It was shown earlier that trypsin inhibitors inhibit sperm-ZP binding and penetration [75-77]. The list of acrosomal proteases identified in mammalian spermatozoa includes a collagenase-like peptidase [78], a cathepsin D-like protease [79], trypsin-like proteases different from acrosin [80-81], a serine protease [82], dipeptidyl peptidase II [83], the TESP proteases [84] and the

26S proteasome [85]. In particular, the 26S proteasome, an ubiquitin-dependent protease with trypsin-like, chymotrypsin-like and caspase-like (peptidyl-glutamyl-peptidase-like) protease activities, has been a focus of intense research in mammals, ascidians and echinoderms. Data show that enzymatically active proteasomes are present in the sperm acrosome and participate directly, by degrading a ubiquitin-tagged sperm receptor on the egg coat [85-87], or indirectly, by facilitating acrosomal remodeling during capacitation and AE [88-89], in the process of sperm-egg coat penetration.

SPERM-OOLEMMA INTERACTIONS

Sperm-Oolemma Binding & Fusion

After spermatozoa penetrate the zona pellucida, they reach the perivitelline space and adhere to and fuse with the oocyte plasma membrane, the oolemma. The ADAM (a disintegrin and metalloprotease) family of integrin-binding proteins present on sperm head surface and integrins on the egg plasma membrane, the oolemma, were the early candidates for mediators of sperm-oolemma binding and fusion [90-93]. Specifically, fertilin (α and β) and cyritestin, ADAM family proteins found on the plasma membrane of spermatozoa, were thought to interact with oolemma integrins $\alpha6\beta1$ or $\alpha v\beta1$ during fertilization. Fertilin-β (ADAM-2) was proposed to participate in sperm binding to eggs through its disintegrin domain, and fertilin-α (ADAM-1) was hypothesized to induce sperm-oolemma fusion through a virus-like fusion domain. However, the importance of fertilin-integrin interaction has not been confirmed by gene deletion/mutation studies, as these mutants are either fertile or have a fertility defect due to failed sperm transport in the female reproductive system, rather than to failed sperm-egg fusion [94]. Consequently, new candidates emerged, including tetraspanin superfamily members CD9 and CD81, initially considered as potential sperm receptors on the oolemma because of CD9's proposed association with integrin $\alpha6\beta1$.

Female *Cd9*-/- mice showed reduced fertility and sperm-egg fusion [95-97]. The *Cd9*-/- mice eggs injected with *CD9* mRNA recovered their sperm-fusion ability. Based on these experiments, the large extracellular loop of CD9 was involved in gamete fusion [98]. In addition, the soluble ligand PSG17 which is a member of the immunoglobulin superfamily (IgSF) has been identified as a CD9 receptor [99-100]. Knockout of a related tetraspanin-encoding gene, *Cd81,* also reduces female fertility and sperm-oolemma fusion, although the effect is only partial. Altogether, it appears that tetraspanins CD9 and CD81 act synergistically during sperm-oolemma adhesion/fusion. Tetraspanins cooperate with integrins within a cortical network composed of cytoskeletal elements, cell surface receptors and signaling proteins, called the tetraspanin web. Such a close association may explain why anti-integrin antibodies and disintegrin domain-mimicking peptides interfering with extracellular domain of integrins hindered sperm-oolemma adhesion in earlier studies.

At the current level of knowledge, the likely binding partners of oolemma tetraspanins are the sperm-expressed proteins from the immunoglobulin superfamily (IgSF). In particular, the IgSF member IZUMO shows testis and spermatid-specific expression, and the monoclonal anti-IZUMO antibodies inhibit sperm-egg fusion *in vitro* [101]. *Izumo*-null males are sterile, as their mating with wild-type females produces no pups; *Izumo*-/- spermatozoa show normal motility and ability to undergo ZP-induced acrosomal exocytosis; the null females are fertile. Consequently, the *Izumo*-/- mutant spermatozoa penetrate the ZP, but accumulate in perivitelline space without fusing with the oolemma [102].

The IZUMO protein is composed of 397 amino acid residues with a single Ig domain in roughly the middle of the extracellular domain [102]. While IZUMO is apparently involved in sperm adhesion to oolemma, it is not clear if or how it participates in membrane fusion. Other IgSF proteins participate in cell-cell adhesion, and CD9, the likely oolemma receptor for IZUMO, is one of its *cis*-partners in tetraspanin web, an intricate network of tetraspanins, integrins and associate cytoskeletal and signaling proteins underlying the plasma membrane [103].

Most of IgSF members act as cell adhesion molecules in *Drosophila* and *C. elegans* [104-105]. Tetraspanin-associated IgSF members, IgSF8 and prostaglandin F2 receptor negative regulator (PTGFRN, or EWI-F and CD9-P-1), have been identified in mouse eggs [106-107]. IgSF *cis*-partners EWI-2 and EWI-F may be linking CD9 to microvilli of the oocyte, and microvilli could support, directly or indirectly, sperm-oolemma adhesion/fusion [107]. The mechanism governing sperm oolemma fusion is not known, although sperm surface ligands, such as viral envelope–like proteins and members of the SNARE hypothesis, discussed with regard to membrane fusion during

acrosomal exocytosis, could be involved. We do know, however, that the fertilizing spermatozoon contributes membrane components to the oolemma and that the fusion of the respective sperm and oocyte plasma membranes has an effect on subsequent fertilization steps. In the absence of sperm-oolemma fusion, mouse ova fertilized by intracytoplasmic sperm injection (ICSI) fail to develop a competent anti-polyspermy defense observed during natural fertilization. There is some evidence suggesting that the interaction between sperm plasma membrane receptors and oolemma integrins elicits this response during fertilization [108].

Sperm Incorporation

After sperm-oolemma adhesion and fusion, the sperm tail motility ceases and the sperm head, and subsequently sperm tail, are incorporated in the cortical ooplasm. The oocyte cortex is supported by actin microfilaments forming microvilli that cover most of the oocyte surface except the smooth surface over the metaphase chromosomes, located cortically in the mature oocytes [109]. In zebrafish eggs, sperm equatorial surface binds to the egg microvilli [110]. In mammals, the equatorial segment and subsequently the post-acrosomal sheath contact with microvilli during sperm binding [107]. With the help of cortical microfilaments, the mouse oocye cortex raises around the sperm head, forming a distinct fertilization cone, a structure that is not seen in mammals. This swelling of the oocyte cortex disappears only after the entire sperm head and flagellum enter the ooplasm [110]. The actin microfilaments around the sperm entry site in zebrafish eggs consist of a tight mesh formed before fertilization. This filament network forms the fertilization cone within 15-20 seconds of fertilization, and actin is rearranged into a thick network around the sperm nucleus during incorporation [111]. The sperm incorporation site of non-rodent mammalian oocytes, in contrast, is small and flat [112-114].

In zebrafish eggs pre-incubated with microfilament disruptor cytochalasin B (CB), spermatozoa fail to enter eggs [115]. Similarly, CB-treatment during fertilization prevents sperm incorporation without affecting sperm-oolemma fusion and oocyte activation during bovine and porcine fertilization [116-117]. Microfilament modulator jasplakinolide (JAS) also inhibits sperm incorporation in the mouse [118]. Reduced sperm incorporation has been shown in mouse zona-free oocytes in the presence of CB, JAS and latrunculin B [119]. However, the rate of polyspermy increased during fertilization in the presence of a related microfilament disruptor, cytochalasin D (CD). Somehow, CD-treatment interrupted cytoskeletal rearrangements and led to polyspermy [119]. Rho protein(s) regulating actin-based cytoskeletal reorganization have been implicated in sperm incorporation [120]. It can be concluded that the activity of microfilaments in the oocyte cortex, including the formation of a protruding fertilization cone in rodents, plays an important role in sperm incorporation. It is during the sperm incorporation step of fertilization that the spermatozoon releases the sperm borne, oocyte-activating factor(s) that convey full oocyte activation. These factors are embedded in the dense matrix of the sperm perinuclear theca that solubilizes completely upon sperm head entry in the oocyte cytoplasm [56].

CONCLUSIONS AND CHALLENGES

To achieve fertilization, gametes adapted, in a unique fashion, many mechanisms also studied in somatic cells. While such mechanisms as membrane fusion, motility and exocytosis are reversible in somatic cells, they are one time, point-of-no-return events during fertilization. Consequently, gametes are irreversibly altered during the fertilization process. Already spermatozoa commit to their fate when they undergo sperm capacitation and hyperactivation during passage through the female reproductive system, which leads to sperm death unless the capacitated spermatozoon meets the oocyte. Sperm-egg coat binding is mediated by glycans decorating the sperm receptor protein(s) on the zona pellucida such as ZPC and ZPB. While multiple candidates have been identified, the essential sperm partner of this receptor is not known with certainty. Sperm-ZP binding induces acrosomal exocytosis via one or more pathways leading to calcium influx into the acrosomal interior. These signaling pathways are only partially understood. Acrosomal exocytosis involves irreversible fusion of sperm plasma membrane with the neighboring outer acrosomal membrane, possibly mediated by proteins similar to those involved in synaptic vesicle fusion. Sperm penetration through the zona is most likely achieved by a combination of enzymatic zona digestion by acrosome-associated proteases and mechanic propulsion provided by the motile force of sperm flagellum. Further work is necessary to characterize acrosomal enzymes involved in this process. Upon completion of sperm-ZP penetration, the adhesion of sperm plasma membrane to oolemma is mediated by IZUMO on the sperm equatorial segment and tetraspanins CD9 and CD81 on the oolemma. It is not clear at present if any of these proteins also has a

role for sperm-oolemma membrane fusion. The functioning of tetraspanins during fertilization is probably supported by the tetraspanin web that is organized in the oocyte cortex with support from plasma membrane integrins connected to the microfilament cytoskeleton and its underlying signaling pathways. The oocyte cortex also plays a central role in sperm incorporation and facilitates the release of sperm-contributed factors necessary for oocyte activation. The complexity of gamete interactions during fertilization will undoubtedly reveal many surprises through focused study of regulatory pathways and protein-protein interactions.
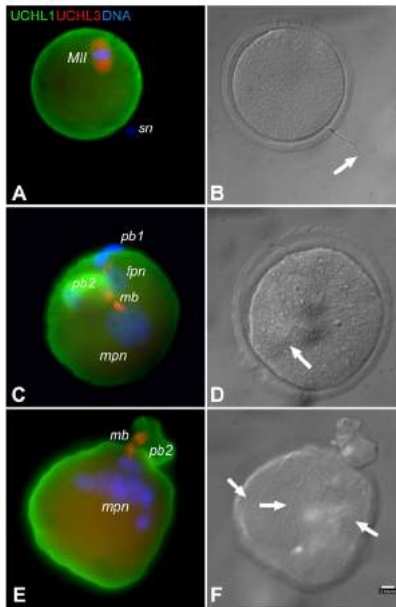
Figure 2: Mouse fertilization visualized by triple immunofluorescence labeling (A, C, E) of ubiquitin-C-terminal hydrolases UCHL1 (green) and UCHL3 (red), and sperm and oocyte DNA (blue; DAPI stain). Corresponding differential interference contrast (DIC) images are shown in panels B, D, F, with arrows pointing to sperm tails. (A, B) Sperm-zona penetration stage; the oocyte is still in metaphase of second meiotic division, showing a typical metaphase-II spindle *(MII)*. (C, D) Normal monospermic fertilization with one male *(mpn)* and one female pronucleus, two polar bodies *(pb1, pb2)* connected with the oocyte through a microtubule-based midbody *(mb),* and one sperm tail inside the oocyte cytoplasm. (E, F) Aberrant, polyspermic fertilization showing multiple male pronuclei *(mpn)* and multiple tails inside the ooplasm, some only partially incorporated.

ACKNOWLEDGMENTS

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Ionic Events at Fertilization

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Abstract: The difference in ionic balance between the cytoplasm and the extracellular environment in all cells is maintained by ion channels and transporters located in the plasma membrane. In this chapter we review the voltage-gated channels and transporters common to both gametes and somatic cells and describe gamete specific ligand-gated channels in spermatozoa and oocytes. In lower deuterostome oocytes the fertilization potential is biphasic. The first event may be the result of gamete fusion, while the second larger depolarization is the result of the activation of several hundred non-specific channels in the oocyte plasma membrane. The fertilization channel is one of the largest channels known in biological membranes with a single channel conductance of upto 400pS and a reversal potential of +20mV. A soluble sperm factor appears to gate this channel via the ADPr/NO pathway. In higher deuterostomes the fertilization channel is a calcium-gated potassium channel. The type, number and topographical distribution of channels and transporters change continually during gameteogenesis, through fertilization to early embryonic cleavage stages indicating both the importance of ionic homeostasis and the role of second messengers in early development.

ION CHANNELS

The difference in ionic constitution of the cytoplasm and intracellular organelles with the extracellular medium is maintained by the hydrophobic lipid bilayer and the trans-membrane proteins - ion channels and transporters. All cell types, including gametes, survive in this manner, in fact one of the first manifestations of cell death is loss of this ionic homeostasis. It has been estimated that 15-30% of all membrane proteins are involved in transport. Transporters have moving parts to transport specific molecules across membranes and may be coupled to an energy source, while channels form a narrow hydrophilic pore, allowing passive movement of small inorganic ions. By generating ionic concentration differences across the lipid bilayer, cell membranes can store potential energy in the form of electrochemical gradients; however, the cell is electrically neutral, e.g., it must contain equal quantities of positive and negative charges. Up to 100×10^6 ions can pass through one open channel each second, 10^5 times greater than the fastest rate of transport mediated by transporters.

Ion transport through channels is passive, i.e., not linked to energy sources, and is often specific for a type of ion. Although channels are essentially hydrophilic pores they are not continuously open, but gated, i.e., they open and close briefly in response to a change in voltage, mechanical stress or the binding of a ligand. Protein phosphorylation and dephosphorylation also regulates the activity of many ion channels. When there is no net flow of ions across the plasma membrane the resulting transmembrane voltage is called the resting membrane potential which, using the Nernst equation, may be calculated knowing the ratio of internal and external ion concentrations. The resting potential in most cells depends on the gradient of K^+ across the membrane together with the characteristics of K^+ ion channels. Since there is little Na^+ inside the cell, this has to be balanced by an increase in cations, mainly K^+, which is actively pumped into the cell by the Na^+/K^+ pump and can also move freely in or out through the K^+ leak channels in the plasma membrane. Very few ions adjacent to the plasma membrane (<1nm) actually contribute to the resting potential and a small flow of ions carries sufficient charge to cause a large change in the membrane potential. Generally, the more permeable the membrane is to a specific ion, the closer the potential will be to the equilibrium potential for that ion. Since, 1 microCoulomb of charge (6×10^{12} monovalent ions) per square centimeter of membrane, transferred from one side of the membrane to the other, changes the membrane potential by roughly 1 V, then in a spherical cell of 10 microns diameter about 1 /100,000 of the total number of K^+ ions in the cytosol have to flow out to alter the membrane potential by 100 mV.

The plasma membrane of many cells also contain voltage-gated cation channels, which are responsible for depolarizing the plasma membrane i.e. to a less negative value inside. Voltage-gated Na^+ channels, allow a small

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amount of Na^+ to enter the cell down its electrochemical gradient and this then depolarizes the membrane further, thereby opening more Na^+ channels, and so on in an auto amplification mode. In an action potential this may shift the membrane from -70mV to +50mV in a fraction of a second. The Na^+ channels then inactivate and voltage-gated K^+ channels open to return the plasma membrane potential back to its original resting potential. The efflux of K^+ is much more powerful that the influx of Na^+ and quickly drives the membrane back toward the K^+ equilibrium potential of -70mV. Voltage-gated sodium channels are primarily responsible for action potential propagation in neurons [1]. The channel family consists of 9 members. These channels have two subunits, α and β and several membrane-spanning regions [2]. The primary effect of voltage-gated sodium channels in the plasma membrane is a depolarisation of the cell through the influx of sodium ions. In contrast to that of sodium channels, the primary effect of the opening of voltage-gated potassium channels is a hyperpolarisation of the plasma membrane. This occurs because potassium ions leave the cell cytoplasm, causing a net increase in negative charge in the cell cytoplasm. The role of these channels is to regulate the depolarisation caused by voltage-gated sodium channels – causing the cell to repolarise after an action potential. Potassium channels have a tetrameric structure consisting of four subunits [3]. Voltage-gated calcium channels allow the entry of calcium ions into the cell after depolarisation. The channel is a complex structure consisting of $α_1$, $α_2δ$, $β_{1-4}$, and γ subunits. Voltage-gated calcium channels are commonly involved in muscle contraction, gene expression and neurotransmitter release. There are four common types of voltage-gated calcium channels; L-type, N-type, P/Q type, R-type and T-type [4]. Voltage-gated chloride channels also exist, and play a role in resetting the action potential caused by the opening of other voltage-gated channels.

Cell membranes contain thousands of ion channels and recording with an intracellular microelectrode allows a qualitative measurement of the membrane potential. To quantify and measure the actual currents underlying these voltage changes it is necessary to voltage clamp the membrane with a second intracellular micro-electrode. In 1976, Neher and Sakmann [5] refined a much superior technique for voltage clamping with a single electrode called patch clamping. This new technique, using a fire polished micropipette of about 1micron in diameter, demonstrated current flow through a single channel and took electrophysiology to the molecular level. Breaking the G ohm seal by suction gives access to the cell interior and enables the researcher to whole cell voltage clamp the cell with a single micropipette. Since that date many channel types have been classified in a variety of cells. Patch-clamp recording showed that individual voltage-gated Na^+ channels open in an all-or-nothing fashion. A channel opens and closes at random, but when open, the channel always has the same conductance, allowing 1000 ions to pass per millisecond. Therefore, the total current across the membrane reflects the total number of channels that are open at any one time [6].

Voltage-gated Na^+, K^+, and Ca^{2+} channels have positively charged amino acids in one of their trans membrane segments that responds to depolarization by opening the channels. Despite their diversity, all these voltage channels belong a large super family of related proteins. Another gene family embraces Cl^- channels and a class of ligand gated channels activated by ATP [7]. Ligand-gated ion channels are relatively insensitive to the membrane potential and therefore cannot by themselves produce a self-amplifying depolarization. The best example, of a ligand-gated ion channel is the acetylcholine receptor, which was the first channel to be sequenced. The acetylcholine receptor of skeletal muscle is composed of five trans membrane polypeptides encoded by four separate genes, and is non-specific for ion selectivity. Na^+, K^+, and Ca^{2+} may pass through the acetylcholine-gated channel [8].

Transporters or Pumps

Transporters are long polypeptide chains that cross the lipid bilayer several times and transfer bound solutes across the membrane either passively or actively. Transporters are often called pumps since they are able to "pump" certain solutes across the membrane against their electrochemical gradients [9]. This active transport is tightly coupled to a source of metabolic energy, such as ATP hydrolysis or an ion gradient [9]. The solute binding sites are alternately exposed on one side of the membrane and then on the other. Transporters may be categorized into uniporters that move the solute from one side of the membrane to the other, symporters, that simultaneously transport two solutes in the same direction or, antiporters that transfer two solutes but in opposite directions [9].

Antiporters, pH and Ca^{2+} Regulation

Marine and mammalian cells need to maintain their pH around a value of 7.2 for the correct functioning of enzymes. To do this they employ one or more Na^+ driven antiporters in their plasma membranes, which use the energy stored in the Na^+ gradient to pump out excess H^+. One example is the Na^+/H^+ exchanger, which couples an influx of Na^+ to

an efflux of H^+, while another is the Na^+ driven Cl^- / HCO_3^- exchanger that couples an influx of Na^+ and HCO_3^- to an efflux of Cl^- and H^+ [10]. Free cytosolic calcium needs to be maintained at very low levels in all cells. Ca^{2+} transporters that actively pump this ion out of the cell are the Ca^{2+} ATPase and the Na^+ / Ca^{2+} exchanger [11].

The Na^+ / K^+ Pump

K^+ is typically 20 times higher inside cells than outside, whereas the reverse is true for Na^+ (Table **1**) The Na^+/ K^+ pump maintains these concentration differences, by actively pumping Na^+ out of the cell against its steep electrochemical gradient and pumping K^+ in [12]. This pump is vital for survival and it has been estimated that 30% of cellular energy is devoted to its activity. Since the pump is an enzyme it can work in reverse to produce ATP [12]. The electrochemical gradients for Na^+ and K^+ and the relative concentrations of ATP, ADP and phosphate determine whether ATP is synthesized or Na^+ is pumped out of the cell. This pump is also involved in regulating osmolarity [12].

Table 1: Distribution of the major ions across the plasma membrane of marine and mammalian cells.

Species of Ion	Mammalian Cell In/Out (mM)	Marine Cell In/Out (mM)
K^+	140/5	400/20
Na^+	10/140	50/450
Cl^-	10/110	50/550
Ca^{2+}	10^{-4}/2	10^{-4}/10

Notes: In/Out refers to the concentration of the ion species (in mM) inside and outside of the cell cytoplasm.

ION REGULATION IN GAMETES

Not surprisingly, gametes possess a wide variety of ion channels and transporters that are essential for their function. Modifying or eliminating, partially, or totally, any of these plasma membrane elements will of course be detrimental to development. Echinoderms have been used extensively to study the electrophysiological properties of the oocyte plasma membrane. In germinal vesicle stage starfish oocytes three types of voltage-dependent currents have been identified. An inward Ca^{2+}, a fast transient K^+ and an inwardly rectifying K^+ current [13]. During hormone-induced *in vitro* maturation, the Ca^{2+} current increases, whereas both K^+ currents become smaller [13-14]. These changes are also associated with a decrease in membrane conductance and a depolarization of the resting potential [15-17]. A similar situation was reported for sea urchin GV oocytes [18]. Ca^{2+} channels were first reported in sea urchin oocytes by Okamoto and collaborators [19].

Block and Moody [20] described a transient inward Na^+ current, a transient inward Ca^{2+} current, and an inwardly rectifying K^+ current in ascidian oocytes. Later, this calcium current was shown to be composed mainly of L-Type calcium channels, suggesting a role in regulating cytosolic calcium during early developmental processes [21]. Common voltage gated channels in the oocyte membrane are sodium, potassium and chloride channels. L-type calcium channels are also widely present [20-25].

In amphibians, K^+ and Cl^- voltage-gated currents present in immature oocytes decrease in mature oocytes, and are replaced by a Na^+ current [26-31]. A voltage-dependent hydrogen current has been described in axolotl oocytes [32]. Ion currents have also been measured in molluscs [33-35], and marine polychaetes [36-37]. Ion channels are known to be activated both during germinal vesicle breakdown and during meiosis. For example, in ascidians, a Na^+ current is present in prophase I oocytes and this current diminishes as oocytes progress towards germinal vesicle breakdown (GVBD). This is accompanied by a Ca^{2+} current that reaches its' peak at the time of GVBD [38-40]. The Ca^{2+} current is driven by L-type calcium channels [38-40]. Prevention of calcium entry reduced the efficiency of both GVBD and the release of calcium after activation [38-40], suggesting that calcium currents during prophase I potentiate GVBD, and fill the internal Ca^{2+} stores of the oocyte in preparation for fertilisation [38-40]. These observations have been made in many species to date [33, 41-48]. Although the calcium current appears common to many species, the sodium current may be specific to ascidians since it has not been yet demonstrated in other species [20,22,24]. In Xenopus and *Caenorhabditis elegans*, a chloride current that increases the efficiency of GVBD has been found [49-50].

Mammalian oocytes are in connection with their cumulus cells via gap junctions [51-52], however the two cell types have different membrane potentials [53]. Gap junctions are in fact low resistance channels [54] and are involved in meiotic maturation of the oocyte [55-60]. During meiosis progression there are changes in ionic permeability of the mammalian plasma membrane [58-59,61-62] which appear to be due to L-type Ca^{2+} channels. In bovine oocytes, the activity of the L-type Ca^{2+} channels decreases throughout meiosis progression [58]. Since Ca^{2+} is necessary for meiotic progression [63-65], there may be a role for external sources of Ca^{2+} in the mobilization of intracellular stores during oocyte activation and fertilization.

In human oocytes, the first studies on membrane potential were performed by intracellular recordings in immature oocytes collected by ovariectomy [68-69], while the patch clamp technique showed that the most frequently observed channel in mature oocytes was a 60 pS non-inactivating, K^+-selective pore, which was activated by depolarization [70]. The membrane potential in mitochondria in situ in human oocytes can be measured indirectly by using fluorescent probes, where it has been shown that this parameter may be used to indicate oocyte competence [71-72].

Despite their size, techniques such as voltage and ion-sensitive fluorescent indicators, immunocytochemistry, pharmacology and DNA recombinant technology, have demonstrated the role for ion channels in sperm function [73-75]. Single channel recording first identified K^+ and Cl^- channel activity in the sea urchin sperm plasma membrane [76] and this was extended to several animals revealing the presence of K^+, Ca^{2+} and Cl^- channels [77]. Immature spermatogenic cells offer a technical advantage, for the patch-clamp technique. Hagiwara and Kawa [78] first demonstrated the presence of K^+ and Ca^{2+} currents in rat spermatogenetic cells, and later it was shown that the negative resting potential of rat spermatids was determined by Cl^- and K^+ conductance with a minor contribution of Na^+ conductance [79]. A role for Cl^- conductance in spermatogenesis was shown in *Caenorhabditis elegans* [80], while in mouse spermatogenetic cells [81], a pH dependent Ca^{2+} permeability factor and a series of K^+-selective currents were correlated with the function of mature sperm [82-83]. Recently, evidence for the regulation of T-type Ca^{2+} channel expression during mouse spermatogenesis [84] was presented. Spermatozoa are less well studied than oocytes due to the small size and difficulty of applying the patch clamp technique. However, in these cells, both voltage and ligand gated channels are now known to be present [73, 85-92]. Voltage gated channels include sodium, potassium, calcium and anion channels. Voltage gated sodium channels appear to be required for motility [93] whereas voltage-gated potassium and anion channels are associated with the poorly understood capacitation process. Calcium channels are required for the motility, the calcium influx that primes hyperactivation and the acrosome reaction necessary for fertilisation [94]. Spermatozoa appear to have two specific calcium channel types. The first – a sperm-specific channel group termed CatSper located on the flagellum of human spermatozoa [95], appears more involved in hyperactivated motility, whereas L- type voltage gated calcium channels located on the acrosome, [85,88] control the rapid influx of calcium required for the acrosome reaction. Ligand gated channels in spermatozoa are almost completely undefined. However, an ATP –gated sodium channel has been defined, although its' function is not known [96]. Given the fact that spermatozoa must complete several events prior to fusing with the oocyte such as chemotaxis and species specificity, it is likely that other ligand-gated channels will be discovered as techniques improve.

Rothschild [97] demonstrated that K^+ concentration, pH and oxygen tension maintain sea urchin spermatozoa quiescent in the testis. After spawning, a change in these physical parameters induced spermatozoa to swim by acting on the axonema in the tail, while peptides from the outer oocyte layers also change sperm motility [98]. These peptides bind to a sperm plasma membrane receptor which then is transduced to change the modulation of the K^+ channel [76, 99]. K^+ efflux causes a hyperpolarization of the membrane potential, a Na^+/H^+ exchange, a rise in pH, increase in cyclic nucleotides, Na^+ influx, depolarization of the membrane potential and Ca^{2+} efflux [74]. In ascidians it has been suggested that K^+ permeability increases when the spermatozoon interacts with the oocyte investment, and this induces a -50 mV hyperpolarization of the plasma membrane which in turn elevates cAMP. A cascade of cAMP dependent kinases may then activate sperm motility [100]. T- type Ca^{2+} channels were also found to be related to the elevation of cAMP [101], suggesting a role for Ca^{2+} in ascidian sperm chemotaxis. Osmolarity and K^+ concentration are the main factors regulating sperm motility in teleosts. K^+ efflux and a rise in intracellular Ca^{2+} both appear to be responsible for initiation of motility in marine and freshwater teleosts, salmonid and rainbow trout [102-105]. Finally, in mammals, where the role of follicular factors as chemo attractants for sperm is less clear [106], there is some evidence to indicate that intracellular Ca^{2+} release from stores in the mid-piece is involved that mediates flagellar beating [107-109].

In order to interact and fuse with the oocyte plasma membrane the spermatozoon must undergo the acrosome reaction. In echinoderms, several compounds with acrosome inducing activity have been isolated. These include, fucose sulphate polymers [110], sulphated fucose, galactose, xylose and the acrosome reaction inducing substance (ARIS) [111-113]. The acrosome reaction occurs following binding of these substances to a specific receptor on the sperm plasma membrane and is an ion channel-regulated event. Ca^{2+} influx is an absolute requirement for the acrosome reaction in the sperm of all species [73], since it seems to be involved in the dehiscence of the acrosomal vesicle and in membrane fusion [74]. Potassium ions also play a role in the acrosome reaction [114-117]. In sea urchin spermatozoa after contact with the jelly layer there is an immediate influx of Na^+ and Ca^{2+} and an efflux of H^+ and K^+ leading to a change in membrane potential and an increase in intracellular pH [73]. Evidence for the presence of two different Ca^{2+} channels and their synergistic action in inducing the acrosome reaction has been reported by Guerrero and Darszon [118]. The Na^+/H^+ exchange and rise in intracellular pH is induced by mobilization of K^+ channels causing a fast and transient hyperpolarization followed by a Ca^{2+}-mediated depolarization [76,119-120]. Cl^- selective anion channels also identified in the sea urchin sperm plasma membrane may also have a role in the acrosome reaction [121].

In mammals, contact of the spermatozoon with the zona pellucida induces the acrosome reaction causing an increase in both Ca^{2+} and pH and also a change in the membrane potential [74,122-123]. Ion channels responsible for this calcium entry and increase include, low and high voltage-activated channels, receptor-operated Ca^{2+} channels, store-operated Ca^{2+} channels [85] and T-type voltage-gated Ca^{2+} channels [73,123]. These channels may also be activated by progesterone or mannose [84,122,124-126]. Although voltage-gated Ca^{2+} channels have been shown in human sperm [127], their function in the induction of the acrosome reaction has not yet been elucidated [for review see 128]. A capacitating Ca^{2+} entry mechanism has been proposed as a possible mechanism for gating plasma membrane Ca^{2+} channels. In mouse sperm, the existence of a Ca^{2+} influx dependent on depletion of Ca^{2+} stores has recently been shown [129]. Rossato and collaborators [130] demonstrated that depletion of Ca^{2+} stores activated gating of Ca^{2+}-activated K^+ channels, with a K^+ efflux causing a hyperpolarization, and the capacitative gating of voltage-gated Ca^{2+} channels, with a subsequent depolarization of the plasma membrane. The mammalian sperm head also contains several type of Cl^- channel [131], while the cytoplasm is rich in Cl^- ions [132].

PLASMA MEMBRANE EVENTS AT FERTILIZATION

The importance of ion fluxes across the plasma membrane in the process of oocyte activation has been recognized for over 50 years [133-136]. Direct measurement of electrical events during oocyte activation using intracellular micro-electrodes have been carried out in ctenophores, echinoderms, annelids, teleosts, amphibians and mammals [137-147]. Not surprisingly, most data has been generated in the sea urchin, where it was noted to be a biphasic depolarization [138].

The Initial Step Depolarization and the Latent Period

Close observation of the initial phase of the activation potential in sea urchin oocytes showed it to be composed of discrete step-like events. Each spermatozoon that entered the egg induced a small, l-2mV, step-like depolarization [148]. A composite shoulder phase indicated polyspermy. In a monospermic situation, of the hundreds of attached spermatozoa, only the fertilizing spermatozoon is capable of reacting with the oocyte, thereby inducing a single step-like depolarization (Fig. **1**) [149]. This report was the first to show how the fertilizing spermatozoon differed from the supernumerary spermatozoa in its capacity to generate a discrete electrical event. This step, which is the earliest detectable event in the egg at fertilization is not seen in parthenogenetically activated oocytes [18]. Voltage-clamp studies confirmed this sperm induced electrical event in the sea urchin [150-151], and a comparable event has been identified both in the ascidian [152,153] and in the anuran *Discoglossus pictus* [146]. Fig. **1** shows that in all these deuterostomes the step event lasts about 5-10 seconds at room temperature.

Owing to the rapid succession of change in the oocyte during activation it is difficult to dissect out the cause of the initial step with the subsequent activation events. Germinal vesicle stage oocytes may also be fertilized. However, since they are immature they do not give rise to the cortical reaction, the fertilization potential, or other autocatalytic events seen in the mature oocyte. When a GV oocyte is fertilized not all of the spermatozoa are capable of penetrating the cell or of producing a fertilization cone. Over a thousand spermatozoa may attach to the GV oocyte,

but usually not more than 10 enter, as demonstrated by the formation of fertilization cones and histological sections. If we record electrically from immature oocytes, successful spermatozoa give rise to an electrical depolarization and conductance increase and induce a fertilization cone some 50 sec later [154]. Other sperm were not capable of inducing either an electrical event or a cone, whilst a third category induced a step depolarization that after several seconds spontaneously reversed. These sperm did not enter the oocyte, nor did they induce the formation of a fertilization cone. These experiments raised the possibility that the step depolarization was the direct result of sperm-egg fusion, the conductance increase being due to the appearance of sperm channels in the newly formed syncytium (Fig. **2**). If fusion is inhibited by the ATPase inhibitor Quercetin [155], or by removing Mg^{2+} [156], spermatozoa are unable to generate electrical changes in the oocyte. Furthermore, the step event may be experimentally reversed by adding a spermicide to inseminated oocytes [154]. Spontaneously or experimentally induced reversible sperm steps have since been seen in a variety of circumstances, [see 151 for review].

Longo and colleagues voltage clamped sea urchin oocytes, fertilized them and then serially sectioned them to locate the fertilizing spermatozoon. Since the authors did not detect gamete fusion until 5 sec after the step depolarization they suggested that the step is a pre-fusion event, suggesting that factors in the sperm plasma membrane cause channel openings in the oocyte through a second messenger mechanism [157-158]. These second messenger systems could be cytoplasmic such as inositol trisphosphate or ADP ribose, or membrane linked such as G-proteins. Since G-protein activators closely mimicked events of activation, these authors proposed a G-protein linked mechanism for the generation of these depolarisations [159]. McCulloh and Chambers [143,160] have data that show the onset of the step event is coincident with an increase in capacitance. Since, in biological membranes capacitance is proportional to surface area, an increase in capacitance at this moment indicates gamete fusion. To estimate the elementary conductance change underlying the step depolarization in sea urchins the ratio of the change in voltage noise variance to the change in potential was calculated. Knowing the cell membrane resistance during this change the single channel conductance was estimated to be about 30-90pS [149]. It is still not clear if the step event is the result of sperm-oocyte fusion, or the release of a channel gating factor into the oocyte cytoplasm, however since the input resistance of the oocyte decreases from 25 MOhms to 15 MOhms at the step and, assuming the sperm membrane is in parallel with the oocyte membrane, then the sperm would have a conductance of 15nS, or in other words, it would have to contain 40 channels of 400pS each to induce the step event.
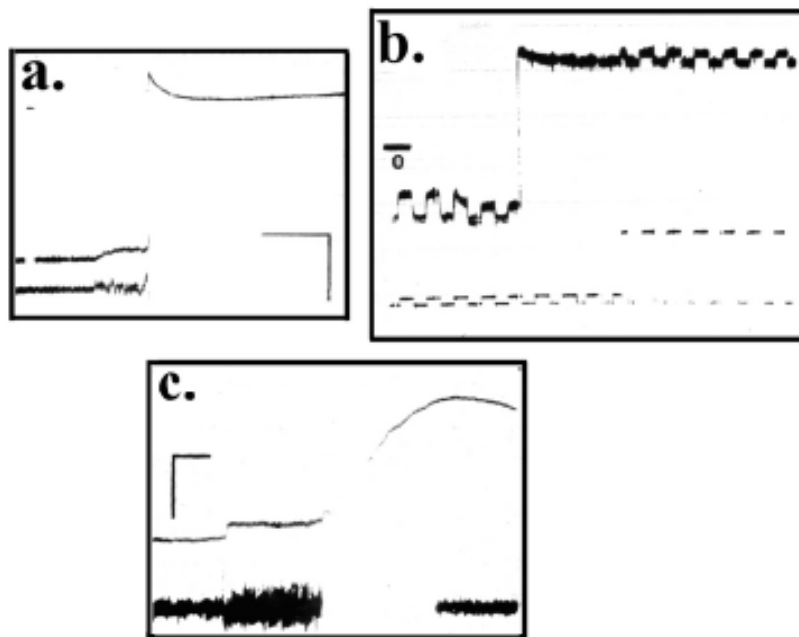
Figure 1: The fertilization potential in an ascidian oocyte (a), a frog oocyte (b) and a sea urchin oocyte(c). Note in all cases the potential is preceded by a small step depolarization, thought to indicate sperm-oocyte fusion, that is accompanied by an increase in voltage noise in (a) and (c) and an increase in conductance in (b). These voltage recordings were made with intracellular micro-electrodes.

In most animals there is a time delay from the moment the spermatozoon attaches to the oocyte surface until cortical exocytosis. The delay between the two corresponds more or less to what has been described in sea urchins as the latent period [see 161 for a review], during which there is no obvious morphological change in the egg surface. Rothschild and Swann in 1952 suggested that a fast propagated change traverses the oocyte surface during the latent period [162]. The sea urchin oocyte is a useful model to study the latent period for two reasons; first because the latent period is relatively long and is temperature dependent [18,149,161], and second because the cortical reaction may be reversibly interrupted by a mild heat shock [163,164], giving rise to "partially fertilized oocytes". In these oocytes, 50% of the surface may be activated, while the rest is undistinguishable from a virgin oocyte. Upon re-insemination spermatozoa are able to interact with the " virgin surface " of such oocytes and therefore it appears unlikely that any major change has traversed this area during the latent period [165]. When fertilization occurs in the presence of the microfilament inhibiting agents cytochalasin B or D, the latent period is increased by up to 100% [166]. In contrast, there is no change if the gametes are pre-exposed to these agents and subsequently fertilized in natural sea water. Together these experiments suggest that a microfilament dependent stage of sperm-oocyte interaction occurs during the latent period. If the preceding arguments are correct this event is post fusion.
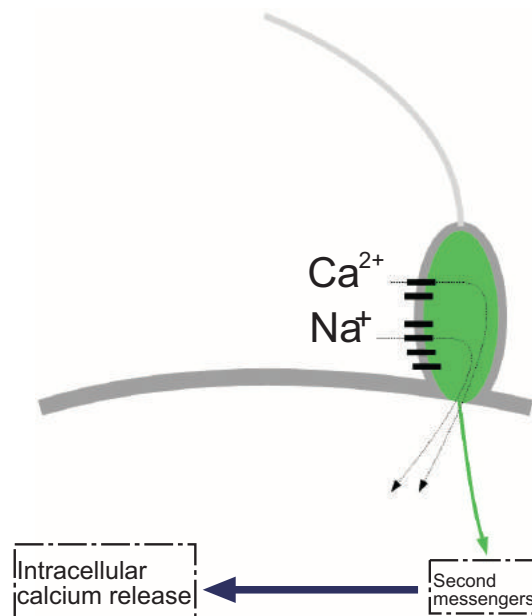
Figure 2: One theory for the step-like depolarization is that it represents sperm-oocyte fusion. This electrical event resulting from the ion channels in the sperm plasma membrane now in parallel with those in the oocyte plasma membrane.

The Fertilization Potential

Mature oocytes are low conductance cells containing a wide variety of both voltage-gated and chemically-gated channels. Approximately 5-10 seconds after the step depolarization there is a much larger depolarization called the 'fertilization potential'. At about the same time there is a massive release of Ca^{2+} from intracellular stores and shortly after a cortical reaction leading to cortical granule exocytosis in the sea urchin or a surface contraction in the ascidian [18,167-169].

The potential remains at a positive value for several minutes and then gradually returns to its original value [170]. Recording voltage with an intracellular microelectrode allows a qualitative measurement of the electrical event. To quantify and define the actual molecular mechanism underling these voltage changes it is necessary to voltage clamp the membrane with a second intracellular micro-electrode or use the patch clamp technique. Using the patch and whole cell clamp techniques a new population of chemically gated channels called "fertilization channels" were identified in the ascidian oocyte plasma membrane at fertilization.

Fertilization channels in Ciona were found to have a single-channel conductance of 400pS [171]. Since the reversal potential was around 0 mV, it was suggested that these channels were not ion specific. To date these channels are

amongst the largest observed in biological membranes. Whole-cell currents in ascidian oocytes also studied during fertilization were shown to peak near -30 mV and approach zero near 0 mV [172], supporting the single-channel data. Knowing the total conductance change at fertilization, the single channel conductance and the probability of a channel being open, we estimated that the fertilizing spermatozoon opens between 200 to 2000 fertilization channels in the oocyte. The fertilization current is a long bell shaped current of about 1000pA over a 60 second period. Since it is inward, and we know that 10^7 ions may flow through an acetycholine channel in one second considerable movement of Na^+ and Ca^{2+} into the oocyte may occur through these channels.

Nude ascidian oocytes may be cut into small fragments and each fragment has the capability of developing into an embryo. By using the whole cell clamp technique on unfertilized and fertilized fragments and inseminating each fragment, it was found that fertilization channel precursors and voltage-gated ion channels are uniformly distributed around the ascidian oocyte surface [173-175]. Since fertilization currents were similar in whole oocytes or fragments, irrespective of their size and global origin, it was concluded that the fertilizing spermatozoon opens a fixed number of fertilization channels limited to an area around its point of entry (Fig. **3**) [174]. The localized ion current through these channels may regulate movements of the cytoskeleton involved in cytoplasmic segregation.

In the sea urchin, the fertilizing spermatozoon triggers an inward current of about -500pA, while the conductance increases from 20 to 40 nS. The I/V curve for this current and the reversal potential of about +10 mV suggest it is non specific for ions [176]. As we have mentioned in the previous section there are also several types of voltage gated ion specific channels in oocytes and these may also be activated during fertilization, however the reversal potential for Ca^{2+} and Na^+ channels does not coincide with the reversal potential for the fertilization current [140,147,177-180].

In amphibians, a Cl^- specific channel is responsible for the fertilization potential. For example, the membrane depolarization at fertilization in *Xenopus laevis* is influenced by the external chloride concentration [181,182] and this is a consequence of Cl^- ion efflux [183]. Similarly, a positive shift in the fertilization potential was shown in *Rana pipiens* associated with an increase of either K^+ or Cl^- and a decrease in Na^+ conductance [29,184]. Membrane potential changes in *Rana cameranoi* oocytes are also based on K^+ as well as on Cl^- conductance [185]. Cl- ions are apparently responsible for the first depolarization phase evoked by sperm, whereas K^+ contributes to the repolarizing phase. The role for Ca^{2+} channels in the amphibian fertilization potential is less clear.
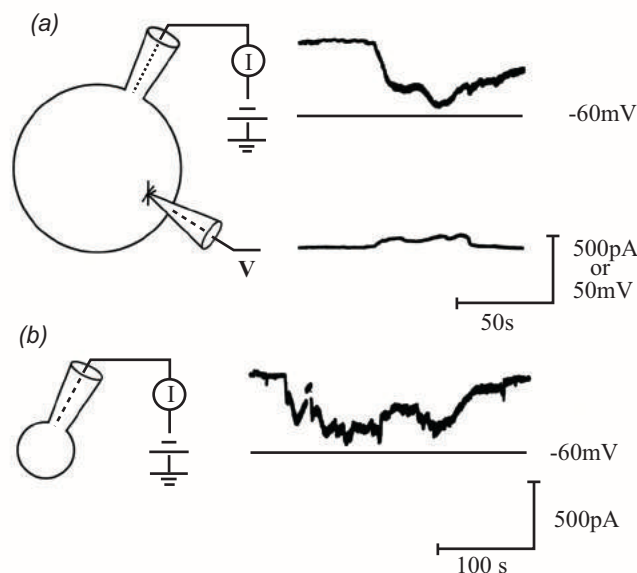
Figure 3: Whole-cell voltage clamping allows quantification of the electrical events at fertilization. The current measured is a direct measurement of the ionic flow into the oocytes. In a whole ascidian oocyte the fertilization current is about -700pA (a). Oocytes may be cut into small fragments and inseminated (b). Note that the current is of similar amplitude, indicating that the spermatozoon gates a fixed number of fertilization channels localized to the area of fusion.

In the anuran *Discoglossus pictus* the sperm entry site is a pre-determined specialized area called the animal dimple [186]. Discoglossus oocytes as other frog oocytes may be activated by pricking with a steel needle. (It should be pointed out that oocytes from other animal groups are not activated upon pricking). At fertilization, the spermatozoon initiates a large regenerative depolarization that is Cl⁻ dependent [146]; pricking elicits a comparable response. When oocytes were pricked outside the dimple area, we saw a wave of contraction spread from the puncture site to the antipode. Since the activation potential was not generated until the wave reached the dimple, it appears that the channels underlying the depolarization are found in the dimple, and that they are gated by a second messenger liberated in the egg cytoplasm that spreads around the oocyte with the contraction wave.

The fertilization potential in mammals is similar in time course to those in invertebrates and amphibians, however inverted in polarity. The first measurements of membrane potential at fertilization in the hamster and mouse showed a series of hyperpolarizations [187-188], while subsequent studies showed the underlying fertilization current to be an outward Ca^{2+} activated K^+ current in the human [189] and bovine [60]. The pattern in rabbit oocytes is different, in that a preliminary depolarization is followed by a repeated diphasic hyperpolarization/depolarization pattern of membrane activity [190].

Gating of the Fertilization Channels

Fertilization channels are specialized ligand-gated ion channels To date, two ligands have been identified, Ca^{2+} and ADP-ribose. In ascidians, the fertilization channels are not Ca^{2+} gated, but by ADP-ribose. In fact, raising the level of intracellular Ca^{2+} in ascidian oocytes, by perfusion or by loading the oocyte cortex (>50uM) with Ca^{2+} through voltage gated channels, did not activate fertilization channels. Alternatively, oocytes exposed to low-Ca^{2+} sea water, perfused with the Ca^{2+} chelator K-EGTA or Ca^{2+} blocking agents to prevent the release of Ca^{2+} from intracellular organelles, and subsequently inseminated, generated fertilization currents [191]. Oocytes exposed to the Ca^{2+} ionophore A23187 were found to contract without generating a fertilization current, while microinjection of InsP₃ or soluble fractions of homogenized spermatozoa induced both a contraction and a fertilization current [192]. Although elevated Ca^{2+} does not gate fertilization channels in ascidians, it appears to be involved in the mechanism of cortical contraction [169,192-193]. Measurements with ion selective electrodes show that the intracellular pH of ascidian oocytes ranges from 7.2- 7.4 and does not vary during activation, making pH an unlikely trigger of early activation events [194].

Nitric oxide has been shown to increase in ascidian oocytes at fertilization [195]. Generating cytosolic nitric oxide in ascidian oocytes with the donor sodium nitroprusside triggers a fertilization-like current and the release of intracellular calcium through a ruthenium-red sensitive mechanism [195]. While, micro-injection of soluble extracts of ascidian sperm cause calcium release in ascidian oocytes but not gating of the fertilization channel [196]. With whole cell and single channel recording it has been shown that the fertilization channel is directly gated by ADP-ribose (Fig. **4**). The channel was shown to be permeable to Ca^{2+} and Na^+, with a reversal potential of 0 to +20mV, and a unitary conductance of 140pS. BAPTA or antagonists of intracellular calcium release did not inhibit the ADP-ribose current showing it is activated in a calcium-independent manner. In situ, the fertilization current is blocked by nicotinamide [197]. In conclusion, ascidian sperm trigger the hydrolysis of nicotinamide nucleotides in the oocyte to ADP-ribose which directly gate the fertilization channels and also assists in the release of intracellular Ca^{2+} (Fig. **5**) [195,197,198]. Since sperm extracts do not trigger all activation events it seems probable that there are multiple pathways at activation, gated by more than one factor from the spermatozoon.

In the sea urchin, the fertilization current may be induce by micro-injection of InsP₃ and inhibited by BAPTA and therefore probably Ca^{2+} gated [176], while in amphibian oocytes there is evidence that intracellular Ca^{2+} contributes to gating the Cl⁻ channels [26]. Glahn and Nuccitelli [199] recorded the fertilization current in Xenopus oocytes and showed it to be generated by Ca^{2+}-activated Cl⁻ channels. In mammals, the fertilization channels are Ca^{2+}-activated K^+ channels [60, 200-201]. Loading mammalian oocytes with EGTA prevents hyperpolarization, while Ca^{2+} injection into the oocyte triggers a hyperpolarization. The oscillations in membrane potential coincide with the oscillations in intracellular calcium [for review see 202] that continue up to pronuclear formation [203]. These oscillations decrease in frequency and amplitude during their progression. The oscillations are large in hamster [204] and smaller in mouse [142, 205] and rabbit [190]. After oocyte activation, plasma membrane Ca^{2+} channels have an increased role in replenishing stores for the continuation of Ca^{2+} oscillations [206], in fact in hamster, external Ca^{2+}

is required for oocyte activation [188]. In the bovine, a clear relationship between electrical properties of the oocyte plasma membrane and intracellular calcium modifications has also been shown following fertilization, as well as following chemical oocyte activation or after exposure to specific Ca^{2+} mobilizers [60]. In the human oocyte, the fertilization current is bell-shaped and outward [189] and may be activated by sperm extracts in the mouse [207] or following ionophore exposure in the human [201].
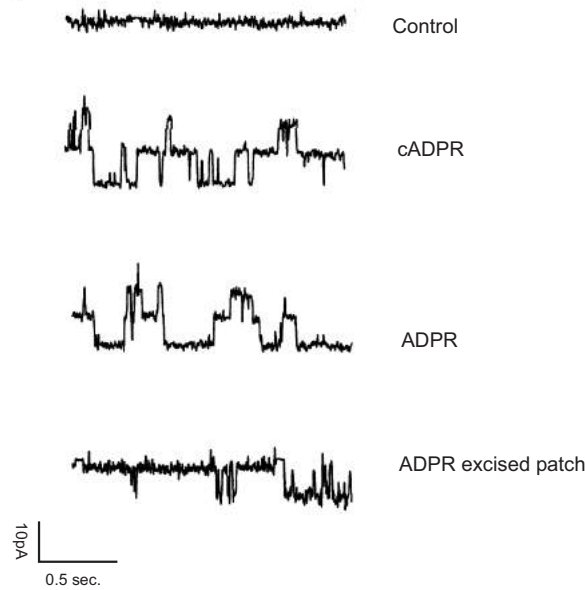
Figure 4: Single channel patch-clamp recordings show that the fertilization channel in the ascidian oocyte plasma membrane is directly gated by the second messengers cADPr and ADPr. The top trace is before addition of the reagents. cADPr was used at a concentration of 5uM and ADPr at 10nM. The lower trace is an outside-out configuration after excision of a patch of membrane from the oocyte. The reversal potential for the channel is +20mV.
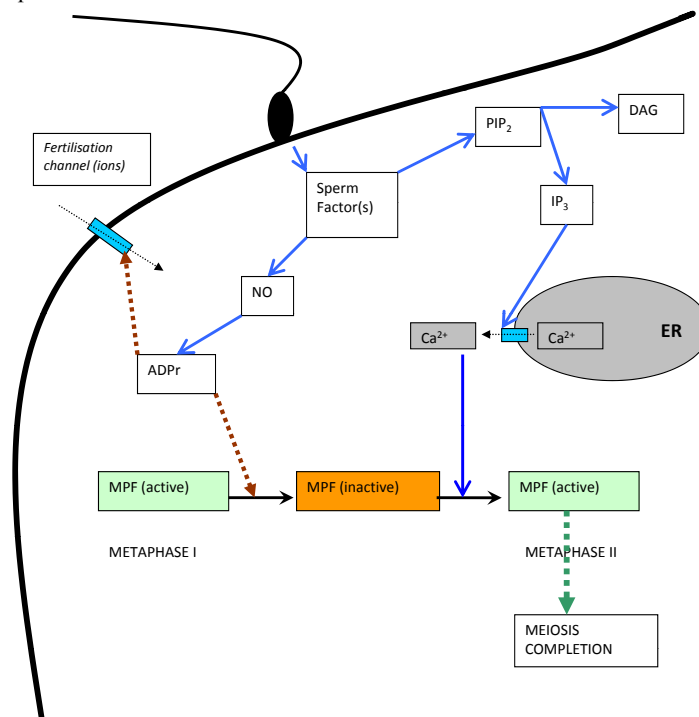
Figure 5: A summary of activation events in the ascidian oocyte. The fertilizing spermatozoon fuses to the plasma membrane and releases sperm factor(s) into the oocyte. These factor(s) stimulate both the production of IP_3 and ADPr, the latter through the production of NO. ADPr gates the fertilization channels, IP_3 gates the release of intracellular Ca^{2+}.

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Recent Advances in the Understanding of the Molecular Effectors of Mammalian Egg Activation

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Abstract: Fertilization is the process by which male and female gametes, and their respective haploid genomes, fuse to form a single cell (the zygote) containing a complete diploid complement of genetic material representative of each parent. The male gamete, the sperm, transduces an activation stimulus to the awaiting female gamete, the egg (also, oocyte or ova), initiating a cascade of events collectively referred to as egg activation. The mechanisms of this process are both complex and tightly coordinated in order to impart a high level of fidelity necessary for initiation of embryonic development and propagation of the species. Although mechanistic discrepancies of this event exist even between species of the same phyla, calcium is the predominant driving force of egg activation in all species studied to date, and is responsible for promoting the resumption and exit of meiosis and the initiation of the developmental program. This chapter will focus on recent discoveries of the molecular mechanisms of egg activation with specific regard to the regulation of calcium signaling as it appears in the oscillatory mammalian system.

INTRODUCTION

In mammals, ovulation occurs after oocytes have reached – and become arrested at – the second meiotic metaphase, or *MII*. Prior to reaching this stage, oocytes undergo a series of processes that last for weeks or months (according to the species) and that entail growth of the oocyte, and transcription / storage of key maternal mRNAs required for cellular metabolism prior to activation of the embryonic genome. Immediately prior to ovulation, fully grown oocytes enter a process termed *oocyte maturation* that spans 12 to 40 hr during which they experience accumulation and activation of metaphase-associated kinases, and a major reorganization of cytoskeletal and key calcium (Ca^{2+})-sensitive elements [1-5]. Such a remodeling of effector molecules and organelles is both necessary and critical for mounting an appropriate response to the fertilizing sperm, as aberrations in maturation events render the oocyte either incapable of fertilization or developmentally incompetent [6].

Shortly after fusion the sperm evokes – in the egg – a series of molecular cascades, collectively referred to as *egg activation* that result in three major groups of events (reviewed in detail in [7-9]): **1.** The release of cortical granule material to create membrane and zona pellucida modifications resulting in a block to polyspermy [10-11], **2.** the destruction of cyclin B [12] and synchronous deactivation of M-phase associated kinases thereby leading to exit from MII arrest and the resumption of meiosis [13-15], and **3.** recruitment of stored maternal mRNAs, formation of the pronuclei, and full initiation of embryonic development [16-17]. These events are made possible by an increase in the concentration of intracellular free Ca^{2+} ions ($[Ca^{2+}]_i$) within the egg. Although the precise mechanism by which the sperm initiates the Ca^{2+} release that is responsible for triggering embryonic development is still under scrutiny, in all species studied to date it has been shown to involve the activation of the phosphoinositide (PI) pathway [18, 19]. Activation of the PI pathway following fertilization results in the production of inositol 1,4,5-trisphosphate (IP_3) and 1,2-diacylglycerol (DAG) via the hydrolysis of phosphatidyl 4,5-bisphosphate (PIP_2) by a phosphoinositide-specific phospholipase C (PLC) isoform [20-23]. A resulting increase in the intracellular concentrations of IP_3 is responsible for mediating Ca^{2+} release by binding and gating its receptor, the type I IP_3 receptor (IP_3R1, [24]), a tetrameric ligand-gated Ca^{2+} channel located on the endoplasmic reticulum (ER) membrane, the main Ca^{2+} store of the cell [25-26]). Additionally, production of DAG may be involved in the regulation of Ca^{2+} influx [27] either directly [28], or indirectly via activation of protein kinase C (PKC, [29, 30]).

Spatiotemporal dynamics of Ca^{2+} release differ markedly in eggs of different species. For example, organisms such as the frog and sea urchin, where fertilization takes place in a very accessible, ex vivo, environment, a single, ~10

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minute $[Ca^{2+}]_i$ wave is propagated almost immediately following sperm-egg interaction (reviewed in [31]). This mechanism no doubt evolved to achieve formation of the fertilization envelope to block polyspermic fertilization in such a promiscuous environment [32]. Conversely, in mammals, where sperm must traverse the oviduct to reach the egg, the potential rate of polyspermy is much lower, and the mechanism to block polyspermy is a slower process involving exocytosis of cortical granule material [10]. In these animals, Ca^{2+} efflux from the ER takes the form of long lasting (>5 hours) and persistent (every 15-60 minutes, species-dependent) oscillations beginning shortly after fertilization ([33]). It is the full complement of this regime of $[Ca^{2+}]_i$ oscillations that is both necessary and sufficient to achieve complete activation of the developmental program [16-17]. This chapter will highlight recent progress in the understanding of the molecular events underlying egg activation in mammals. For more comprehensive reviews on the subject, readers are referred to excellent recent reviews by Parrington et al ([34]), Horner and Wolfner ([35]) and Ducibella and Fissore ([9]).

$[CA^{2+}]_I$ OSCILLATIONS, CA^{2+} EFFECTORS AND EGG ACTIVATION

The certainty that Ca^{2+} was the mediator of egg activation led to the search for effectors capable of transducing the elevations in $[Ca^{2+}]_i$ into events of egg activation. A kinase that immediately emerged as a candidate in participating in the biochemical changes leading to egg activation was the type II Ca^{2+}/calmodulin-dependent protein kinase (CaMKII). Earlier studies have implicated CaMKII in the exit of MII in *Xenopus* eggs, as studies showed that its activation or injection of a constitutively active (CA) form of the protein induced MPF inactivation and exit from the MII arrest [36-37]. At the time of these findings, the mechanism underlying MII arrest and its abrogation by CaMKII were still unknown. Nonetheless, subsequent studies have shown that active CaMKII is required to promote the activation of the anaphase promoting complex (APC) [38] through destruction of a member of the early mitotic inhibitor 2 (Emi2) protein. The APC, an E3 ubiquitin ligase, targets cell cycle proteins such as cyclin B and securin for proteasomal degradation following their poly-ubiquitination [39-44]. The loss of cylin B, the regulatory subunit of MPF [45] and limiting factor for the maintenance of the metaphase state, results in the exit from M-phase and thus the resumption of meiosis. Therefore, at the MII arrest, the activity of the APC complex is held in check by the action of Emi2, whose destruction depends on a CaMKII priming phosphorylation [46]. Interestingly, this Ca^{2+}-mediated cell-cycle transition is thought to be unique to eggs [42], and requires stable levels of cytostatic factor (CSF) [12, 47], which is itself, unique to eggs [48] and that is responsible for stabilizing MPF.

As previously noted, while it was evident that Ca^{2+} and CaMKII were indispensable for egg activation, the molecular partners that made possible this cellular transition were not known. Furthermore, an additional puzzling finding was the discovery that polo-like kinase 1 (Plk1), a kinase originally described to be involved in spindle and chromosome organization [49], was also required for egg activation. Descombes and Nigg [50] had shown that elimination of Plk-1 from *Xenopus* egg extracts prevented the ability of Ca^{2+}-CaMKII complex of promoting MII exit. Interestingly, these findings were not reconciled until recently when the functional association between these two kinases was clarified. Research shows that in addition to phosphorylation by CaMKII, Emi2 requires a subsequent phosphorylation by Plk1 [51], after which it is targeted for degradation by the SCF (Skp1-Cullin F-box) ubiquitin ligase complex allowing MII exit. Further, several recent studies have consolidated the primary role of CaMKII as the main Ca^{2+} effector responsible for MII exit after fertilization in mammals [29]. Using expression of cRNAs encoding for a CA-CaMKII protein these studies showed that this protein alone can promote exit of MII as well as support pre-implantation development to the blastocysts stage [52]. Although the developmental competence of these embryos was not tested further, i.e. development to term, these studies highlight the ability of CaMKII to integrate and translate the majority of the signals encoded by the fertilization-initiated $[Ca^{2+}]_i$ oscillations.

Several important questions remain regarding the role of Ca^{2+} and CaMKII in egg activation including whether $[Ca^{2+}]_i$ oscillations are required or offer an advantage over single $[Ca^{2+}]_i$ rises for development to term. To this end, elegant studies by Ozil and co-workers initially appear to support this paradigm. In their studies, activation methods that mimicked, more closely, the sperm-like patterns of oscillations resulted in higher rates of implantation and development [17, 53, 54]. In these studies the amplitude, frequency, and number of $[Ca^{2+}]_i$ rises were modulated by electroporation of extracellular calcium ($[Ca^{2+}]_e$) into the ooplasm of rabbit and mouse eggs. Notably, each parameter tested had a marked effect on the rates of egg activation as well as post-implantation development. In line with the developmental benefits of Ca^{2+}, more recent studies revealed that different events of egg activation have dissimilar requirements regarding their initiation and completion, with the completion of each event needing a higher number of rises [16-17]. For instance, it was shown that recruitment of maternal RNAs, as evidenced by the detection of new

protein synthesis, was initiated by the administration of 8 $[Ca^{2+}]_e$ pulses but only became fully apparent, or fertilization-like, in zygotes receiving a total of 24 $[Ca^{2+}]_e$ pulses [16]. While these results imply a correspondence between $[Ca^{2+}]_i$ oscillations and protein synthesis, it is important to discriminate whether the observed changes in protein profiles were not simply due to progression into zygotic interphase, which seemed to exhibit similar requirements for $[Ca^{2+}]_e$ pulses [16]. Nonetheless, the approach used in this study, followed by identification of the proteins regulated by Ca^{2+} pulses, could prove invaluable in elucidating yet uncharacterized signaling pathways during egg activation. Follow up studies by the same authors have challenged the initial view that egg activation by $[Ca^{2+}]_i$ oscillations offer developmental advantages. In these studies, the authors show that a single, large Ca^{2+} pulse where $[Ca^{2+}]_i$ remains elevated approximately the same amount time than that attained by repeated oscillations accomplishes similar rates of embryo development to that induced by sperm-initiated oscillations [55]. Therefore, these studies raise the prospect that for all events of egg activation to unfold in a manner that is consistent with embryo development, the egg must receive a "threshold Ca^{2+} signal"; whether it is in the form of a single rise or multiple oscillations is not important for the final outcome [55]. While this interpretation and findings are helpful to gain insight into how eggs "read" or "decode" the Ca^{2+} input initiated by the sperm, it is worth noting that in those studies $[Ca^{2+}]_i$ increases were attained without stimulation of the PI pathway, the activation of which with the subsequent, and possibly persistent, production of DAG and activation of PKC, may have detrimental consequences for development given the persistent Ca^{2+} influx that might elicit [29]. Therefore, it is possible that under natural conditions of fertilization in mammals, $[Ca^{2+}]_i$ oscillations might be the only way for the sperm to deliver the "threshold Ca^{2+} signal".

An additional benefit of $[Ca^{2+}]_i$ oscillations, and the subsequent activation of CaMKII, might be in the regulation of zygotic gene expression. Two recent studies have demonstrated that the gene expression profile of late pre-implantation embryos is susceptible to the egg activation procedure. In one study, both premature termination of $[Ca^{2+}]_i$ oscillations or excessive Ca^{2+} stimulation, while it did not affect embryo development to the blastocyst stage, influenced gene expression and pre- and post-implantation development, respectively [17]. Another study found that activation in the absence of $[Ca^{2+}]_i$ elevation compromised pre-implantation development and gene expression [56]. It remains to be demonstrated whether the effects of Ca^{2+} on gene expression are mediated directly by the $[Ca^{2+}]_i$ rise acting on transcription/translation regulators [57] or if it may be due to the downstream activation of CaMKII activity. In this regard, it is interesting to note that the activity of CaMKII not only increased in response to augmenting levels of $[Ca^{2+}]_i$, but oscillated in tight coordination with each $[Ca^{2+}]_i$ transient after fertilization, as determined in single eggs by simultaneous monitoring of $[Ca^{2+}]_i$ levels and kinase activity [58-59]. Importantly, in hippocampal dendrites, CaMKII directly phosphorylates the cytoplasmic polyadenylation element binding protein (CPEB) that is directly responsible for the polyadenylation, recruitment, and translation of mRNAs [60]. A similar mechanism therefore, could potentially function in the egg. This would provide a direct link between the results mentioned above in which repetitive $[Ca^{2+}]_i$ pulses modulated protein expression patterns from stored maternal mRNAs [16-17], as some of the early recruited mRNAs may be critical for the activation of the zygotic genome [61].

It is worth noting that other kinases such as PKC [27], or proteins such as actin [62, 63] and calreticulin [64], may also serve as effectors of $[Ca^{2+}]_i$ rises during egg activation. For example, conventional PKCs, which are activated by DAG and $[Ca^{2+}]_i$ rises may play an important role in regulating Ca^{2+} influx that is required to maintain oscillations during fertilization [27, 29]. Another kinase that deserves a closer look is the myosin light chain kinase (MYLK2). Recent studies using CA-CaMKII show that of all the egg activation events, the only one not recapitulated by CA-CaMKII was the exocytosis of cortical granules (CG; [52, 65]). Interestingly, inhibition of MYLK2 reduced CG exocytosis and prevented extrusion of the second polar body, suggesting that this kinase is also an effector of Ca^{2+} during mammalian egg activation [62]. Therefore, while CaMKII may be the master translator of the $[Ca^{2+}]_i$ oscillations during mammalian fertilization, other proteins and kinases may act more subtly to impart full developmental competence. The generation of isoform-specific CaMKII null mice should be used to precisely ascertain what events of egg activation are exclusively regulated by CaMKII and how activation with and without $[Ca^{2+}]_i$ oscillations impact gene expression and developmental competence[1].

INITIATION OF $[CA^{2+}]_I$ SIGNALING

There has been much debate and speculation as to the mechanism(s) that triggers activation of the PI pathway and $[Ca^{2+}]_i$ oscillations during mammalian fertilization, and this has led to the proposal of several theories to explain this

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[1] Note. A recent manuscript using isoform-specific CaMKII null mice demonstrated that two events of egg activation, cortical granule exocytosis and recruitment of maternal mRNAs, are not under the direct control of CaMKII. Backs J et al. Proc Natl Acad Sci USA 2010; 107:81-6.

phenomenon. One early hypothesis, referred to as the "conduit hypothesis", proposed that sperm fusion allows Ca^{2+} to passively enter the egg. However, the findings that the initial fertilization Ca^{2+} responses proceed unaltered in the absence of $[Ca^{2+}]_e$ [13, 66], clearly demonstrated that $[Ca^{2+}]_e$ is not necessary for the initiation of $[Ca^{2+}]_i$ oscillations. The "receptor hypothesis" suggests that upon sperm-egg membrane contact, receptor-ligand interactions on the surface of the gametes relay intracellular signaling events that initiate Ca^{2+} release in the egg. One of the signaling cascades thought to be engaged by the interaction of gametes is that mediated by protein tyrosine kinases (PTKs). Specifically, the Src-family of PTKs (SFKs) may activate PLCγ [67-73], thereby triggering Ca^{2+} release through the production of IP$_3$. In accordance with this notion, PTK and PLCγ activity are up-regulated shortly following fertilization in echinoderm and fish eggs [21, 74, 75], and fertilization-triggered Ca^{2+} signaling in egg extracts was reconstituted by adding activated membrane raft fractions [70], which suggests the presence of a receptor-mediated SFK/PLCγ activation model in Xenopus fertilization. Furthermore, inhibition of PLCγ activation by a dominant negative approach using over expression of PLCγ SH2 domains prevented the sperm-induced $[Ca^{2+}]_i$ rise in sea urchin [76] and starfish eggs [77]. A recent study demonstrated the presence and participation of additional SFK isoforms in sea urchin fertilization and found direct interaction between SFK1 and PLCγ in this species [78]. Nonetheless, in mammals, extensive pharmacological studies [79] along with dominant negative approaches [80-81] and injection of recombinant PLCγ [82] failed to show any involvement of this pathway in evoking/preventing fertilization-like $[Ca^{2+}]_i$ responses. This is in spite the fact that PLCγ and SFK isoforms are expressed in mouse eggs [81, 83], and that stimulation of its activity in these cells by exogenous expression of tr-kit, a sperm tyrosine kinase which activates Fyn, a SFK present in mouse and rat eggs [84], was able to induce exit from meiosis and pronuclear formation [85]. Therefore, available evidence argues against a role for SFKs in the initiation of $[Ca^{2+}]_i$ oscillations in mammalian fertilization. Importantly, more recent studies have demonstrated pivotal functions for these kinases in a wide variety of events involving oocyte maturation, MII exit and zygotic progression in the mouse [86-88]. Lastly, extensive studies, which initially relied on the injection of activators and inhibitors of alpha subunits of G$_q$ proteins, implicated the activation of PLCβ isoforms in the initiation of Ca^{2+} release in mammalian fertilization [89-91]. Although later findings demonstrated that inhibition of Gα$_q$ subunits by injection of a function-blocking antibody was without effect on fertilization-induced $[Ca^{2+}]_i$ oscillations [92], and mice lacking one of the PLCβ isoforms are fertile [93], more recent studies utilizing RNAi knockdown of PLCβ1 in mouse eggs showed that the amplitude, but not the frequency or duration of $[Ca^{2+}]_i$ rises was impacted in these eggs [94].

As the above mentioned experiments could not physiologically reproduce the initiation of $[Ca^{2+}]_i$ oscillations in mammals, the need for a novel mechanism to explain the initiation of oscillations in these species was warranted. The hypothesis that emerged, "the fusion hypothesis", proposed that upon gamete fusion the sperm delivers a factor, commonly referred to as the sperm factor (SF), into the ooplasm capable of activating the PI pathway and oscillations [95]. The initial, and sole, experimental support for this hypothesis was the demonstration that injection of sperm extracts into mammalian eggs was able to replicate the pattern of oscillations initiated by the sperm [66, 96-98]. Curiously, the first demonstration of this mechanism was obtained in sea urchin eggs [99], a species in which, paradoxically, the hypothesis under discussion may not account for the mechanism of fertilization (see above). Remarkably, earlier evidence in mammals that could have offered support for the fusion theory seemed to have been overlooked, as injection of human and hamster sperm heads into mouse oocytes resulted in pronuclear formation, implying the occurrence of oocyte activation without sperm-oolema interaction [100], which are the basis of the sperm factor hypothesis. Nonetheless, the subsequent use of the same technique in humans, aptly named intracytoplasmic sperm injection (ICSI), which resulted in the birth of young [101] and was also shown to initiate fertilization-like $[Ca^{2+}]_i$ responses [102-104] consolidated the concept that a sperm product was responsible for initiating oscillations in mammalian eggs. While the success of ICSI unquestionably implicates the sperm as the harbor of the factor that triggers oscillations, close examination of the ICSI-induced $[Ca^{2+}]_i$ responses underscores the notion that events that take place prior to or during interaction of the gamete membranes are pivotal for normal fertilization. For instance, in mouse eggs, ICSI-initiated oscillations occur less frequently after the first hour and show premature termination [102]. More revealing still are the findings in large domestic species where fertilization by ICSI fails altogether to initiate fertilization-like $[Ca^{2+}]_i$ oscillations [105, 106].

IDENTIFICATION OF THE 'SPERM FACTOR'

While early studies assumed that all SF activity was rapidly released into the ooplasm [96-97], subsequent studies revealed that the Ca^{2+}-inducing activity was also present in detergent-resistant sperm domains, most likely the sperm

perinuclear theca [107-111]. Consistent with the concept of SF distribution to several sperm compartments was the demonstration that complete release of SF activity into the ooplasm required ~2 hours [108]. Second, consistent with its perinuclear localization in the sperm, *in vitro* fertilization and ICSI studies showed that sperm's Ca^{2+}-releasing activity could be recovered after fertilization, as it associated with the pronuclei of the developing zygotes [108, 112-113]. Third, *in vitro* PLC assays using sperm extracts showed that these extracts possessed high PLC activity, nearly twice as high as the activity present in other tissues known to express several PLC isoforms [22, 114]. Importantly, the PLC activity of sperm extracts is prominent even in the presence of basal $[Ca^{2+}]_e$, which is very relevant given that this molecule is expected to initiate oscillations in mammalian eggs, which at the time of fertilization show $[Ca^{2+}]_i$ basal levels of ~0.1 μM. Therefore, since several PLC isoforms are expressed in mammalian sperm [93, 115-116], these enzymes surfaced as logical candidates to be the SF. Importantly, injection of recombinant proteins representing most of the isoforms expressed in sperm failed to initiate oscillations, or it did so at non physiological concentrations [82]. Furthermore, chromatographic fractionation of sperm extracts revealed that none of the known PLCs were present in the fractions with $[Ca^{2+}]_i$ oscillation-inducing activity [116-117]. Hence, if a sperm PLC were to be the SF, it had to be a novel PLC. To this extent, a novel sperm-specific PLC, PLCζ [23], was identified in a PLC homology screen of mouse testis expressed sequence tags. Initial studies revealed that PLCζ exhibits $[Ca^{2+}]_i$ oscillation-inducing activity ascribed, thus far, only to the sperm or SF.

PLCζ is to date, the most elementary of PLC isoforms identified. In concurrence with the modular organization of other PLCs [118], PLCζ consists of 2 Ca^{2+}-binding EF hands, X and Y catalytic domains, and the Ca^{2+}-dependent phospholipid-binding C2 domain [119]. Notably, PLCζ lacks the typical pleckstrin homology (PH) domain, which has been found in all previously identified PLC isoforms [120]. In support of its purported role as the SF, injection of recombinant PLCζ [121-122] or PLCζ cRNA has been shown to evoke sperm-like- oscillations in mouse [23, 121-124], rat [125], human [126], bovine [127-128], porcine [129], and equine [130] eggs. In addition, *in vitro* PLC assays using recombinant PLCζ revealed high enzymatic activity at basal $[Ca^{2+}]_e$ concentrations [122]. Moreover, PLCζ cRNA-induced oscillations in mouse eggs cease at approximately the time of pronuclear formation, which is comparable to what is observed after natural fertilization [124, 131]. Further, zygotes activated by injection of PLCζ cRNA showed high rates of *in vitro* development to the blastocyst stage [23, 123]. Lastly, a report in mouse sperm has localized PLCζ to the post-acrosomal region of mouse sperm [121] and to the equatorial region of bull and human sperm [132-134], which are the first sperm areas thought to come in contact with the ooplasm after the fusion of gametes, respectively [111]. Collectively, the evidence in support of PLCζ as the mammalian SF is compelling. Nevertheless, important questions remain to be addressed regarding the expression, localization and storage of PLCζ in sperm, its mechanism of release into the ooplasm, and mechanism(s) of activation once in the egg. Additionally, it needs to be demonstrated whether or not PLCζ represents the sole inducer of $[Ca^{2+}]_i$ oscillations during fertilization and whether its absence impacts fertility.

In regards to the last points, several recent reports highlight the indispensable role of PLCζ in the initiation of $[Ca^{2+}]_i$ oscillations and fertility. First, using transgenic-mediated RNA interference, male mice were created with decreased levels of PLCζ protein. *In vitro* fertilization (IVF) using sperm from these males resulted in attenuated $[Ca^{2+}]_i$ responses, and one of the males produced greatly reduced litter sizes despite inducing normal rates of *in vitro* embryonic development [135]. Second, a report showed that following fractionation of sperm extracts, the presence of immunoreactive 72-kDa PLCζ correlated with the ability of these fractions to induce egg activation [121]. Importantly, several of the active fractions were devoid of 72-kDa PLCζ, and others had greatly reduced amounts of immunoreactive PLCζ. Interestingly, a more current report has demonstrated the presence of N- and C- terminal fragments of PLCζ in chromatographic protein fractions devoid of full-length PLCζ [136]. Furthermore, when the authors coinjected cRNA encoding both 'halves' of PLCζ, but not cRNA encoding the individual fragments, $[Ca^{2+}]_i$ oscillations were initiated. This finding not only validated the results from prior reports that demonstrated activity in fractions lacking PLCζ, but provided some new insight into the action of PLCs in general. Third, two recent reports have associated the absence of PLCζ with infertility. In the first study, it was shown that repeated ICSI failure among human patients is associated with the sperm of these patients being incapable of initiating $[Ca^{2+}]_i$ oscillations. Moreover, the sperm of these patients lacked/have reduced levels of PLCζ by immunofluorescence or Western blotting [134]. Importantly, the activation defect of these sperm when tested in a heterologous system was bypassed by co-injection with PLCζ cRNA. A second study, also found reduced presence of PLCζ in ICSI patients with fertilization failure [137]. In addition, this study identified a point mutation in the PLCζ gene that causes an amino acid change in the catalytic region of the protein that severely compromises the ability of the injected cRNA to initiate $[Ca^{2+}]_i$ oscillations.

Recent research has brought to light some intriguing features of PLCζ function whose elucidation may offer important insights in the mechanism of fertilization in mammals. For example, while the most studied PLCζ, mouse PLCζ, accumulates into the nucleus following PN formation [23, 131], none of the other PLCζ isoforms tested thus far display this localization despite sharing a nuclear localization signal in the linker region of the protein [138]. Further, there seems to be a significant variation among species regarding specific activity. For instance, human PLCζ seems many-fold more active than the mouse form of the enzyme, which itself is several-fold more active than the rat enzyme [123, 125-126]. The role of these species-specific variation in localization and activity of the enzyme are unknown, although it is revealing that rat PLCζ, which shows the weakest capacity to initiate oscillations in mouse oocytes, is responsible for activating rat oocytes, which are notoriously susceptible to undergo spontaneous parthenogenetic activation [139].

Altogether, research evidence suggest that PLCζ might be the long sought-after sperm factor responsible for the initiation of $[Ca^{2+}]_i$ oscillations in mammals. Nonetheless, the generation of PLCζ-null mice and analysis of their phenotype is required to unequivocally establish its role as the sole trigger of $[Ca^{2+}]_i$ oscillations in these species.

THE CELL CYCLE AND $[CA^{2+}]_I$ OSCILLATIONS

In mammals, oocyte maturation is a dynamic process integral to the developmental competence of an egg. While only taking approximately 12 hr to one day to occur, the oocyte undergoes massive changes, both structurally and biochemically. From the onset of the first maturation initiation signal (still under intense study and debate, but see [140]), there is a marked increase in the accumulation and activity level of both MPF and MAPK as the cell transitions from prophase I to metaphase II (reviewed in [141-142]). During this time, there is a key structural reorganization of cytoskeletal actin and in-turn, ER and IP_3R1, from the perinuclear region of the germinal vesicle to distinct and punctate clusters that congregate at the cortex of the MII egg [143-148], and this is thought to contribute to maximal sensitivity of the egg to IP_3-induced $[Ca^{2+}]_i$ release following fertilization [149-151]. Furthermore, MPF and MAPK, as well as the rate-limiting kinase involved in MPF autoamplification, polo-like kinase 1 (PLK1), have all been shown to interact with (either directly or indirectly), and phosphorylate regulatory elements of IP_3R1 [145, 152-155]. Thus, the establishment of cytostatic factor in arrested MII eggs leads to a cell that is structurally and biochemically harmonized to initiate $[Ca^{2+}]_i$ oscillations after sperm entry.

Just as M-phase-associated kinases as well as other events of maturation sensitize eggs to IP_3-induced $[Ca^{2+}]_i$ release, undoubtedly one the reasons that $[Ca^{2+}]_i$ oscillations occur most often during metaphase stage of meiosis [31], so too does cell cycle progression and exit from metaphase result in a protracted desensitization of the Ca^{2+}-releasing machinery that results in the termination of oscillations. As mentioned above, $[Ca^{2+}]_i$ oscillations in eggs begin shortly after sperm-egg fusion and terminate around the time of pronuclear formation. While, in mice, this phenomenon is tethered with entry into interphase and the recently validated localization of PLCζ to the pronuclei [124, 131], its association is not absolute. For example, although oscillations persist up until the first embryonic interphase, $[Ca^{2+}]_i$ transients do become less frequent as the embryo enters telophase [156], and in many fertilized zygotes oscillations terminate ahead of pronuclear formation [138], suggesting that a gradual desensitization of the $[Ca^{2+}]_i$ oscillation-inducing machinery contributes to the termination of oscillations independently of PLCζ sequestration. Several changes after fertilization can be envisioned to affect IP_3 production or IP_3R1 function, which may contribute to the termination of the oscillations. For example, it is now well demonstrated that IP_3R1 becomes dephosphorylated following egg activation, and this might modify the pattern of $[Ca^{2+}]_i$ oscillations [153]. This association is further implied by several studies that used inhibitors to block/stimulate the activity of these meiotic kinases [153, 157-159], although the precise site of action of these inhibitors was not determined in those studies. Further, a recent study showed that the IP_3R1 degradation that accompanies fertilization causes overt changes in the pattern of $[Ca^{2+}]_i$ oscillations, although by itself it cannot cause the cessation of the oscillations [160]. Altogether, evidence suggest that at least in the mouse, several factors contribute to entrain the oscillations with the cell cycle and that PLCζ is not the sole determinant of the pattern of oscillations.

The role of PLCζ PN sequestration as the key determinant of cell cycle-associated oscillations becomes even more tenuous in the rat, a species where despite oscillations terminating around the time of PN formation, sequestration of PLCζ to the PN does not occur. While it is certainly possible that negative feedback may decrease PLCζ activity, IP_3 production and cause cessation of oscillations in these eggs [138], it is important to note that total PLCζ activity in

rat sperm is greatly reduced compared to other mammals (Lee et al., unpublished observations). Further, bovine PLCζ also fails to translocate to the PN [161] and consistent with this, oscillations reportedly continue up to the 2-cell stage [162]. Interestingly, close examination of IP$_3$R1 sensitivity at the PN stage in these zygotes reveals greatly desensitized IP$_3$R1, which might underlie the reduced amplitude of $[Ca^{2+}]_i$ oscillations initiated by injection of bovine PLCζ cRNA at this stage (Malcuit et al., unpublished).

Regulation of Ca^{2+} influx is one other cellular mechanism that might enhance Ca^{2+} release in matured oocytes/eggs and whose function may be differentially regulated during maturation and following fertilization. For example, it is well documented that the Ca^{2+} content of the ER changes dramatically during maturation [8, 163], which occurs in conjunction with increased IP$_3$R1 sensitivity, although it is still unclear what molecules/molecular mechanism are responsible for the filling of the Ca^{2+} stores in mammalian oocytes. Toward this end, the recent discovery of proteins that effect Ca^{2+} entry in somatic cells (for review see [164-165]), stromal interaction molecule 1 (Stim 1) and calcium release-activated calcium modulator 1 (Orai1), should prove useful in investigating the regulation of Ca^{2+} homeostasis in oocytes and zygotes. Remarkably, in *Xenopus* oocytes, research has shown that the Store Operated Ca^{2+} Entry (SOCE), which is operated by Stim1/Orai1, was responsible for the filling of the ER during maturation [166]. In mammals, however, although two recent studies demonstrate that following emptying of the stores by pharmacological agents, Stim1, the presumed Ca^{2+} sensor of the complex, undergoes redistribution and forms puncta, it remains to be demonstrated whether Stim1-Orai1 participate in the filling of the stores during maturation or in their refilling during the sperm-initiated oscillations. Noteworthy are two recent reports highlighting how the function of the Stim1-Orai1 complex and Ca^{2+} influx is affected during the cell cycle. Both reports concur that Ca^{2+} influx is cell cycle regulated and that it undergoes severe down regulation in mitosis [167] and at MII stage in Xenopus oocytes [168]. Also, both studies point to the inability of Stim1 to reorganize to the cell cortex and activate Orai1 as a possible cause of the Ca^{2+} influx inhibition at M-phase stage. However, the changes in Stim1 that are responsible for incapacitating its function remain controversial. Importantly, whatever the mechanism, mammalian oocytes are likely to also carefully regulate Ca^{2+} influx during maturation and following fertilization, although it is unlikely that MII causes complete suppression of external Ca^{2+} influx, as in its absence $[Ca^{2+}]_i$ oscillations cease prematurely [169]. Therefore, elucidating the molecules that control Ca^{2+} influx and their regulation may provide important insight into oocyte maturation and the development of activation competence.
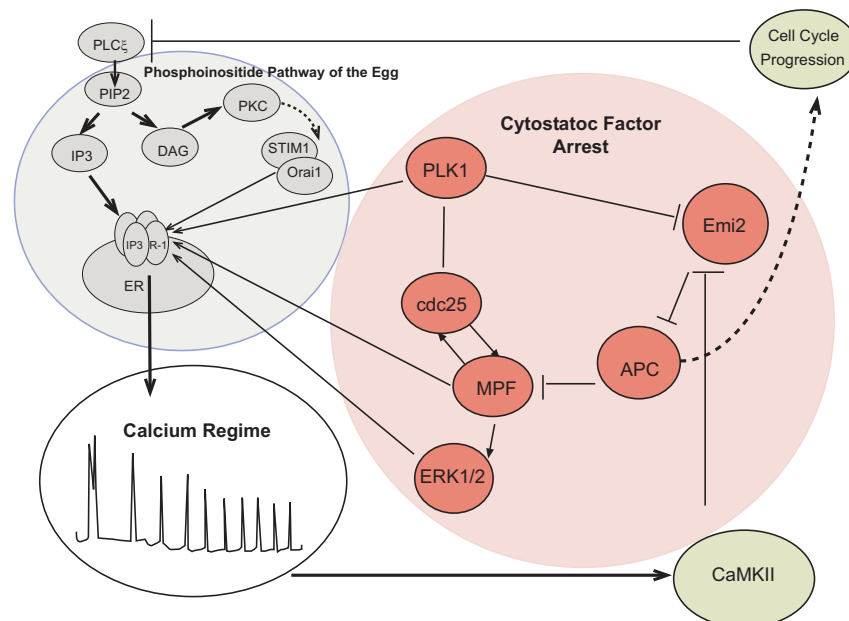
Figure 1: A system poised to self-destruct. A cartoon depicting the interplay between the phosphoinositide (PI) system of the egg (blue) and MII arrest by cytostatic factor (CSF, red). Elements of CSF, including PLK1, MPF, and ERK1/2 all confer IP$_3$ sensitivity to IP$_3$R-1 resulting in a maximal response to activation of the PI system by the incoming sperm's PLCζ. IP3, generated via PLCζ's hydrolysis of PIP2 initiates Ca^{2+} release by gating its receptor, the IP$_3$R-1. Concomitantly, DAG and Ca^{2+} promote PKC activation and membrane translocation, possibly enhancing store-operated Ca^{2+} entry through Stim1 and Orai1. The PI system, and in-turn, $[Ca^{2+}]_i$ oscillations act as a driver to promote continuous bursts of CaMKII activity through IP$_3$-

mediated Ca^{2+} release. Activated CaMKII triggers degradation of Emi2, the molecule responsible for holding the APC inactive. Over the full complement of the calcium regime (white), which persistently stimulates CaMKII activity, complete deactivation of MPF (via cyclin B degradation by the APC) and ERK1/2 results in cell cycle progression. Interestingly, in mice, entry into interphase is accompanied by nuclear sequestration, and therefore inhibition of PLCζ activity. However, in all species studied, loss of M-phase associated kinase activity deregulates Ca^{2+} release by desensitizing IP_3R-1 to IP_3, thereby providing a mechanism by which to shut down excessive Ca^{2+} stimulation and aberrations in developmental programs.

In summary, over the past 20 years significant progress has been made in the elucidation of the molecules and signaling cascades that initiate the $[Ca^{2+}]_i$ oscillations responsible for egg activation in mammals. During this period, it has been demonstrated that sperm PLCζ is the key trigger of IP_3 production in the egg, and that IP_3R1 is the indispensable channel that mediates all the release required for egg activation. We have also learned that CaMKII is the main transducer of $[Ca^{2+}]_i$ oscillations into events of egg activation, but not the only one, as cortical granule exocytosis, and possibly the membrane block to polyspermy, appear to occur independently of the activity of this Ca^{2+}-dependent kinase. Thus, future studies should uncover the downstream molecules and pathways that translate $[Ca^{2+}]_i$ oscillations into egg activation events as well as the molecules and mechanisms responsible for rendering oocytes/eggs activation and developmentally competent.

ACKNOWLEDGEMENTS

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In Vitro Fertilisation

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Abstract: The historic birth of Louise Brown in 1978, the world's first "test tube baby" justifiably ranks as a major milestone in the history of both medicine and science. This remarkable achievement represented the culmination of several different lines of research and investigation carried out by Patrick Steptoe, Robert Edwards and their respective colleagues: (i) More than two decades of laboratory research into the science of oocyte maturation and fertilization; (ii) Clinical observation and studies of the endocrinology and physiology of ovulation and implantation; (iii) Technological advances in the use of laparoscopy to observe the pelvic organs and rescue mature oocytes just prior to ovulation.

On the medical side, the results of their unique achievement offered treatment to couples who previously had no hope of having a child of their own; scientifically, the ability to initiate the creation of new life in the laboratory brought a revolution in biotechnology, and opened new vistas in our understanding of cell biology, the regulation of cell growth, and the events and control mechanisms surrounding fertilization and early preimplantation embryo development. This chapter will outline the current steps and procedures that are required for the successful establishment of a pregnancy when a couple undertake a cycle of assisted reproductive treatment by *In Vitro* Fertilization.

HISTORY OF IVF

Advances in reproductive endocrinology, including identification of steroid hormones and their role in reproduction, contributed significantly to research in reproductive biology during the first half of the 20th century. During the 1930's–40's, the pituitary hormones responsible for follicle growth and luteinization were identified, and a combination of FSH and LH treatments were shown to promote maturation of ovarian follicles and to trigger ovulation. Urine from postmenopausal women was found to contain high concentrations of gonadotrophins, and these urinary preparations were used to induce ovulation in anovulatory patients during the early 1950's.

Parallel relevant studies in gamete physiology and mammalian embryology were underway by this time, with important observations reported by Austin, Chang and Yanagimachi. In 1951, Robert Edwards began working towards his PhD project in Edinburgh University's Department of Animal Genetics under the direction of Alan Beatty. Here he began to pursue his interest in reproductive biology, studying sperm and eggs, and the process of ovulation in the mouse. He continued to explore his interest in genetics, mammalian oocytes and the process of fertilization at the Medical Research Council in Mill Hill, London, and during this period started expanding his interests into human oocyte maturation and fertilization, utilizing human oocytes retrieved from surgical biopsy specimens. In 1962 he observed spontaneous resumption of meiosis in a human oocyte *in vitro* for the first time [1]. By this time Chang [2] had successfully carried out *in vitro* fertilization with rabbit oocytes and sperm, and Yanagimachi subsequently reported successful IVF in the golden hamster [3]. Edwards published his observations about maturation *in vitro* of mouse, sheep, cow, pig, rhesus monkey and human ovarian oocytes in 1965 [4-5]. Whittingham reported successful IVF in the mouse in 1968 [6], and during that year Edwards began his historic collaboration with Patrick Steptoe, the gynaecologist who pioneered and introduced the technique of pelvic laparoscopy in the UK. Edwards and his colleague Jean Purdy traveled from Cambridge to Oldham in order to culture, observe and fertilize fresh oocytes obtained via laparoscopy *in vitro*. The team began to experiment with culture conditions to optimize the *in vitro* fertilization system, and tried ovarian stimulation with drugs in order to increase the number of oocytes available for fertilization. After observing apparently normal human embryo development to the blastocyst stage in 1970 [7-8], they began to consider re-implanting embryos created *in vitro* into the uteri of patients in order to achieve pregnancies: the first human embryo transfers were carried out in 1972.

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Despite the fact that their trials and experiments were conducted in the face of fierce opposition and criticism from their peers at the time, they continued in their efforts, with repeated failure and disappointment for the next 6 years. Finally, their 10 years of collaboration, persistence and perseverance were rewarded with the successful birth of the first IVF baby in 1978 [9], and this spectacular achievement heralded the birth of the modern field of Assisted Reproductive Technology (ART). By the late 1980s, IVF treatment for infertility was available to patients worldwide. The first babies born after transfer of embryos that had been frozen and thawed were born in 1984-85, and cryopreservation of embryos as well as semen became routine. Advances in technique and micromanipulation technology led to the establishment of assisted fertilization via Intracytoplasmic Sperm Injection, ICSI [10]. Gonadal tissue cryopreservation, *in vitro* oocyte maturation and embryonic stem cell culture are now under development as therapeutic instruments and remedies for the future.

In the field of animal science, the application of assisted reproductive techniques has helped to unravel the fundamental steps involved in fertilization, gene programming and expression, regulation of the cell cycle and patterns of differentiation.

The first live calves resulting from bovine IVF were born in the USA in 1981 [11]. Further refinement in techniques led to the integration of IVF into domestic species breeding programmes by the 1990s. Equine IVF has also been introduced into the world of horse breeding, and China used artificial insemination to produce the first giant panda cub in captivity in 1963; assisted reproduction/IVF is now used in the rescue and propagation of endangered species in specialist zoos throughout the world, from pandas and large cats to dolphins.

CLINICAL IVF TREATMENT

IVF was originally developed as a means of overcoming simple mechanical blockage of the fallopian tubes that prevented sperm reaching and fertilizing oocytes released from the ovary. Mature oocytes were rescued from the ovaries by laparoscopy, cultured and fertilised *in vitro*, and resulting embryos re-transferred to the patient's uterus for potential implantation. However, by the early 1980's, it was evident that the techniques could be extrapolated to the treatment of infertility resulting from numerous causes, including male subfertility. The introduction of vaginal ultrasound-guided techniques for oocyte recovery, together with refinement of drugs used for superovulation treatment, converted the treatment cycle into a procedure that can now safely and effectively be carried out on an out-patient basis.

Female Infertility Treatment

Table **1** summaries the main causes of female infertility successfully treated by IVF, divided into 3 main categories. In the majority of IVF patients, mature oocytes can be retrieved: approximately 25% of patients referred for IVF treatment continue to present with tubal blockage as the primary disorder. Approximately 20% may present with combined aetiologies, including tubal blockage as well as ovulatory or immunological disorders, cervical factors, coital problems, and endometriosis. Seventeen percent present with "unexplained" infertility where no specific aetiology has been identified. Donation of oocytes depends upon the availability of willing oocyte donors, which is restricted in many countries. IVF surrogacy can be used for patients who can produce mature oocytes for fertilization, but are unable to carry a pregnancy to term: the patient's own oocytes are fertilized with her partner's sperm, and resulting embryos transferred to a host surrogate after appropriate counselling and informed consent.

Table 1: Causes of female infertility

1.	**Factors that allow successful recovery of mature fertilizable oocytes**
	Tubal blockage
	Ovulatory disorders, including polycystic ovarian disease (PCO)
	Immunological disorders such as antisperm antibodies
	Endometriosis
	Coital problems
	"Unexplained" infertility, with no aetiological factor identified
2.	**IVF using donated oocytes can be used in cases where mature oocytes can not be recovered from the patient**
	Premature ovarian failure

		Gonadal dysgenesis, including Turner's Syndrome
		Ovarian failure due to medical treatment such as chemotherapy
		Surgical oophorectomy
		Genetic disorders
3.	**IVF Surrogacy**	
		Congenital absence of the uterus
		Post-hysterectomy patients
		Carcinoma of the uterus
		Severe uterine haemorrhage or ruptured uterus
		Patients with recurrent miscarriage
		Patients with repeated failed implantation after IVF
		Medical conditions in which pregnancy could be life-threatening

Male Infertility Treatment

Table **2** summaries the main causes of male infertility. ICSI was initially developed as the treatment of choice in cases where only extremely low numbers of sperm could be found in an ejaculate; its use has since been extended to cover a variety of infertility disorders of both male and female origin. Couples who have suffered recurrent failure of fertilization after IVF-ET may have one or more disorders of gamete dysfunction, in which there is barrier to fertilization at the level of the acrosome reaction, zona pellucida binding or interaction, zona penetration, or fusion with the oolemma. ICSI is always indicated for patients who have unexplained failure of fertilization in two or more IVF-ET cycles.

Until the mid-1990's, virtually all testicular pathologies resulted in untreatable male sterility; this situation was completely reversed by combining ICSI with surgical techniques to recover samples from the epididymis and directly from the testis [12-13].

In cases of obstructive azoospermia, sperm can be recovered by a simple outpatient procedure carried out under local anaesthetic, percutaneous epididymal sperm aspiration (PESA). Testicular biopsies, either by needle aspiration (TESA, testicular sperm aspiration, or TEFNA, testicular fine needle aspiration) or open biopsy (TESE, testicular sperm extraction) also allow sufficient numbers of sperm to be recovered in cases of non-obstructive azoospermia, or when sperm cannot be recovered from the epididymis in cases of obstructive azoospermia.

Table 2: Causes of male infertility

A.	**Pre-testicular - deficient gonadotropin drive - low FSH**	
	1. Congenital	partial or complete Kallman's syndrome GnRH deficiency associated with agenesis of the first cranial nerve & thus anosmia. Low FSH and LH, small but potentially normal testes.
	2. Acquired	space-occupying lesions pituitary tumours craniophraryngioma trauma, meningitis, sarcoidosis Cushing's syndrome (adrenal hypoplasia) congenital adrenal hyperplasia haemochromatosis
B.	**Testicular failure - no spermatogenesis - raised FSH** **Testicular biopsy can show a wide variation in appearance.**	
	1. Congenital	Klinefelter's Syndrome (XXY) autosomal abnormalities torsion (maturation arrest) cryptorchidism, anorchia sickle cell disease myotonic muscular dystrophy Noonan's syndrome (male Turner's)
	2. Acquired	mumps orchitis epididymo-orchitis testicular trauma inguinal/scrotal surgery radiotherapy

C.	Post testicular	- duct obstruction - functional sperm usually present
	1. Congenital	congenital absence of the vas deferens (CAVD) cystic fibrosis Young's syndrome Zinner's syndrome: congenital absence of the vas deferens, corpus and cauda epididymis, seminal vesicle, ampulla and ejaculatory duct
	2. Acquired	TB gonococcal or chlamydial infection surgical trauma smallpox bilharziasis filariasis vasectomy

Indications for ICSI include**:**

1. Severe oligospermia: if as many normal vital sperm can be recovered as there are oocytes to be inseminated, fertilization can be achieved in approximately 90% of these patients.

2. Severe asthenozoospermia, including patients with sperm ultrastructure abnormalities such as Kartagener's syndrome, or "9+0" axoneme disorders.

3. Teratozoospermia, including absolute teratozoospermia or globozoospermia.

4. Ejaculatory dysfunction, such as retrograde ejaculation - a sufficient number of sperm cells can usually be recovered from the urine.

5. Paraplegic males have been given the chance of biological fatherhood using electroejaculation and IVF; they may also be successfully treated using a combination of testicular biopsy and ICSI.

6. Immunological factors - couples in whom there may be antisperm antibodies in female sera/ follicular fluid, or antisperm antibodies in seminal plasma following vasectomy reversal or genital tract infection can be successfully treated by ICSI.

7. Oncology - male patients starting chemotherapy or radiotherapy should have semen samples frozen for use in the future. Although the quality of this frozen-thawed sperm may be grossly impaired, ICSI offers the patient an excellent chance of eventually achieving fertilization.

THE IVF TREATMENT CYCLE

The IVF treatment cycle is a complex, multidisciplinary procedure, which requires carefully co-ordinated teamwork and collaboration between physicians, nursing staff, and scientists. A typical cycle consists of a number of phases:

1. Consultation

2. Ovarian stimulation, "superovulation"

3. Monitoring of follicular growth by ultrasound

4. Induction of ovulation by administration of HCG to complete oocyte maturation

5. Oocyte retrieval by vaginal ultrasound-guided follicular puncture

6. Identification of oocytes in follicular aspirates and incubation in culture medium

7. In vitro fertilization or ICSI using a sample of prepared sperm isolated from the ejaculate, epididymis, or testis

8. Identification of fertilized oocytes (zygotes) and further culture for embryo development

9. Evaluation and selection of embryos for transfer to the uterus

10. Cryopreservation of supernumerary zygotes or embryos.

CONSULTATION

Careful and detailed history and examination, review of investigations, and assessment of both partners is necessary before deciding upon the appropriate course of treatment.

Assessment of the male partner always includes a current semen assessment. Patients must be provided with detailed information about the entire treatment cycle, and given a realistic assessment of their prognosis. Counselling should be offered, and is mandatory if donor gametes are to be used. Consent forms must be issued with sufficient time and information for their careful consideration.

Superovulation Treatment

The chances of achieving an ART pregnancy are significantly improved if a number of mature oocytes are available for fertilization and embryo development, and therefore protocols for ovarian stimulation are used in order to recruit several follicles into the final stages of oocyte maturation. Current strategies use a GnRH agonist or antagonist to suppress endogenous pituitary FSH and LH secretion, combined with an FSH preparation to stimulate folliculogenesis. The protocol should be tailored to the individual, in order to avoid excessive stimulation and the possibility of ovarian hyperstimulation syndrome (OHSS), an exaggerated response to gondadotropin stimulation which can result in a life-threatening syndrome. An appropriate protocol will yield an average of 10 to 15 mature oocytes retrieved per cycle of stimulation, whereas an over-response in some patients, particularly those of a young age or with polycystic ovaries, can produce more than 30 follicles and oocytes.

In Vitro Maturation (IVM)

Women with polycystic ovaries are at high risk of developing OHSS in response to gonadotropin stimulation, and therefore an alternative strategy has been developed in order to circumvent the problem, by harvesting oocytes from Graafian follicles in the unstimulated ovary at the immature, germinal vesicle stage. This approach has the advantage of reducing the costs, inconvenience and risks associated with gonadotropin treatment, and may also provide an alternative for preserving the fertility of women who are about to undergo treatment for cancer.

Spontaneous maturation of GV oocytes *in vitro* was first observed in the rabbit in 1935 [14], and IVM has been well established in animal systems for many years. Immature oocytes can be retrieved from slaughterhouse ovaries and matured *in vitro* prior to fertilization in order to create embryos for research purposes; however, concerns about transmission of pathogens make embryos from this source unsuitable for breeding purposes. In animal breeding programmes that use IVF, it is common for immature oocytes to be retrieved and matured *in vitro* using medium supplemented with LH and FSH.

Edwards observed spontaneous maturation of human GV oocytes *in vitro* in 1969 [5], and he was the first to successfully fertilise human IVM oocytes [7]. However, the first pregnancies following IVM were not achieved until nearly 20 years later [15]. The techniques were refined during the 1990s, and currently many groups routinely employ a strategy of IVM for selected patient indications [for review see 16]. However, although human GV oocytes do progress through meiosis to metaphase II, their subsequent developmental competence remains relatively low, and of the embryos that are transferred, less than 15% implant to form a viable fetus [17]. Clearly, the fundamental processes (as described in Chapter 3 in this book) that lead to successful oocyte maturation remain to be elucidated [18-19], and research continues towards developing an appropriate culture system specifically for the maturation of human oocytes [20]. Current protocols suggest that an initial priming of the ovaries with low doses of FSH from days 2-6 improves success, followed by administration of hCG and co-culture of the oocytes with granulosa/cumulus cells in a medium that is supplemented with FSH and LH or hCG [21].

Poor Response to Stimulation

In some patients, ovarian function is compromised due to a reduced ovarian reserve, resulting in an inadequate response to gonadotropin stimulation. This is particularly common in older women or in patients who suffer premature ovarian failure with reduced ovarian reserve at an early age. Although a variety of different ovarian stimulation strategies have been tried (including natural cycle IVF), the prognosis is invariably poor for a successful outcome. However, the prognosis can be considerably improved with the use of donor oocytes for IVF.

OOCYTE RETRIEVAL AND CULTURE

Oocytes are retrieved from the ovary by aspirating follicles under vaginal ultrasound guidance, using specific disposable needles that fit into a needle guide attached to the ultrasound probe. Aspirates are collected into heated 15ml Falcon tubes. Oocyte retrieval can be safely carried out as an outpatient procedure, using local anaesthesia, intravenous sedation, or light general anaesthesia. An experienced operator can collect an average number of oocytes (i.e., 8-12) in a 10 to 20 minutes time period, and the patient can usually be discharged within 2 to 3 hours of a routine oocyte collection.

Follicular aspirates are examined under a stereo dissecting microscope with transmitted illumination base and heated stage. Low power magnification (6x - 12x) can be used for scanning the fluid, and oocyte identification verified using higher magnification (25x - 50x). After identification, the oocytes are placed into a culture system and incubated at 37ºC in an incubator with appropriate gas mixture to maintain the correct pH, e.g. 5%CO_2 in air, or a gas mixture containing O_2, N_2 and CO_2.

Tissue Culture Systems

Vessels successfully used for in-vitro fertilisation include test tubes, four-well culture dishes, organ culture dishes, and Petri dishes containing microdroplets of culture medium under a layer of paraffin or mineral oil. Whatever the system employed, it must be capable of rigidly maintaining fixed stable parameters of temperature, pH, and osmolarity. Human oocytes are extremely sensitive to transient cooling *in vitro*, and modest reductions in temperature can cause irreversible disruption of the meiotic spindle, with possible chromosome dispersal [22]. Analyses of embryos produced by IVF have shown that a high proportion are chromosomally abnormal [23-24], and it is possible that temperature-induced chromosome disruption may contribute to the high rates of preclinical and spontaneous abortion that follow IVF. Therefore, it is essential that temperature fluctuation is controlled by using heated microscope stages and heating blocks or platforms from the moment of follicle aspiration and during all oocyte and embryo manipulations.

An overlay of equilibrated oil as part of the tissue culture system confers specific advantages as oil acts as a physical barrier, separating droplets of medium from the atmosphere and air-borne particles or pathogens. It prevents evaporation and delays gas diffusion, thereby keeping pH, temperature, and osmolarity of the medium stable during gamete manipulations, protecting the embryos from significant fluctuations in their micro-environment.

Tissue Culture Media

A great deal of scientific research and analysis has been applied to the development of media which will successfully support the growth and development of human embryos, and many controlled studies have shown fertilization and cleavage to be satisfactory in a variety of simple and complex media [25]. Commercially prepared, pre-tested high quality culture media is available for purchase from a number of suppliers world-wide, and there is no scientific evidence that any commercially prepared media is superior to another in routine IVF. Choice should depend upon considerations such as quality control and testing procedures applied in its manufacture, cost, and, in particular, guaranteed efficient supply delivery in relation to shelf-life.

Media containing HEPES, which maintains a stable pH in the bicarbonate-buffered system, can be used for sperm preparation and oocyte harvesting and washing. However, HEPES is known to alter ion channel activity in the plasma membrane and may be toxic to gametes and embryos. The gametes must therefore subsequently be washed in HEPES-free culture medium before insemination and overnight culture. Media specially designated for "sperm washing" is also commercially available.

When an oocyte/cumulus complex (OCC) is identified in a follicular aspirate, its stage of maturity can be approximately assessed by noting the volume, density and condition of the surrounding coronal and cumulus cells. If the egg can be seen, the presence of a single polar body indicates that it has reached the stage of metaphase II.

The following scheme can be used routinely for assessment (Fig. **1**):

1) *Germinal vesicle* - the oocyte is very immature. There is no expansion of the surrounding cells, which are tightly packed around the egg. A large nucleus (the germinal vesicle) is still present and may occasionally be seen with the help of an inverted microscope. Maturation occasionally takes place in-vitro from this stage, and germinal vesicles are pre-incubated for 24 hours before insemination.

2) *Immature* - A tightly apposed layer of corona cells surrounds the oocyte, and tightly packed cumulus may surround this with a maximum size that it approximately 5 times greater than the size of the oocyte. If the oocyte can be seen, it no longer shows a germinal vesicle. The absence of a polar body indicates that the oocyte is in metaphase I, and are not suitable for ICSI.

3) *Pre-ovulatory* - this is the optimal level of maturity, appropriate for successful fertilization. Coronal cells are still apposed to the egg, but are fully radiating; one polar body has been extruded. The cumulus has expanded into a fluffy mass and can be easily stretched.

4) *Very mature* - the egg can often be seen clearly as a pale orb; little coronal material is present and is dissociated from the egg. The cumulus is very profuse but is still cellular. However, the latest events of this stage involve a condensation of cumulus into small black (refractile) drops, as if a tight corona is reforming around the egg.

5) *Luteinised*- the egg is very pale and often is difficult to find. The cumulus has broken down and becomes a gelatinous mass around the egg. These eggs have a low probability of fertilization.

6) *Atretic* - granulosa cells are fragmented, and have a lace-like appearance. The oocyte is very dark, and can be difficult to identify.

Gross morphological assessment of oocyte maturity is highly subjective, and subject to inaccuracies. Denuding oocytes from surrounding cells using hyaluronidase in preparation for ICSI allows accurate assessment of the cytoplasm and nuclear maturity, and it is apparent that gross OCC morphology does not necessarily correlate with nuclear maturity. Alikani *et al.* [26] suggest that although aberrations in the morphology of oocytes - possibly a result of ovarian hyperstimulation - are of no consequence to fertilization or early cleavage after ICSI, embryos generated from dysmorphic oocytes may have a reduced potential for implantation and further development.
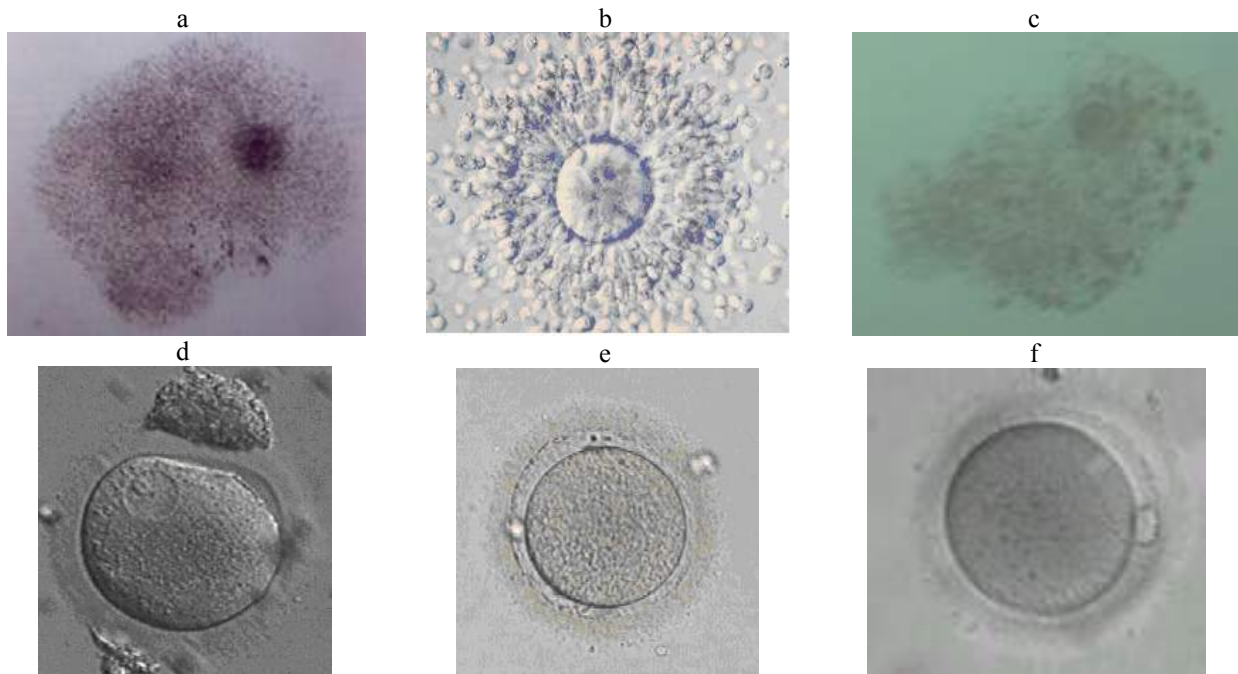
Figure 1: Oocytes at different stages of maturity in vitro. (a) Oocyte cumulus complex in follicular fluid, at the time of oocyte retrieval, low magnification (x12); (b) Phase-contrast micrograph of oocyte cumulus complex at the time of oocyte retrieval, showing expanded coronal cells (x25); (c) Oocyte cumulus complex showing signs of luteinisation at the time of oocyte retrieval; (d) Denuded Germinal Vesicle stage oocyte, with acentric intact nucleus; (e) Denuded oocyte at Metaphase I stage – polar body not extruded; (f) Denuded oocyte at Metaphase II – first polar body extruded.

Many different factors can have an effect on oocyte quality, including morphology, chromosome anomalies, age, follicular microenvironment (in relation to ovulation induction protocol), and endocrine factors [27]. Nuclear maturity is crucial, and must be in synchrony with the maturity of cytoplasm, ensuring that all substrates and reserves are available to support the early stages of development with correct chronology and timing. LH plays a central role in the maturation process of the oocyte, and an imbalance in the secretion of LH may upset the mechanisms involved. Chapter 3 (in this book) outlines the complex and sophisticated combinations of factors and pathways that are essential to achieve a competent mature oocyte that is capable of fertilization and embryo development.

PREPARATION OF SPERM FOR IVF/ICSI

Ejaculated semen is a viscous liquid composed of testicular and epididymal secretions containing spermatozoa, mixed with prostatic secretions produced at the time of ejaculation; seminal plasma contains substances that inhibit capacitation, and prevent fertilization. Aitken *et al.* [28] also demonstrated that white cells and dead sperm in semen are a source of reactive oxygen species which can initiate lipid peroxidation in human sperm membranes. Peroxidation of sperm membrane unsaturated fatty acids leads to a loss of membrane fluidity, which inhibits sperm fusion events during the process of fertilization. Therefore, semen samples must be treated in order to separate the sperm fraction from seminal plasma, leukocytes and dead sperm.

Methods used for preparation include using an overlay of medium and allowing the sperm to "swim up", discontinuous buoyant density gradient centrifugation, or in cases of severe oligo or cryptozoospermia, high-speed centrifugation and washing.

The choice of sperm preparation method or combination of methods depends upon assessment of:

- the motile count
- ratio between motile/immotile count
- volume
- presence of antibodies, agglutination, pus cells or debris

Buoyant density gradients apparently protect the sperm from the trauma of centrifugation, and a high proportion of functional sperm can be recovered from the gradients. Discontinuous two- or three- step gradients are simple to prepare and highly effective in preparing motile sperm fractions from suboptimal semen.

Obstructive Azoospermia - Epididymal and Testicular Sperm

Epididymal sperm can be obtained by open microscopic surgery or by percutaneous puncture, using a 21g. "butterfly" or equivalent needle to aspirate fluid. If large numbers of sperm are found, they can be processed by buoyant density gradient centrifugation or even by swim-up techniques. If only a few sperm are found, the sample may be put into drops under oil, and washed in medium drops using the micromanipulator.

Testicular sperm can be obtained by open biopsy or by percutaneous needle biopsy, and there are a variety of approaches to sample processing, shredding the tissue either with glass slides, needle dissection, dissection using micro-scissors, or maceration using a micro-grinder. The debris is concentrated by centrifugation and examined under high power microscopy to look for spermatozoa. Further processing steps will depend upon the quality of the sample: it may be loaded onto a small single-step buoyant density gradient, or sperm simply harvested "by hand" under the microscope, using a large needle, or assisted hatching or biopsy pipette to collect and pool live sperm in a clean drop of medium.

All preparation methods are adaptable in some way: it is important to tailor preparation techniques to fit the parameters of the semen specimen, rather than to have fixed recipes. In cases where the semen parameters are very poor, a trial preparation prior to oocyte retrieval may be advisable in choosing the suitable technique for particular patients.

INSEMINATION

Oocytes are routinely inseminated with a concentration of 100,000 normal motile sperm per ml. If the prepared sperm shows sub-optimal parameters of motility or morphology, the insemination concentration may be accordingly increased - some reports have suggested that use of a high insemination concentration (HIC) may be a useful prelude before deciding upon ICSI treatment for male factor patients.

ICSI

The ICSI procedure involves direct microinjection of a single sperm into the cytoplasm of a mature Metaphase II oocyte. The oocytes must first be denuded of all surrounding cumulus and coronal cells, by brief incubation in a hyaluronidase solution followed by repeated aspiration through a fine-bore pipette to remove all adherent cells. Morphologically intact oocytes that have extruded the first polar body are suitable for ICSI. A single spermatozoon with normal morphology is selected, and immobilized by crushing its tail using the tip of the microinjection needle. This "tail crushing" impairs motility, and destabilizes the cell membrane, which may be required for sperm head decondensation. The selected immobilized sperm is aspirated, tail first, into the injection pipette, and positioned approximately 20 µm from the tip. After injection, the oocytes are washed in culture medium, transferred to a prepared, warmed culture dish, and incubated overnight (Fig. **2**)

Figure 2: Intracytoplasmic sperm injection

SCORING OF FERTILIZATION ON DAY 1

Approximately 18-22 hrs following insemination, the oocytes must be dissected to remove surrounding coronal and cumulus cells in order to clearly visualise the cell cytoplasm and assess fertilization. Oocytes that have been injected by ICSI have all cells removed prior to injection, and these can be directly scored for fertilization without further treatment. Scoring for fertilization should be done between 17 - 20 hours after insemination, before pronuclei merge during syngamy (Fig. **3**). Normally fertilised eggs are identified by the presence of two pronuclei and two polar bodies, regular shape with intact zona pellucida, and a clear healthy cytoplasm. However, a variety of different features may be observed: the cytoplasm of normally fertilised eggs is usually slightly granular, whereas the cytoplasm of unfertilised eggs tends to be completely clear and featureless. The cytoplasm can vary from slightly granular and healthy-looking, to brown or dark and degenerate. The shape of the egg may also vary, from perfectly spherical to irregular.

a   b
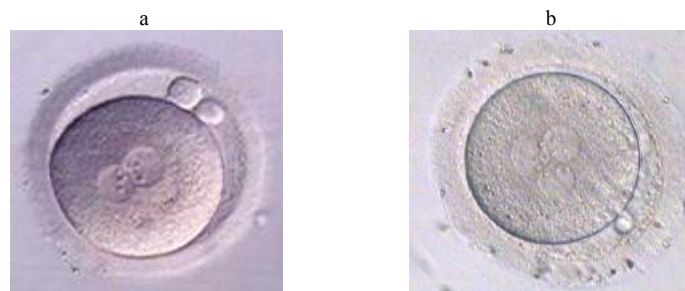
Figure 3: Fertilized oocytes showing: (a) two pronuclei; (b) three pronuclei

Zygotes with normal fertilization at the time of scoring are removed from the insemination drops or wells, transferred into new dishes or plates containing pre-equilibrated culture medium, and returned to the incubator for a further 24 hours of culture. Those with abnormal fertilization such as multipronucleate zygotes must be cultured separately, so that there is no possibility of their being selected for embryo transfer - after cleavage these are indistinguishable from normally fertilized oocytes. Although the presence of two pronuclei confirms fertilization, their absence does not necessarily indicate fertilization failure, and may instead represent either parthenogenetic activation, or a delay in timing of one or more of the events involved in fertilization.

Delayed fertilization with the appearance of pronuclei on day 2 may also be observed, and these embryos tend to have an impaired developmental potential, probably due to morphological, endocrine or sperm defects.

EMBRYO ASSESSMENT AND SELECTION FOR TRANSFER

On day 2 -3 (approximately 48 - 72 hours post oocyte retrieval), oocytes with normal fertilization and cleavage may contain from two to eight blastomeres. The embryos should then be carefully evaluated in order to select those with the highest implantation potential. The limitations of evaluating embryos based on morphological criteria alone are well recognised - correlations between gross morphology and implantation are weak and inaccurate, unless the embryos are clearly fragmenting. Many studies have researched more objective criteria for judging embryo viability and implantation potential, including delayed embryo transfer with culture to blastocyst stage, measurement of metabolic activity and secretions by assaying culture medium, and embryo biopsy followed by preimplantation genetic diagnosis. These methods are excellent tools for research in specialised laboratories, and may eventually lead to the development of accurate embryo assessment. It is also possible that in the future the techniques will be simplified and refined to the extent that they may be more widely accessible and available. However, currently it is difficult to incorporate these selection procedures into a routine IVF practice, and we are left with subjective morphological assessment, which, although unsatisfactory, is quick, non-invasive, and easy to carry out in routine practice (Fig. **4**). Standard morphological critera used in evaluating embryo quality include the rate of division judged by number of blastomeres, size, shape, symmetry, and cytoplasmic appearance of the blastomeres, and the presence of anucleate cytoplasmic fragments.

Fragments

Some degree of blastomere fragmentation is the norm in routine IVF, but it is not clear whether this is an effect of culture conditions and follicular stimulation, or a characteristic of human development. The degree of fragmentation varies from 5 or 10 % to 100%, and the fragments may be either localised or scattered. Alikani *et al.* [29] used an analysis of patterns of cell fragmentation in the human embryo as a means of determining the relationship between cell fragmentation and implantation potential, with the conclusion that not only the degree, but also the pattern of embryo fragmentation determines implantation potential.

1. Five distinct patterns of fragmentation that can be seen by day 3 have been identified:
2. <5% of the volume of the perivitelline space occupied by fragments
3. All or most fragments localised, concentrated in one are of the perivitelline space (PVS), with 5 or more normal cells visible.
4. Fragments scattered throughout, and similar in size
5. Large fragments, indistinguishable from blastomeres, and scattered throughout the PVS; usually associated with very few cells.

Fragments throughout the PVS, appearing degenerate such that cell boundaries are invisible, associated with contracted and granular cytoplasm.

Surprisingly, fragmented embryos can implant and often come to term. This demonstrates the highly regulative nature of the human embryo, as it can apparently lose over half of its cellular mass and still recover, and also confirms the general consensus that the mature oocyte contains much more material than it needs for development. The reasons why part, and only part of an early embryo should become disorganised and degenerate are a mystery.

Different degrees of fragmentation argue against the idea that the embryo is purposely casting off excess cytoplasm, somewhat analogous to the situation in annelids and marsupials that shed cytoplasmic lobes rich in yolk, and favours the idea of partial degeneration. Perhaps it involves cell polarisation, where organelles gather to one side of the cell. It is certain that pH, calcium, and transcellular currents trigger cell polarisation, which may in certain cases lead to an abnormal polarisation, and therefore to fragmentation. These areas are for the moment open to speculation, and continuing studies are in progress.
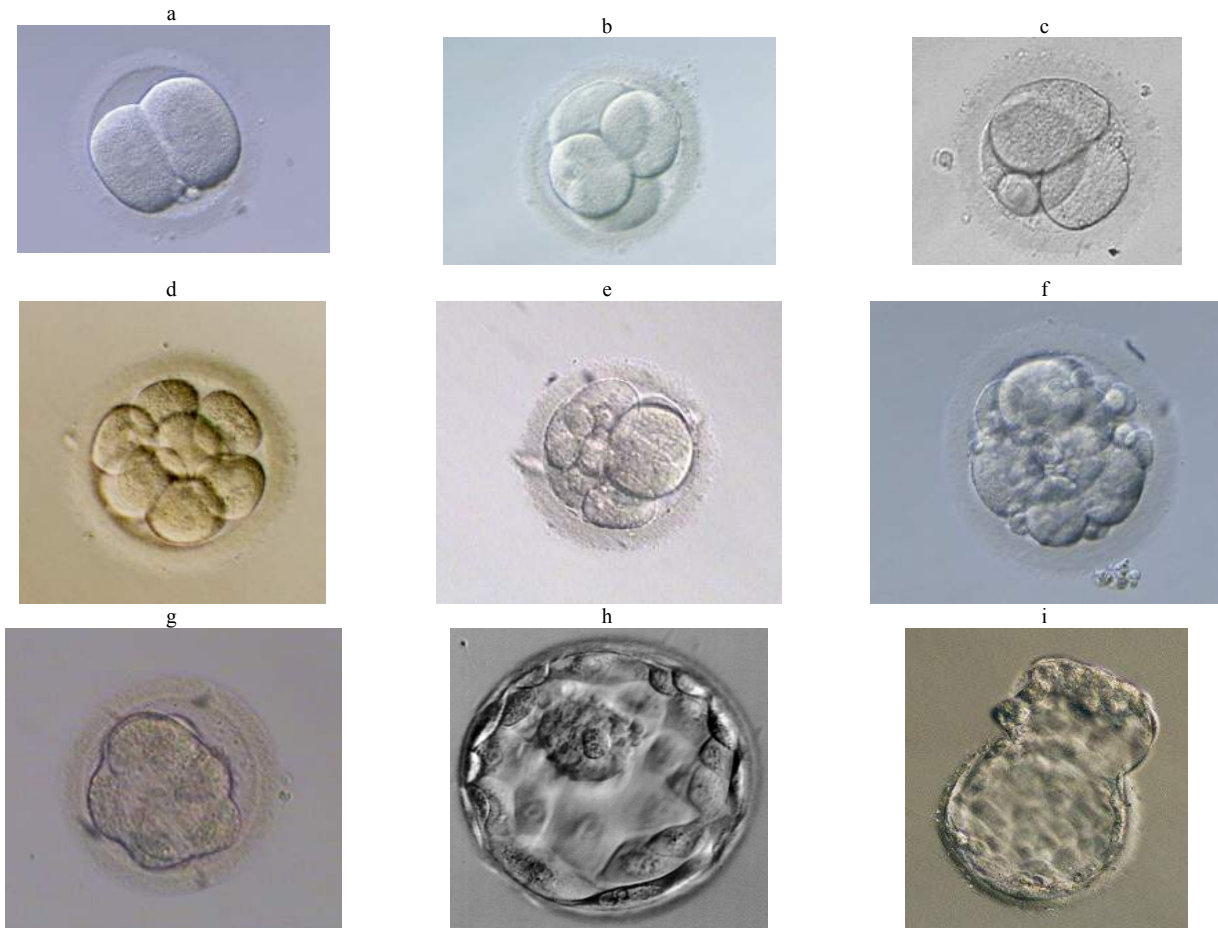
Figure 4 Morphological assessment of embryo quality. (a) 2 cell embryo at the end of Day 1, Grade1; (b) 4 cell embryo on Day 2, Grade 1; (c) 4 cell embryo showing unequal blastomeres on Day 2, Grade 2; (d) 8-cell embryo on Day 3, Grade 1; (e) 6 cell embryo on Day 3, Grade 2; (f) 6-cell embryo on Day 3, Grade 3; (g) Compacting morula, Day 4; (h) Expanded blastocyst, Day 5; (i) Hatching blastocyst, Day 6

Embryo Transfer

Historically, routine embryo transfer was carried out on Day 2, approximately 48 hours after oocyte retrieval. However, delaying embryo transfer until Day 3 has shown no detrimental effects on pregnancy rates, and may be of benefit in selecting embryos with a better prognosis for development. When selecting embryos for transfer, we must appreciate that the time during which this observation and judgement is made represents only a tiny instant of a rapidly evolving process of development. Embryos can be judged quite differently at two different periods in time, as may be seen if a comparison is made between assessments made in the morning, and later in the day immediately before transfer. In general, those embryos at later stages and of higher grades are preferred, but the choice is often not clear-cut. Attention should also be paid to the appearance of the zona pellucida and to the pattern of fragmentation.

The recent application of preimplantation genetic diagnosis by FISH analysis of biopsied blastomeres has shown a surprising discrepancy between gross morphology and genetic normality of the embryos, in that even the most

"beautiful" embryos may have genetic abnormalities, whilst those with less aesthetic qualities, including the presence of fragments, may in fact have normal implantation potential [30-31].

Blastocyst Transfer

Extended culture with the transfer of blastocysts on day 5 or 6 may confer advantages with respect to better synchrony between the embryo and endometrium, as well as eliminating those embryos that are unable to develop after activation of the zygote genome due to genetic or metabolic defects. The aim of extended culture is to produce blastocysts with better implantation potential than cleavage stage embryos, so that transfer of only one or two blastocysts may result in successful pregnancy, thus reducing the number of multiple gestations and increasing the overall efficiency of IVF. Blastocyst culture also allows trophectoderm biopsy and assessment of embryo metabolism as further tests of embryo viability. An important prerequisite for blastocyst culture is an optimal IVF laboratory culture environment; there is no advantage in extended culture unless satisfactory implantation rates are already obtained after culture to day 2 or 3. This strategy has also been used in the treatment of patients who carry chromosomal translocations; these translocations cause a delay in the cell cycle, and abnormal or slowly developing embryos are eliminated during *in vitro* culture. Normal pregnancies have been successfully established after transfer of healthy blastocysts in a group of patients carrying translocations [32].

The ability to identify healthy viable blastocysts is an important factor in the success of blastocyst transfer, and a grading system has been devised which takes into consideration the degree of expansion, hatching status, the development of the inner cell mass and the development of the trophectoderm [33].

Embryo Transfer Procedure

There is no doubt that the technique of embryo transfer, although apparently a simple and straightforward procedure, is absolutely critical in safe delivery of the embryos to the site of their potential implantation. Studies repeatedly show that pregnancy rates can vary in the hands of different operators, and with the use of different embryo transfer catheters. In a study of embryo transfer procedures under ultrasound-guided control, Woolcott and Stanger [34] observed guiding cannula and transfer catheter placement in relation to the endometrial surface and uterine fundus during embryo transfer: their results indicated that tactile assessment of embryo transfer catheter was unreliable, in that the cannula and the catheter could be seen to abut the fundal endometrium, and indent or embed in the endometrium in a significant number of cases.

Luteal phase support with progesterone supplements is started the day before the oocyte retrieval and continued daily until pregnancy is assessed on day 15 post OCR. If pregnancy is confirmed, luteal phase support may be continued until day 77, after which it is gradually withdrawn.

A positive pregnancy test on D15 after oocyte retrieval is confirmed by ultrasound scan on day 35 to confirm the presence of (hopefully) a single intrauterine gestation sac with visible foetal heartbeat.

"The development of a new individual is a long and complex process, which can fail at any step. Each step can only occur if all of the previous ones have been completed correctly, and every link in the chain is essential" [27].

Despite the progress that has been made over a 30-year period, with IVF practiced in every country of the world resulting in the birth of hundreds of thousands of healthy children, many scientific and medical questions and problems remain to be solved in order to optimise the chance of delivering a single, healthy baby after IVF treatment. Current emerging technologies aimed towards embryo assessment will hopefully reduce the risks associated with multiple births by allowing the transfer of a single embryo [35]. *In vitro* maturation of oocytes obtained either from Graafian or from primordial follicles is a further area of current research that may significantly alter the future of ART and its applications [36].

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Current State of the Art in Large Animal Cloning: Any Lesson?

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Abstract: Following fertilisation, major changes occur in the organisation of chromosomes and genes in the zygote that contributes to the formation of totipotent cells and their ensuing differentiation to the diverse cell lineages in the organism. The aim of animal cloning through Somatic Cell Nuclear Transfer (SCNT) is to re-establish the reprogramming that occurs in the fertilized embryo. SNCT often leads to abnormal development with a majority of concepti lost before birth. Imprinted genes are amongst the most frequently affected genes, and their altered expression could in part explain the observed developmental phenotypes. Here, we report our experience in sheep cloning. We describe the major phenotypic abnormalities observed in the extraembryonic tissues of sheep clones and suggest some possible strategies to improve SCNT.

INTRODUCTION

Dolly the sheep demonstrated that it was possible to multiply genotypes though nuclear transfer. In that epochal experiment, a single lamb was produced by the reconstruction of enucleated sheep eggs with cultured epithelial cells obtained from the mammary gland of an ewe, and transferring the resulting embryos into the womb of surrogate mothers [1]. This remarkable accomplishment broke a major dogma in developmental biology, which asserted that cells from adult animals (frogs in the specific case) are irreversibly locked into their differentiated status [2] and became one of the hottest scientific issues of the last 10 years. So far, 11 mammalian species have been cloned [3], and micromanipulation procedures have been simplified allowing the production of large numbers of embryos [4-5]. However, despite significant technical improvements, the frequency of offspring production in Somatic Cell Nuclear Transfer (SCNT) remains essentially comparable to the original report.

SCNT has also been applied to non-reproductive cloning. The demonstration of the full reversibility of the differentiated state of a cell suggested that nuclear transfer could be applied to the production of patient-tailored stem cells for therapeutic purposes [6]. The potential of therapeutic cloning has been suggested by the successful treatment of an animal model of disease through integrating nuclear transfer, gene therapy and stem cell biology [7]. Since then, the progress achieved has been truly spectacular if we consider that "oocyte free" nuclear reprogramming of somatic cells to a condition of multipotency can be induced directly through the transfection of 4 pluripotency-associated genes (induced Pluripotent Stem Cells, iPS), [8-10]. Accordingly, SCNT has lost appeal comparing to iPS, which is of paramount importance for basic science and particularly for biomedicine.

Reproductive cloning has been the main aim of the scientists that cloned the first mammal and still stands as a powerful technology with multiple applications. SCNT allows scientists to "copy and paste" a specific genotype; a strategic tool with important applications in farm animal breeding [11], the production of transgenic animals [12-15], and for the multiplication of threatened species [16-17]. Thereby, the development of efficient nuclear reprogramming approaches for the production of normal cloned animals remains central in the agenda of reproductive biologists.

SHORTCOMINGS IN SCNT

With the technique of SCNT, the aim is recapitulate in 24-48 hours the epigenetic reformatting that occurs separately in the male and female germ line over a much greater timespan. Hence, we should not be surprised by the abnormal phenotypes arising in clones [18]. The main factor responsible for the frequent demise of clones is the oocyte's failure to restore a totipotent state to the transplanted nucleus, a process defined as "Nuclear Reprogramming" [19].

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Nuclear reprogramming remains phenomenological under the current state of art [20] and, as a consequence, epigenetic deregulation and abnormal gene expression are observed in pre/post implantation embryos [21-22] as well as in newborn animals [23-25].

The extra-embryonic tissues are the most affected in all the stages of foetal development [26-27] and even in full term clones [25] with lesions that differ according to the timing and mechanism of implantation between the species studied. In mice [28], and also partially in cattle [29], a diffuse placentomegaly has been described. An increased number of glycogen cells and enlarged spongiotrophoblast cells are also commonly found in mice [28], sometimes associated with an enlargement of trophoblast giant cells and disorganization of the labyrinth layer [28]. The placentae in cattle clones display fewer placentomes, often larger than normal and irregular in size [30]. Histological examination revealed a hypotrophic trophoblastic epithelium and reduced vascularization [31]. The latter two alterations were also found in sheep cloned placenta [25].

Why the placenta is the organ where genes are often deregulated in clones is not clear. A plausible explanation might be the short functional life of extraembryonic tissues. Hence, the epigenetic marks securing its developmental genes might be not safely locked. A confirmation of the "light" epigenetic control can be seen in early embryos, where the trophectoderm, from which predominantly originates the placenta, has a significantly more labile level of DNA methylation comparing to the Inner Cell Mass (ICM) which generates instead the foetus [32].

Apart from epigenetic deregulation, chromosomal anomalies such as aneuploidy and/or endoreduplication are also quite common in extraembryonic tissues. An extreme example might be found in tetraploid embryos. These embryos, artificially produced by electrofusion of the first two blastomeres or inhibition of the completion of the first mitotic division, develop a functional placenta able to nourish a foetus derived entirely from stem cells till term [33]. Even normal cleavage stage embryos have a high proportion of aneuploid cells [34, Ptak and Iuso, unpublished]. It is therefore very likely that the cells with abnormal chromosome constitutions are allocated within the trophectoderm.

ABNORMAL PLACENTATION IN SCNT ARISES FROM THE UNBALANCED EXPRESSION OF IMPRINTED GENES.

A paternal and a maternal set of chromosomes are required for normal development in mammals, due to the allele-specific expression of imprinted genes [35]. The phenotype of the SCNT clones and particularly the corresponding placenta indicates that imprinted genes are deregulated.

Genomic imprinting is controlled by epigenetic modifications which are added to the DNA through DNA methylation and histone modifications at the loci of imprinted genes [36]. These epigenetic marks (imprints) are present on the DNA sequence of elements regulating the mono-allelic expression of imprinted genes, named 'Imprinting Control Regions' (ICR). The establishment of the imprinting marks in the germ cells and their relative maintenance in the somatic compartment is discussed in detail elsewhere [37].

The epigenetic milestone in all ICR is the addition of a methyl group in methyl cytosine in GpC sequences through the recruitment of a repressive protein component [38]. Some ICRs are methylated on the maternal allele, whereas others are methylated on their paternal allele. These imprints are essential for the expression of imprinted genes. Since placental development and function relies largely on the correct expression of imprinted genes, any alteration of the epigenetic asset might exert its effects early in development [39]. The establishment and the maintenance of the epigenetic marks appear to be highly sensitive and easily destabilized by external stressors. It has been established beyond any doubt that the exposure of the embryo to non physiological *in vitro* culture conditions result in abnormal imprinted gene expression with compromised development, often characterized by unbalanced foetal growth [40-41]. This phenotype is particularly severe in clones, whose production requires invasive manipulation. The question is: do the abnormalities observed in SCNT clones result only from the abnormal expression of imprinted genes? Probably not.

IMPRINTED GENES ARE NOT ONLY DEREGULATED IN SCNT

Imprinted gene loci are not the only differences in DNA methylation that exist between the sperm-derived and the egg-derived genome. The DNA brought about by the fertilizing spermatozoon is highly methylated, but following

fertilisation, most of this DNA methylation is removed [42] by a nuclear demethylase which is just starting to be examined [43]. This global DNA demethylation does not affect imprinted gene loci. Thus, the egg and sperm-derived genomes are remodelled very differently in the zygote and during early cleavage stages.

DNA methylation is a landmark for epigenetic regulation, so one should expect that embryonic development, essentially similar between mammals, should rely on a highly conserved mechanism. Apparently this is not the case. Similar studies conducted on other mammalian embryos unveiled remarkable differences in methylation patterns between different species. Rats, pigs and bovines follow the mouse pattern, with zygotes displaying active demethylation of the paternal pronuclei, followed by a passive demethylation during the morula stage and asymmetric methylation in TE and ICM cell lineages, although with quantitative and temporal differences between species [44-45]. Sheep and rabbit zygotes do not demethylate the paternal pronuclei, and the passive demethylation which is typical of early cleavage is also absent [46-47]. Human zygotes are somehow in the middle, since asymmetric demethylation of the two pronuclei is shown in only half of the processed zygotes, whereas in maternal and paternal pronuclei, a low level of methylation has been found in the remaining half [48]. The extent of which these chromatin remodelling mechanisms present in the egg act on the somatic cell-derived chromosomes remains poorly understood. In SCNT, the somatic cell which is introduced into the enucleated egg has a chromatin which is already nucleosomally organised with DNA methylation levels typical of differentiated cells, and most of this methyl mark remains unchanged. The main message from the published data is that the oocyte fails to reprogram, as far as DNA demethylation is concerned, the genome of a somatic cell. Given the gross abnormalities in the methylation patterns observed in cloned embryos, it is likely that even non-imprinted genes are affected by SCNT. Genes associated with pluripotency, like Oct4, Nanog, and Sox2, are also down regulated in pre-implantation early SCNT embryos [49-50]. The evidence that other genes are also aberrantly expressed in SCNT was first described in a short paper describing the expression profile of imprinted and non imprinted genes in cloned mice foetuses and placenta [51]. This concept contrasts somehow with the dominant view that abnormalities in SCNT arise from the deregulation of imprinting.

Recently, we have analysed by RT-PCR the expression of 22 imprinted and non-imprinted genes, including the main angiogenetic factors and the components of the Notch signalling pathway, which are involved in vascular development and differentiation [52] in the extra-embryonic tissues of sheep clones. As expected, we have found significant differences for most of the imprinted genes analysed in clones. Interestingly however, non-imprinted genes were also deregulated in SCNT extra-embryonic tissues. Particularly, deregulation in the expression of angiogenetic and Notch, such as NOTCH 1, 2 and 4 and JAG1 was observed. The expression in extra-embryonic tissues of the enzyme DeNovoMethyltransferase 1 (DNMT1), one of the master genes for the establishment of imprinting marks in the genome, was strongly decreased in SNCT in comparison to control conceptuses (Fig. **1**, Toschi *et al.*, unpublished).

These data suggest that we must take into account the expression of imprinted and non-imprinted genes for the elaboration of effective reprogramming strategies.

Few of the solutions proposed so far to improve nuclear reprogramming and consequently to increase the frequency of normal offspring achieved by SCNT are actually effective. Only two approaches tested in mice resulted in an effective benefit. These were the use of chromatin modifiers like Tricostatin A (TSA, an inhibitor of histone de-acetylases), and the use of ES cells as nuclei donors [53]. But there are other approaches that might be worth testing.

The best proof that a complete nuclear reprogramming has been achieved is the delivery of a normal offspring after SCNT. However, this is a massive waste of time and money for the developmental biologist dealing with large animals. In addition, we believe that it is unethical to transfer a cloned embryo for development to term unless we have objective evidence that our cloning strategy gives an improvement over the current state of art. Therefore, a molecular fingerprint that allows us to "quantify" a nuclear reprogramming strategy has to be established.

HOW AND WHEN CAN THE IMPACT OF A NUCLEAR REPROGRAMMING STRATEGY BE ASSESSED?

A reasonable number of genes indicative of nuclear reprogramming can be monitored at the single blastocyst level [54], and DNA microarrays for detecting large scale gene expression profiles of individual SCNT embryos are now

available [55]. Another important tool is the monitoring of the expression of critical reprogramming genes in living embryos (OCT4, for instance) through their coupling to fluorescent tags [56].

The results obtained in the few publications to date are not clear. Analyses carried out at the transcriptome level are indicative of reprogramming following SCNT [57]. However, this finding contrasts with previous data on the global epigenetic reorganization of SCNT clones, namely DNA methylation [46], and does not justify the phenotype observed in foetuses and extraembryonic tissues in clones. Probably, minor epigenetic changes not detected by these techniques exert their effect during organogenesis, as demonstrated in the mouse [58]. Hence, it is crucial to identify a developmental stage that provides us with a molecular fingerprint indicative of the "normality" of the clones. It is also likely that, due to species specific differences in the mechanisms of implantation, the developmental window, as well as the gene panel, might vary among the different animals.

Regarding sheep, the animal model on which we have been working, we decided to focus on early post-implantation stages, around day 20-22. This is the stage where placental vascularization, a fundamental process for further foetal and extraembryonic tissue development, starts. Therefore, we monitored the expression pattern of a suitable panel [22] of marker genes (Table **1**) between day 20 and 24 of pregnancy, thus establishing a reference model against which to plot the expression profiles of cloned placenta. *In vivo* development to term should be carried out only in cases where the gene expression profile obtained in clones is similar to the one observed in control foetuses.

Table 1: Panel of genes used as markers of placental vascularization/development in early post implantation sheep normal conceptuses (days 20-22)

Notch pathway	Angiogenetic Factors	Maternally imprinted	Paternally imprinted
NOTCH 1, 2, 4; DLL 1, 4; JAG 1, 2; HEY 1, 2. EPHRIN-B2.	VEGF; FGF-2; FGF2-R2; ANG1; ANG2; Tie-2;	IGF2; MEST.	H19; CDKN1C; PHLDA2;
Function			
Vascular vein/artery differentiation	Vascular formation Vascular proliferation	Growth factors production Nutrients transport	

HOW TO IMPROVE SCNT?

The solution so far suggested such as DNA demethylation and TSA treatment [59] rely on bulk, non specific effects which might lead to positive as well as negative effects. The use of ES cells as nuclei donors is also effective in mice, but it cannot be extended to other mammals where ES are not available. Therefore, a reliable cloning protocol has yet to be established. SCNT is a multi-step procedure, therefore, every passage required for embryo reconstruction could be improved, including aspects related to oocyte/embryo biology. The current state of art of *in vitro* embryo production is now satisfactory in all farm animals; however, the media formulated for normal embryos may not meet the requirements for cloned ones. There is evidence that SCNT embryos develop better in complex media, suggesting that some of the metabolic pathways of the differentiated cell are still active after nuclear transfer [60]. The development of "compromise" (embryo/somatic cells) media may therefore improve the viability and in turn the level of nuclear reprogramming in cloned embryos [61-62].

However, the core of the problem remains nuclear reprogramming. Promising indications come from the data from induced pluripotency, the iPS field. The mechanism of nuclear reprogramming is being carefully dissected at the molecular level, and the complete control of this process appears to be within reach. The easiest thing to try should be the use of iPS as nuclei donor, which, being pluripotent, should be more "reprogrammable". In addition, work carried out in iPS cells has shed new light on the role of the tumour suppressor gene p53 during nuclear reprogramming [64]. This finding suggests that p53 silencing in somatic cells prior to nuclear transfer might result in the improvement of nuclear reprogramming. Whereas the induced expression of only four totipotent genes in somatic cells reversed the state of differentiation restoring the multipotent status of somatic cells [8], we recently suggested a reverse approach for "oocyte based" nuclear reprogramming, where somatic cells are induced to acquire the chromatin configuration of a terminally differentiated cell, the spermatozoa. This chromatin reorganization is induced through the transfection of testis-specific reprogramming/remodelling proteins (DRBT) in somatic cells

prior to nuclear transfer [45]. In a preliminary study, we showed an improvement in reprogramming in somatic cells (skin fibroblasts) transiently transfected with DRBT, at least at the blastocyst stage (Loi, unpublished).

We can conclude this short review by stating that nuclear reprogramming is no longer confined to the oblivion of empiricism, as it used to be, but targeted nuclear reprogramming strategies are now available, letting foresee significant advancements in SCNT in the short term.
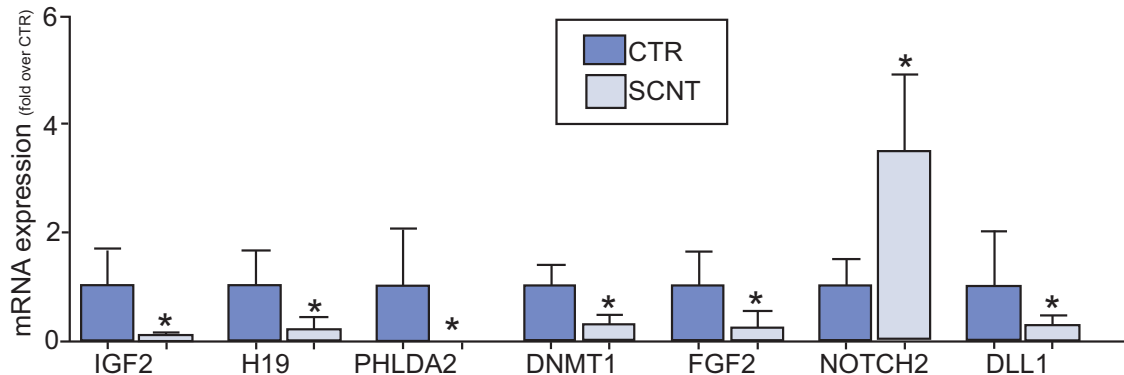
Figure 1: mRNA expression levels of genes differentially expressed between control and clone conceptuses. Data reported in this graph are the mean (±SEM) of at least three independent determinations, each in triplicate. Statistical analysis was performed by the non parametric Mann-Whitney T test, elaborating experimental data by means of the InStat 5 program (GraphPAD Software for science). Differences were considered significant for P value <0.05.

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Stem Cells from Oocytes and Oocytes from Stem Cells

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Abstract: Full oocyte competence is the indispensable requisite for embryonic development and, at present, no ways are known to restore competence if, for any reason, it has been even slightly compromised. The same is not true for sperm cells that can initiate and sustain development even if severely abnormal or damaged as long as the DNA is intact. This points clearly to the uneven burden carried by the two gametes and indicates clearly the essential role of the oocyte. Parthenogenesis is the obvious consequence of such disparity with a number of lower species capable of giving birth to new individuals without any paternal contribution. Mammals are an exception to the rule due to epigenetic mechanisms limiting the parthenotes developmental potential. However, the blastocyst stage is easily reached and parthenogenetic stem cells can be generated whose differentiation potential seems to be much wider than that of whole parthenotes. Switching perspective, we move from stem cells originated from oocytes to oocytes originated from stem cells. Since embryonic stem cells can colonize the germ cell lines when chimeras are generated, it was not so surprising that oocytes can be obtained from stem cells. Although there is still a long way to go before full competence is reached it clearly opens the way to the hypothesis of having an unlimited source of oocytes. Finally, a recent and highly controversial set of results suggests that oocytes are not in such a limited supply as it is generally believed but post-natal oogenesis takes place at a surprisingly high rate.

THE POTENCY OF THE OOCYTE AND ITS LIMITS

Oocyte competence is a familiar concept that has been extensively investigated and refers to the capacity of a single oocyte to sustain embryonic development. The basic concept of oocyte competence is that the first requirement for embryonic development is that the female gamete has completed its growth and accumulated all the components of different nature that will be used during embryogenesis. A successful transition from maternal to embryonic transcription is the first obvious step but evidence exist that oocyte influence extends well beyond this stage into embryogenesis. A wide consensus exists that even if several things can go wrong during embryonic development this will not even begin in the absence of a fully competent oocytes and no ways are known to restore such competence once it has been compromised. Therefore, it comes with no surprise that development can progress to a great extent without any paternal contribution as demonstrated by parthenogenetic activation.

Parthenogenesis is a form of reproduction common to a variety of lower organisms such as ants, flies, lizards, snakes, fish, amphibians, honeybees that may routinely reproduce in this manner. Mammals are not spontaneously capable of this form of reproduction. However, mammalian oocytes can successfully undergo artificial parthenogenesis *in vitro* and can be activated by mimicking the calcium wave induced by the sperm at fertilization, with a variety of chemical, mechanical or electrical stimuli and begin to cleave. Mammalian parthenotes can develop into different stages after oocyte activation, depending on the species, but never to term [1].

Parthenogenetic activation can be induced at different stages along oocyte meiosis resulting in parthenotes with different chromosome complements.

When parthenogenetic activation is performed in oocytes at the second metaphase it results in the extrusion of the second polar body and leads to the formation of a haploid parthenote. This method is rarely used since, in this case, the developmental competence is reduced compared to normal embryos and to diploid parthenotes).

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Diploid parthenotes can be obtained in two different ways. The most common consists in combining the activation of metaphase-2 oocytes with exposure to an actin polymerization inhibitor, usually cytochalasin B [3].

Alternatively, a diploid parthenote can be generated by preventing the extrusion of the first polar body. This protocol leads to the formation of tetraploid oocytes [4] and the diploid status is then re-established at the end of oocyte maturation with the extrusion of the second polar body.

Using one or the other method has important consequences on the parthenotes genetic make up. In fact, performing oocyte activation before the extrusion of the second polar body determines the formation of highly homozygous parthenotes, since the diploid status is obtained after the segregation of sister chromatids. On the contrary, when the extrusion of the first polar body is inhibited parthenotes are genetically identical to each other but have the same heterozigosity of their mother [4].

The occurrence of a high degree of homozigosity in parthenotes has been evaluated in contrasting ways in the perspective of using these entities as a source of embryonic stem cells.

Homozygosity can be seen as a potential benefit when the reduction of immunogenicity of a stem cell derivative is considered. At the same time, it must be remembered that homozigosity can represent a severe risk. Loss of heterozigosity, in fact, may amplify any negative genetic component potentially present in the genotype.

Irrespective of how activation has been performed and of what ploidy has been generated, mammalian parthenotes are unable to develop to term as opposed to their lower vertebrates counterpart. In the mouse the most advanced parthenotes survive to the early limb bud stage, have little extra-embryonic tissue and almost no trophoblast [5]. As shown in Table **1**, parthenotes will not develop and will arrest development by day 10 in the mouse, day 11.5 in rabbit, day 21 in sheep and day 29 in pigs. This arrest in development does not seem to be due only to the fact that they develop a small trophoblast since, even when supplied with trophoblast cells, parthenotes will stop and die [6]. Indeed studies with chimeras between mouse normal (zygotic) embryos and parthenotes show that parthenotes fail because of some cell-autonomous defects that affect parts of the embryo proper, including skeletal muscle, liver and pancreas [7].

Table 1: Parthenotes are unable to develop to term. The table summarizes the day of pregnancy when the different mammalian species considered arrest development following parthenogenetic activation (maximum development) and the related length of pregnancy (pregnancy length).

Species	*Max Development*	*Pregnancy Length*	*Reference*
Mouse	10	21	[91]
Rabbit	10-11	31	[92]
Pig	29	114	[93]
Sheep	25	150	[94]
Bovine	48	280	[95]
Marmoset Monkey	10-12	144	[96]

The reason for this arrest in development is believed to be due to genomic imprinting. Genomic imprinting appears to be restricted to eutherian mammals, and has evolved as a result of the conflicting concerns of the parental genomes during the growth process. It has been shown that normal mammalian development requires genomic contributions from both the mother and the father. As described by Surani [8] although oocytes are potentially totipotent in many organisms, this is not so in mammals. The reason is that the maternal genome is epigenetically modified in the germ line to contain only the maternal 'imprints', which will normally result in the repression of certain maternally inherited imprinted genes (Fig. **1**). A paternal genome is therefore essential to 'rescue' the oocyte, as the maternal genes are imprinted reciprocally to paternal imprints. This explains why both genomes are needed in mammalian development. Maternal and paternal genomes are complementary but not equivalent therefore both sets are required for a correct growth process [9]. This implies that mono-parental duplications of regions of a number of chromosomes may therefore be lethal or detrimental to the embryo.
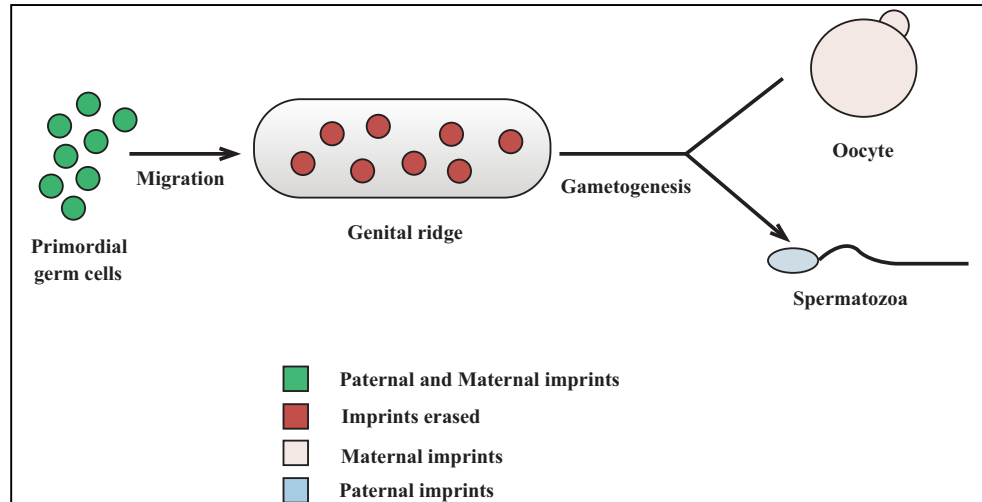
Figure 1: Imprinting pattern. Primordial germ cells have contributions from both parental genomes (paternal and maternal). After migration in the genital ridge all imprints are erased. During differentiation specific genes are reciprocally repressed in spermatozoa or oocyte making them complementary.

At present more than 50 genes have been identified as imprinted in the human. These genes display a vast range of functions, ranging from splicing factors, such as *Snrpn*, to growth factors, such as insulin (*Ins1* and *Ins2*) and *Igf-2*, to genes that are functional as RNAs, such as *H-19* and *Xist* [reviewed by 10].

The birth of mice without a paternal contribution [11] should not be confused with parthenogenesis and, therefore, be considered a sort of possible dispensability of genetic imprinting.

On the contrary, bi-maternal mice, as these animals are defined, represent the elegant demonstration of how parental methylation imprints exert their function [12]. In their simplest form, bi-maternal mice are generated by putting together the genome from a fully-grown (fg) oocyte (with maternal imprints) and one from a non-growing (ng) oocyte (with no maternal imprints yet) but restoring normal expression of the Igf2 gene by deletion of the H19 ICR in the non growing oocyte. This manipulation allowed the development of bi-maternal embryo till birth, and, very rarely, till adulthood [13]. Further studies demonstrated that the restoration of normal expression levels at both the Igf2-H19 and the Dlk1-Gtl2 domains (by deletion of the two corresponding ICRs in the ng oocyte) is the minimum manipulation of the imprinting pattern that allows the birth of many live-born animals that are viable and fertile [11].

Taken together, these studies indicate that the aberrant expression of genes at the paternally-imprinted domains is the only obstacle to the progression of parthenogenesis and, as a consequence, that sperm-derived proteins and RNAs are not required for full development to term, at least in mouse. Therefore, there are no apparent biological restrictions to the generation of an entire organism exclusively from an oocyte not only in lower species but in mammals as well. The ultimate reason for the evolution of imprinting at all remains an open question. Besides obvious reasons like avoiding high levels of homozygosity that can be harmful to the single individual but less so to a species, recent theories hypothesize that genomic imprinting has played a significant part in two major developments that have influenced the evolution of sexually dimorphic reproductive strategies of mammals: viviparity and development of a placenta on one side, and the massive expansion of the brain through growth of the neocortex on the other [14].

STEM CELLS FROM OOCYTES

Having established that mammalian oocytes are actually totipotent with no need for a paternal contribution but are limited in their development by mechanisms that act primarily on the development of a functional placenta, it is not surprising that pluripotent cell lines can be derived from parthenogenetically activated oocytes. Whether or not parthenogenetic, embryonic stem cells (ESC) can escape the limits imposed by imprinting to the development of a full individual is an open question.

The first cell lines derived from parthenogenetic embryos were established in mice more that 20 years ago [15]. These pioneering results were followed more recently by the derivation of parthenogenetic cell lines in *Macaca fascicularis* [16-17] and in rabbit [18-19]. These results were very promising since, in all three species, cell lines exhibited the fundamental properties that characterize normal bi-parental embryonic stem cells. Cells lines were stable in culture, maintain a normal karyotype, can be differentiated *in vitro*, form embryoid bodies when cultured in suitable conditions and form teratomas, when injected in immune-suppressed mice. All these data support the hypothesis that it should be possible to derive parthenogenetic stem cells also in the human species and that these cells could theoretically be suitable for therapeutic applications.

However, more detailed studies performed in mouse cell lines suggest that cell lineages derived from parthenogenetic cells may be restricted in their differentiation potential. Diploid mouse parthenogenetic lines were able to form chimeras when injected into normal blastocysts with a participation ranging from 5% to more than 70% but the contribution to skeletal muscle and testis was considerably lower than in the other tissue tested [20]. If mouse ES are aggregated with tetraploid host embryos, tetraploid host cells contribute fully to the development of the extra-embryonic membranes while being gradually selected against in the embryo proper. This enables the generation of mice entirely derived from ES cells [21]. When the same procedure is performed with parthenogenetic stem cells, development ceases between day 13 and 15, indicating that parthenogenetic stem cells are unable to form a complete individual [20]. The restricted developmental potential of parthenogenetic stem cells was further indicated by the analysis of teratomas produced by transfer of aggregates under the kidney capsule since very little skeletal muscle could be found [20]. Nevertheless germ-line chimeras could be obtained when parthenogenetic ES were injected in normal embryos [20].

However, the reduction of totipotency, observed in parthenogenetic ES cells, is significantly lower if it is compared to that observed when parthenogenetic embryos are aggregated in chimeras with normal bi-parental embryos [7, 22]. The improvement of parthenogenetic ES cell developmental potential compared to parthenogenetic embryos may be attributed to the disruption of normal imprinting observed during *in vitro* culture, thus suggesting that these cells, despite some alterations, may retain the potential to be used for therapeutic purposes.

Research in the field of human embryology presents a series of specific limits related to ethical concerns. Many people regard in vitro-obtained embryos as human beings even though there is a complete lack of consensus on when the moral and legal status of the developing conceptus should be recognized. Numerous national legislatures or guidelines have therefore banned the use of embryos, even supernumerary or discarded, for research purposes. The International Federation of Fertility Societies (IFFS) Surveillance 2007 reports that about half of the 57 surveyed nations indicated that experimentation on human "pre-embryos" is unacceptable [23]. Authorized researches are always subjected to time limits with respect to embryonic developmental stage and special cautions are mandatory for studies on embryos. Ethical, legal and political concerns on the value of human embryos regulate embryological studies and, similarly to any other study involving patients, impose that research projects are preliminary examined by Institutional Review Boards, use informed consent forms, follow international guidelines, such as the Declaration of Helsinki [24] or World Health Organization Ethical Guidelines [25], founded on the widespread belief that human embryos should not be created and studied for research purposes only.

On this basis, parthenogenesis has been proposed as a possible way to generate a source of embryonic stem cells that may overcome many of the ethical limitations to research on human embryos [26].

Until a few years ago the only data available in the literature indicated that human oocytes can be parthenogenetically activated *in vitro* but do not proceed beyond the 8-cell stage; only recently the development of human parthenotes to the blastocyst stage was reported and not many data are available because of the limited accessibility of unfertilized human oocytes [reviewed by 1]. Due to the Italian legislation (no more than 3 oocytes can be fertilized per IVF cycle) our laboratory had access to high number of human oocytes to be used for developing an efficient protocol of parthenogenetic activation and for directly comparing their developmental competence with that of oocytes derived from the same patients but undergoing ICSI [26]. These experiments allowed us to learn that the combined use of ionomycin and 6-DMAP constantly enabled the development of parthenotes to the blastocyst stage. We think that this is an interesting finding, indicating that 6-DMAP better mimics a kinetic similar to that occurring after fertilization and leads to high *in vitro* development rates in human oocytes, consistent with what already demonstrated in several other species [27].

Having established a reliable source of human parthenotes also enabled the derivation of a number of cell lines (Fig. **2**) that have been proposed as an alternative, less controversial source of embryonic stem cell lines [27-31].
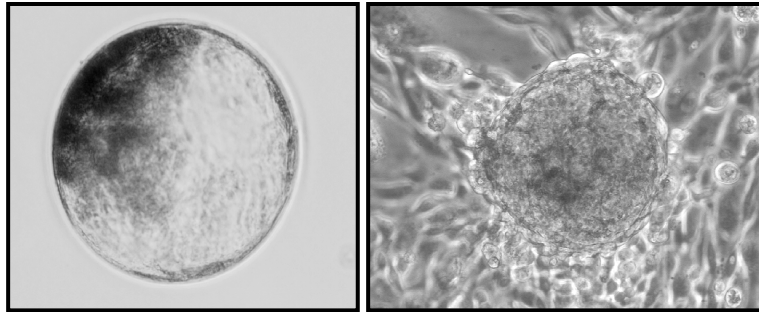
Figure 2: Early parthenogenetic blastocyst (left) and ES cell colony (right). Inner cell mass was isolated from blastocyst and plated on feeder layer cells to form a colony. Original magnification 400x.

However many aspects related to the biology of parthenogenetic embryos and parthenogenetic derived cell lines still need to be elucidated. The decreased extent of heterozygosis may amplify any negative genetic component potentially present in the genotype [32-33]. The very high incidence of chromosome instability and aberrant chromatid separation in oocytes retrieved from IVF patients, especially when over 34 year old [34-36] also represents a concern, given the fact that these represent a large part of the population accessing assisted reproductive therapy and, hence, are a major potential source of oocytes for parthenotes derivation.

Human parthenogenetic (HP) cell lines derived in our laboratory showed typical ESC morphology, expressed a panel of pluripotency genes and had high telomerase activity [37]. Expression of genes specific for different embryonic germ layers was detected from HP cells differentiated upon embryoid body (EB) formation. Furthermore, when cultured in appropriate conditions, HP cell lines were able to differentiate into mature cell types of the neural (Fig. **3**) and hematopoietic lineages. However, the injection of undifferentiated HP cells in immunodeficient mice resulted either in poor differentiation or in tumour formation with the morphological characteristics of myofibrosarcomas. Further analysis of HP cells indicated aberrant levels of molecules related to spindle formation as well as the presence of an abnormal number of centrioles and autophagic activity. These results confirm and extend the notion that human parthenogenetic stem cells can be derived and can differentiate in mature cell types, but also highlight the possibility that alteration of the proliferation mechanisms may occur in these cells, suggesting great caution if a therapeutic use of this kind of stem cells is considered.
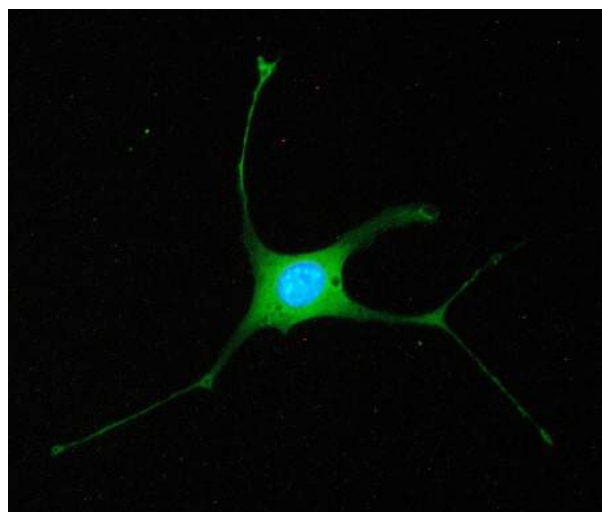
Figure 3: Neural differentiation of human parthenogenetic (HP) cells. When cultured in appropriate conditions, HP cell lines were able to differentiate into mature cell types of the neural lineage. Detail of a cell stained for nestin, a marker of neural differentiation (green) and counterstained with DAPI (blue). Original magnification 600x.

OOCYTES FROM STEM CELLS

A central dogma of mammalian reproductive biology is that females are born with a finite, non-renewing pool of germ cells, all of which are arrested in the prophase of meiosis I (oocytes) and are enclosed by somatic cells in follicles [38-41]. Oocyte numbers decline throughout postnatal life [42-44] through mechanisms involving apoptosis [45-46], eventually leaving the ovaries deprived of germ cells [47]. This is the recognized cause that limits female fertility with age through the exhaustion of the oocyte reserve during the years. The limited and finite number of fully grown oocytes is also the main limit to the application of somatic cell nuclear transfer for the generation of immunologically matched stem cells or to the implementation of assisted reproduction programs for the conservation of endangered species.

However, in recent years, different experimental approaches as well as unexpected findings are providing a completely new perspective and the promise of an infinite source of oocytes.

Oocytes from embryonic stem cells. Embryonic stem cells injected into a blastocyst have the capability to contribute not only to any somatic tissue but also to the germ line [48].

When cultured in vitro, ESC differentiate into a heterogeneous mixture of cell types representing the three somatic germ layers if the pluripotency-maintaining factors are withdrawn from the culture media and the feeder layer is removed. Differentiating ESC can be cultured either in suspension or as adherent monolayer cultures. Suspension cultures will generate embryoid bodies, three-dimensional structures mimicking gastrulation-like process [49]. ESC cultured as adherent cultures form a monolayer of cells that quickly overgrow the culture plate and form multiple layers.

Therefore it was a logical extension to try to differentiate, both mouse and human ESC also into germ cells.

Reports using mouse ESC described oocyte-like [50-54] and sperm-like differentiation [55-58], or both [59], as well as primordial germ cell-like development [60-61]. Human ESC studies reported both the spontaneous and induced differentiation of germ cells but at present, it has not been possible to obtain more mature gametes [62-67].

Mouse ESC studies. Hubner *et al.* [50] were the first to develop a strategy for mouse ESC differentiation into oocytes using a ESC line genetically modified with a germ cell-specific Oct4 promoter driving a GFR reporter construct. In the appropriate culture conditions ESC differentiated into c-KIT/GFP positive cells expressing low levels of VASA representing migratory stage germ cells. Further culture induced a down-regulation of c-KIT together with the increased expression of VASA, as expected in post-migratory germ cells. After 16 days of culture the meiotic marker SCP3 was found in the nucleus of oocyte-like cells but no distinct chromosomal alignment was detectable. Irrespective of this abnormality, the culture for further 10 days lead to the formation of follicle-like structures that released oocytes of 50-70 μm expressing some specific markers like Zp2/3 and Figlα. But, even more surprisingly, a few days later, pre-implantation embryos were observed, which were likely to be the result of a spontaneous parthenogenetic activation. Further studies based on the same protocol [52] lead to the formation of follicle-like aggregates capable to secrete high level of estrogens into the medium but unable to progress beyond an abnormal meiotic profase.

Recent attempts were based on a two-step strategy which included an initial phase where ESC were induced to differentiate into germ cells with retinoic acid, followed by the co-culture of the resulting EBs with granulosa cells [51]. This allowed the formation of oocytes resembling cells with a diameter of up to 25 μm expressing specific genes like Vasa, Scp3 and Gdf9 but with no sign of follicular organization.

Possibly the most striking results has been described by Kerkis *et al.* [59] who obtained both types of gametes from male mouse ES cells without any genetic manipulation or preselection. The differentiation of both gametes was induced by retinoic acid within non-adherent embryoid bodies, as determined by the expression of early and late germ cell-specific genes in the correct order. At the end of the culture period even a putative blastocyst-like structure was observed but it was not possible to determine whether it was originated by fertilization or parthenogenetic activation.

Even if these results are very encouraging it must be noted that oocyte-like cell maturation, oocyte functionality or their ability to be fertilized and produce offspring was not demonstrated in any of the above reports. A normal progression through meiotic prophase I and/or meiotic arrest seems to be beyond the reach of the current methods.

Human ESC studies. The possibility to obtain gametes from human ESC was suggested by the observation that a subpopulation of undifferentiated human ESC express markers common to both inner cell mass cells and germ cells like OCT4, STELLAR and NANOS1 as well as the gonocyte-specific DAZL [62,68]. Further differentiation into EB determined a down-regulation of these genes accompanied by an increased expression of VASA, a later stage marker of germ cell differentiation. Several studies reported that upon various intervals of EB culture, with or without specific inducing factors like BMP, it was possible to observe the expression of the oocyte specific marker GDF9 and the meiotic markers SCP1 and SCP3 but chromosomal alignment indicative of meiotic prophase I progression was not observed [63-64].

It is worth to notice that only a limited number of ES cells became primordial germ cells (PGCs) in culture. This is consistent with the situation present in normal development, where a limited number of the cells in the proximal epiblast are allowed to differentiate into germ cells. The "choice" of germ cell fate may depend on differences among the ES cells, and the choice is then reinforced by interactions between the cells becoming germ cells and those retaining the somatic cell fate [69]. Tilgner et al. [65] recently presented a promising new protocol to differentiate two human embryonic stem cell lines that maximizes the numbers of primordial germ cells based on cell selection by FACS for SSEA1. The cells obtained in this way have high-level expression of germ cell-specific VASA, SCP1 and SCP3 genes and a small subpopulation appeared to be haploid further supporting their primordial germ cell identity. Furthermore, analysis of methylation patterns showed a series of changes suggesting that these putative hESC-derived primordial germ cells may have begun the epigenetic reprogramming process typical of these cells in vivo.

The lower efficiency of human ESC to differentiate into putative germ cells and their current inability to progress towards more mature cell types is likely to reflect the longer physiological interval of human gamete differentiation compared to the mouse. Furthermore, it must also be reminded that human ESC are not the equivalent of mouse ESC but rather the equivalent of mouse epiblast stem cell (EpiSC) [70-71]. The difference is likely to be very relevant since, in contrast to mouse ESCs, mouse EpiSCs do not express germ cell markers, suggesting a non-germ cell origin [70-71]. However, even if an assessment of their germ cell developmental potential through germ line transmission *in vivo* is not possible because EpiSCs are unable to reconstitute blastocysts, evidence indicates that *in vitro* germ cell differentiation is possible also from EpiSCs but BMP4 is required [71].

Oocytes from Tissue Specific Stem Cells

Even if ESC are the most likely candidates as a source of oocytes data are available indicating that tissue specific stem cells may be a possible source as well. In particular fetal porcine skin stem cells and adult rat pancreatic stem cells were reported to differentiate into oocyte-like cells in follicle-like aggregates that expressed germ cell markers [72-73].

The porcine skin stem cell differentiation protocol looks particularly promising since the oocyte-like cells could bind sperm and support the development of parthenogenetic embryo-like structures; however, meiotic progression and fertilization were not reported [74].

The logical and desirable evolution of these studies would be the differentiation of oocytes from induced pluripotent stem cells (iPSCs) derived from any adult tissue of any individual.

The first results towards this ambitious goal have recently been published in a study which described that the differentiation of human iPSCs into PGCs was obtained by co-culture with human fetal gonadal stromal cells [75]. Gene expression analysis and bisulfite sequencing determined that these cells correspond to committed first trimester germ cells, although the differentiation efficiency of iPSC was lower and less complete than that of normal hESC.

Physiological Oogenesis in Postnatal Ovaries

All the above studies are based on the differentiation of stem cells of different origin with no suggestion that such phenomena can happen physiologically. On the contrary, a series of recent studies resurrected the idea of continuous

oogenesis in postnatal ovaries by showing regeneration of oocytes from putative germ cells in bone marrow and peripheral blood [76-77]. Following the initial observation that the quantitative analysis of healthy and atretic follicles in ovaries sterilized with chemotherapy showed almost no difference in follicle count between control and treated ovaries at 2 months [78], Tilly's group showed that bone marrow transplantation (BMT) in mice chemo-ablated with combined chemotherapy that kills all existing germ cells restored oocyte production. However, since all offsprings were derived from the donor germline mice the Authors suggested that the most likely mechanism of action of BM derived cells is to reinstate recipient oogenesis [76].

These results as well as the idea itself that adult oogenesis actually exists has stirred a heated debate which is currently still ongoing [79-83].

Support to the concept that the oocyte pool endowed in the ovaries of female mammals at birth is fixed and non renewable, initially, came from the study of follicle dynamics in juvenile and adult mouse ovaries, using accurate morphological quantification of oocyte numbers [78, 84-85]. Old [86] and recent [87] studies also described that 400–500 new oocytes are produced during each reproductive (estrous) cycle in the adult female mouse, with the highest and lowest ovarian reserves observed during metestrous/diestrous and estrous, respectively. These estimates of cyclic primordial follicle renewal imply the formation of thousands of new oocytes during the prime reproductive period in females. Since this may be a higher rate than that taking place during foetal development, it sounds difficult to accept. Tilly's group defend their calculations taking into account the loss of hundreds of developing oocytes on a daily to weekly that routinely takes place in the adult mouse ovary through follicular atresia according to their own estimates [78]. According to this view the follicle reserve begins its age-associated decline when oocyte renewal no longer counterbalances loss due to atresia [77,88].

As mentioned above, all these data have been vigorously contested and no consensus has been reached. Finally, should post-natal oogenesis be confirmed, an interesting question remains: is the bone marrow the only source of oocytes in adult life?

A strong alternative candidate as possible source of oocytes has been described in the form of ovarian stem cells (OSC) isolated from the ovarian surface. These cells appear to have the capacity of totipotent germ line ‑ competent embryonic stem cells [89]. They were shown to be capable of differentiating into oocytes, fibroblasts, and epithelial and neural cell types and are known to have the capability of self-renewal, maintaining their undifferentiated state [90].

CONCLUSIONS

This brief summary of a somehow unorthodox series of research on the oocyte opens several exciting possibilities and perspectives. Parthenogenetic stem cells may lead to unleashing the full oocyte potency also in mammals, obtaining every tissue without a paternal contribution. Even more astounding is the possibility to obtain fully competent oocytes directly from the Petri dish eliminating the major limitation of all reproductive biotechnology and of many fertility therapies. And, finally, the well established dogma of the finite amount of oocyte reservoir present in each female is being seriously challenged. Irrespectively of what the general consensus in the future will be this debate has brought a certainly fruitful very accurate screening of oogenesis. Once again facts are more surprising than fantasies.

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