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Preface

The oocyte is one of the largest and most mysterious of cells in mammalian organisms. After many years of inactivity in a primordial state, the oocyte progresses through growth and maturation achieving on the one hand a remarkable level of specialization, while maintaining throughout development a state of totipotency. Most telling of all is the unique capability of the oocyte, in collaboration with the spermatozoon, to give rise to a fully developed organism formed from hundreds of different tissues and myriads of individual cells – a feat that has inspired intellectuals of all ages. Aristotle theorized that "the embryo originates from a gradual development of undifferentiated material (the oocyte)," forerunning epigenesis views of development that emerged early in the seventeenth century. Around this period, preformistic (ovistic) viewpoints appeared that considered embryonic development as an unfolding and growth of parts already assembled in the oocyte. These perceptions and predispositions have withstood the test of time (through centuries) and are buttressed in a biological and clinical context by the work of contemporary scientists who share a passion toward understanding oogenesis. In the human, for example, meiotic errors initiated by events taking place in the fetal ovary can be propagated into the fully grown oocyte and impart a dark shadow on the genetic integrity of a resultant embryo decades after the original aberration took place. On a different time scale, the continuum of the growth and maturative phase of oogenesis, entailing the progressive accumulation and positioning of organelles, RNAs, and proteins, establishes the molecular foundations of the preimplantation embryo that will guide it in both time and space. With the advent of and continued application of assisted reproduction technology (ART) in human medicine, the biology of the oocyte has gained stature and prominence that few would have anticipated 60 years ago. We hope this book brings the reader a sense for why and how this extraordinary cell has become such a mainline focus in the biomedical research enterprise.

This book finds impetus and purpose in casting scientific perspective toward this unique cell for the benefit of scientists and ART specialists. The authors of the chapters are distinguished authorities in their respective areas of competence, some of whom have collaborated with the editors over the last several years. From the opening of the book, the reader is led on a fantastic voyage from the formation of the primordial oocyte to the development of the early embryo, passing through crucial processes of oogenesis, such as

co-ordination of oocyte and follicle growth, gene expression and organelle reorganization during growth and maturation, epigenetic mechanisms, regulation of meiosis, totipotency, cell polarity, oogenesis in vitro, and maternal regulation of early development.

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Part I

Imaging

Imaging Strategies for Studying Mammalian Oogenesis

1

S. Darlene Limback and David F. Albertini

Abstract

Advances in biomedical imaging are now impacting the evaluation of gonads and gametes in experimental animal model systems and human clinical specimens. This chapter summarizes the rationale for both live cell and fixed sample imaging using light microscopy. Useful protocols, detailed methods, and tools for data extraction are covered for a variety of applications pertaining to the analysis of oocytes, embryos, and intact ovarian tissue.

Keywords

Oogenesis • Confocal microscopy • Sample fixation • Sample processing Vital staining • Image processing • Image archiving

Introduction

Oogenesis in mammals involves a complex series of morphogenetic changes that occur within the ovarian follicle. The somatic cells of the follicle, the dominant functional unit of the ovary, provide a platform for achieving the two major attributes of steroidogenesis and ovulation. Teasing apart the contributions of various ovarian cell types, comprising both follicular and nonfollicular compartments, has required the deployment of a variety of experimental tools. Morphological, genetic, biochemical, and cell culture approaches have been adopted to evaluate the process of oogenesis in the intact mammalian ovary, as well as with fractionated and reconstituted cell types, or by the use of follicle isolation and in vitro culture. Each approach offers advantages and disadvantages depending on whether the experimental objective is aimed at improving our understanding of oogenesis, steroidogenesis, or both. It is now appreciated with some degree of certainty that for the process of oogenesis to be more completely understood, multiple strategies are ultimately the most likely to uncover the mechanisms that underlie the remarkable integration of

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folliculogenesis and oogenesis within the context of ovarian physiology [1].

In this chapter, we review and explain the theoretical and practical principles for imaging strategies employed over the past decade to study the process of oogenesis in mammals. Analyzing the structure of the mammalian ovary is complicated by the fact that during developmental and adult functional periods, the ovary is a dynamic organ undergoing de novo remodeling of the vasculature, patterns of innervations and compartmental transformations such as the conversion of the follicle into the preovulatory follicle and corpus luteum that provide the immediate microenvironment in which oogenesis proceeds [2]. Moreover, adoption of newer techniques to analyze intact ovarian tissues in a spatial and temporally accurate fashion has required development and implementation of microscope-based imaging strategies for the study of both living and fixed samples. The goal of this chapter is then to inform and guide students of oogenesis seeking to understand the intricacies of oocyte-somatic cell interactions in the context of the intact mammalian ovary [3].

Extending the Utility of Traditional Histological Approaches

Traditional histological approaches are routinely used for the analysis of intact ovary. This has been especially evident in the many recent papers documenting ovarian phenotypes in transgenic murine animal models [4, 5]. While standard fixation, dehydration, embedment, and sectioning protocols have been extensively reviewed and applied, several new parameters have come to surface that increase the sensitivity and applicability of histological sections for purposes of molecular definition[6]. Adding molecular details by being able to detect both mRNAs and protein in a histological context has considerably extended many of the classical aspects of follicular and oocyte development first recognized in the classical studies of Peters and her colleagues [7].

The ability to identify multiple epitopes in the same or sequential sections makes possible a bewildering array of labeling combinations when used in conjunction with monospecific or polyspecific antibodies. Coupled with the recognition of specific growth factors of oocyte or granulosa cell origin, many key components of paracrine signaling pathways have been revealed [3, 8–10]. As more specific methods for controlling gene and protein expression are developed [11], it can be expected that future experimental strategies will necessitate the adoption of internal standards or markers using small molecule reagents that exhibit the properties of binding directly or specifically localizing to compartments such as the nucleus, plasma membrane, or extracellular matrix. Efforts to achieve an increased level of sensitivity and specificity are currently aimed at applications to histological approaches and thus prompt a closer examination of protocol modifications that have evolved over the past two decades as illustrated in Fig. 1.1. When coupled with the widespread commercial availability of immunological probes for specific proteins or metabolic status, several critical factors emerge that must be addressed in well-controlled and meaningful studies including specimen preparation prior to fixation, choice of fixation, antigen retrieval on sections, counterstaining options, and choice of imaging modalities. In the context of recent efforts to quantify various aspects of ovarian function during aging and in the context of paracrine signaling, these approaches will be drawn upon heavily [12–17].

Specimen preparation prior to fixation should take into account the objectives of the experiment with respect to desired outcomes. If the outcome is purely histological, there is general consensus that Bouin's is the superior method of fixation for germ cells. On the other hand, classical studies on the histochemistry of the ovary and other tissues identified Bouin's as damaging to the retention of enzymatic activity such that if the latter is the objective (i.e., preservation of enzyme function for purposes of localization), then freshly prepared paraformaldehyde (2–4 %) or glutaraldehyde (1 %) can be used followed by preparation of frozen sections. As the aim of studies presently emphasizes the use of immunodetection strategies, fixation becomes a key issue in terms of the preservation of epitope integrity, something that can be broadly and adversely affected by Bouin's and which is generally retained after formaldehyde fixation. Table 1.1 summarizes our experience over the past 7 years using a variety of immunological reagents. It

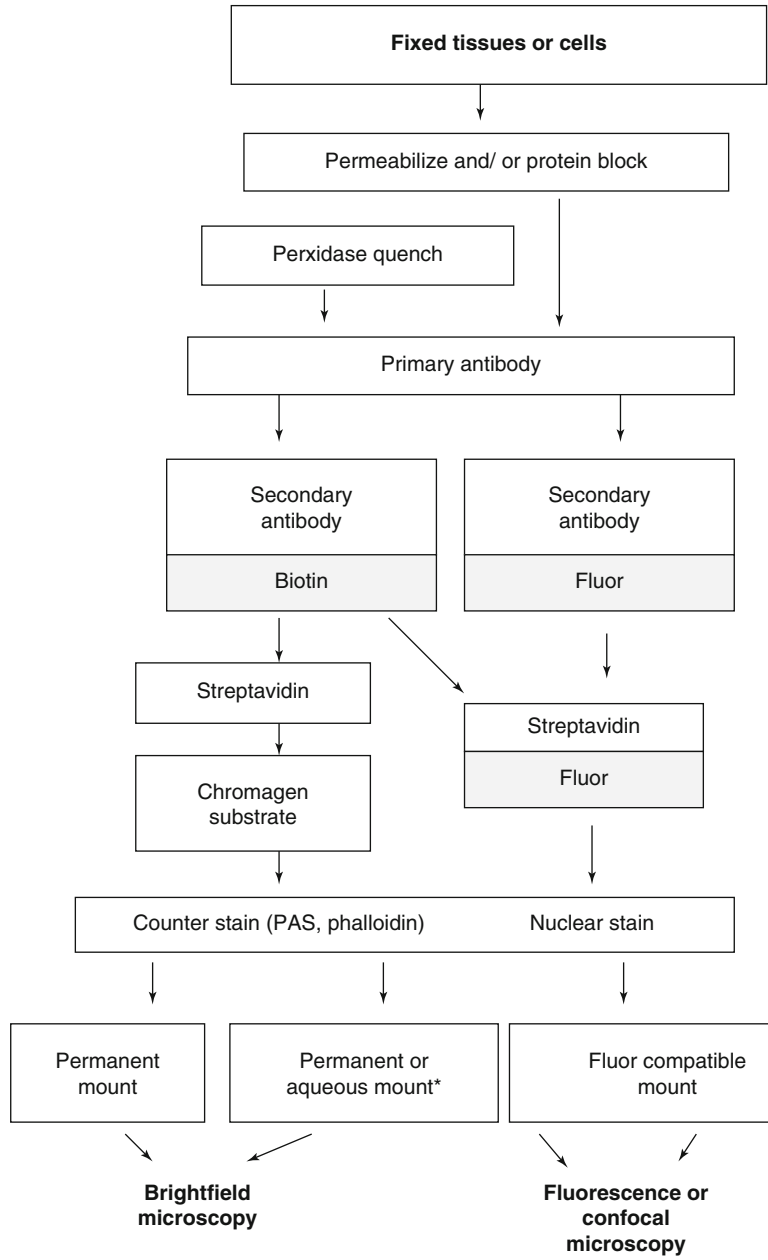

Fig. 1.1 Flow sheet summarizes sequential steps for processing of fixed ovarian tissues or isolated cells. Note that a "conditioning" step is required for all samples prior to exposure to primary antibody. The *arrows* indicate pathways in processing steps that should be followed depending upon the type of microscopic detection (bright field or confocal/fluorescence) that will be used

should be appreciated that while compromising optimal histological integrity, paraformaldehyde fixation, with or without antigen retrieval, provides the most reliable and predictable choice of fixation especially if retention of in vivo signals generated by the use of vital stains or endogenous fluorophores is anticipated.

Conventional histology has also been rendered practical for combination with immune-detection using opaque substrate deposition due to enzyme-coupled secondary antibodies such as peroxidase or alkaline phosphatases. An example of achieving multiple labeling in such material was developed by one of us (SDL) and is illustrated in Fig. 1.2. Combining immunocytochemistry with conventional stains such as PAS makes possible rendering of distinct stages of follicle development with high resolution. In the example shown, mouse vasa homologue, a germ cell-specific RNA helicase also known as DDX4, labels the

Table 1.1 Processing recommendations for ovarian samples

Preparation	Antigen retrieval	Fixation		Extraction		Wash interval
		Solution	h	Detergent (%)	h	
Paraffin sections	Yes	4% PFA Bouin's	18 4	None	None	3×5 min
Isolated follicles	No	PFA/XF	2–6	0.1–1	12–24	3×30 min
Isolated COC's	No	PFA/XF	1–3	0.1	1–6	3×15 min
Denuded oocytes or embryos	No	PFA/XF	0.5–1.0	0.1	0.5	3×15 min
Whole ovary (rodent)	No	PFA/XF	18	1	18	3×1 h
Cortical strips (large mammal)	No	PFA/XF	0.5–4	0.5–1	24–48	3×8 h
Granulosa cell monolayers	No	XF	0.5	0.1	0.5	3×5 min

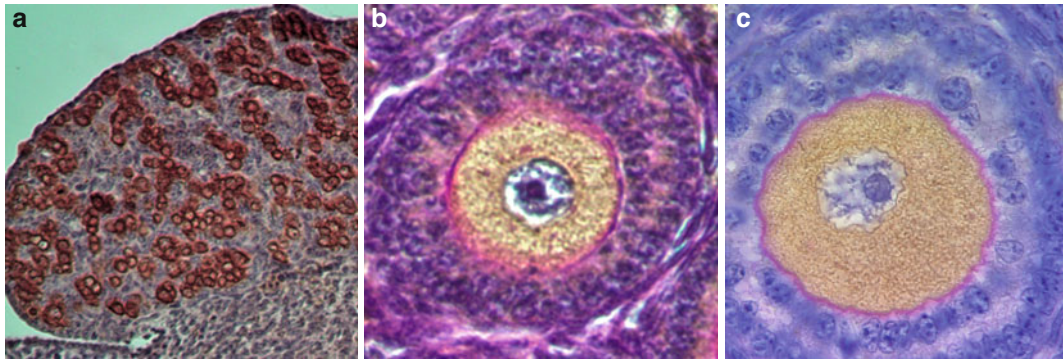

Fig. 1.2 Illustration of triple stain bright field technique applied to conventional histological sections of mouse ovary. Samples stained with PAS (*pink*), mouse vasa homologue (MVH, *brown*), and hematoxylin (*blue*). Examples highlight detection of germ cell cysts in an E15 mouse ovary (**a**) and MVH positive oocytes within preantral follicles (**b** and **c**); note the deposition of PAS-positive zona material surrounding oocytes

cytoplasm of oocytes at three different stages of ovarian development. Retaining hematoxylin in such preparations also provides detailed information about the patterns of chromatin organization in intraovarian oocytes.

One of the less exploited ways to extract high-resolution information from conventional histological sections is to employ illumination strategies such as polarization and differential interference contrast microscopy (DIC). Using hematoxylin and eosin (H&E)-stained samples and imaging under these modalities can bring out contrast details especially due to the hematoxylin labeling of nucleic acids. Figure 1.3 illustrates this point for sections of bovine ovary. The highly organized connective tissue of the tunica albuginea is best appreciated when sections are viewed under polarized light compared to conventional bright field illumination (compare b to a). A further enhancement of tissue structure is obtained when viewing the same materials with DIC optics, a perspective that renders significant detail to the chromatin in dictyate-arrested oocytes of primordial or primary follicles (c and see inset). These simple enhancements in microscopic imaging can provide a level of detail especially with regard to changes in chromatin patterning that are associated with in vivo manipulations of ovarian function. While the thickness of typical sections for histology can range between 5 and 10 um, even here there is sufficient depth to gather three-dimensional information that is often useful when conventional markers for chromatin or cytoplasmic structures are combined with fluorescence microscopy as will be described next [18, 19].

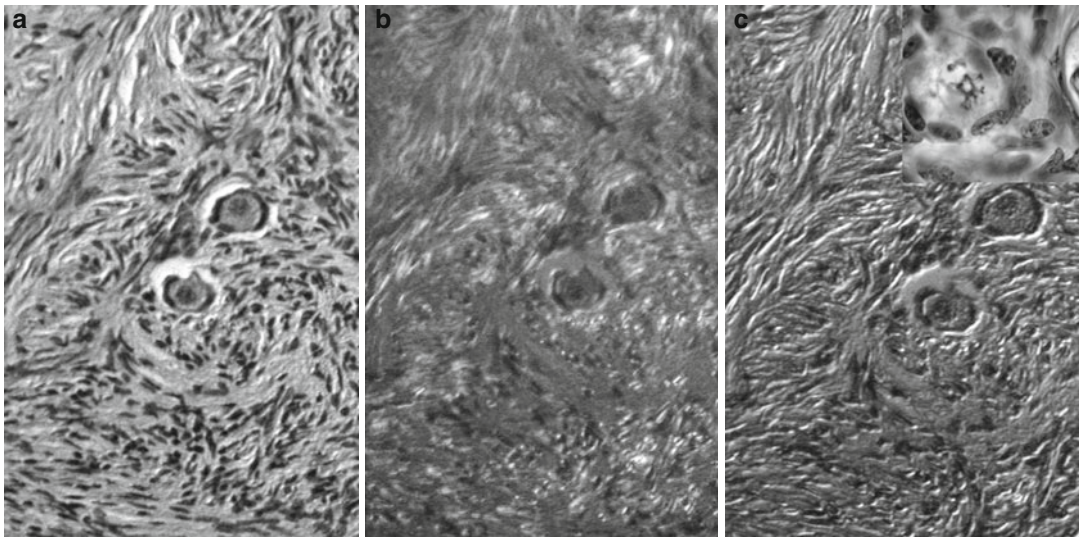

Fig. 1.3 Conventional histological H and E sections of bovine ovary viewed under bright field (**a**), polarization (**b**), or differential interference contrast (**c**) optics. *Inset* shows oocyte chromatin patterns within a primordial follicle (bovine) in which borders of the nuclear envelope are apparent

Fluorescence Microscopy: The Power and the Problems

The ability to localize several discrete components within cells or tissues has been revolutionized by the introduction and development of fluorescence microscopy. For nearly 50 years now, improvements in fluorescence imaging at the level of probe chemistry, low light level detectors, and both wide-field and confocal microscopy have led to advances in biology never imaginable during the peak years of electron microscopy. In achieving the ability to detect and monitor many kinds of molecules, through either direct detection of fluorophore-coupled molecules or indirectly through the use of conjugated antibodies, experimentalists have benefitted from the ability to design multifactorial or multiparameter assays and evaluate a variety of multicomponent interactions. While the power of this technology is well recognized, some drawbacks remain, and those pertinent to the subject matter of this chapter will be considered.

Among the most serious limitations encountered in the use of fluorescence microscopy for studying ovarian tissues or isolated oocytes are properties that confer nonspecific background fluorescence or autofluorescence as well as conditions intrinsic to tissues that form a barrier to detecting specific binding. In the former case, tissues especially present concerns owing to the autofluorescence attributable to erythrocytes or other cell types associated with the vasculature of the ovary. Other lipid-rich compartments also yield high backgrounds as is often seen in corpora lutea or thecal-interstitial tissue. While there is no straightforward way to eliminate signals from endogenous tissue sources, the advent of longer wavelength emitting dyes provides one way for avoiding noise introduced by these typically lower wavelength species of molecules. Moreover, harsher extraction conditions can be introduced especially those designed to eliminate lipidic substances by use of detergents or organic solvents. As with any modification in technology that intends to improve the signal to noise ratio, samples subjected to extreme extraction conditions are likely to introduce artifacts of various kinds that can compromise the original labeling strategy sought to answer a specific question.

The second and more unique problem for investigators of the ovary and oocytes in particular derives from the properties of the zona pellucida. The zona pellucida is a specialized extracellular

matrix that contains a high density of terminal carbohydrates on zona proteins that effectively serve as an ion exchange column for positively charged molecules. Dealing with the zona for labeling purposes requires distinct strategies depending on whether the material of choice is histological or frozen sections or any isolated structures such as follicles, oocytes, or embryos. Many studies dating from the 1980s and 1990s sought to eliminate this problem by removing the zona enzymatically (pronase, trypsin), chemically (acid Tyrode's dissolution), or mechanically by passing oocytes through a narrow-bored micropipette. Such studies often focused on internal structures within oocytes such as chromosomes and the meiotic spindle, and after zona removal, a high signal to noise ratio was obtained. During the mid-1990s, we developed alternative fixation and labeling strategies that would allow for the retention of the zona and other accessory structures such as cumulus cells or follicle cells, and in this way were able to preserve the complex cellular interactions that exist at the interface of the oocyte and surrounding somatic cells. These strategies will be discussed in a later section with respect to imaging intact tissues and the importance of specimen fixation and processing for this application.

Sectioned materials also suffer from the unusual properties of the zona pellucida, and this problem is illustrated in Fig. 1.4. A panel is shown depicting mouse ovary sections that have been labeled for immunocytochemistry to a membrane compartment (a, lamp 1), the cytoskeleton (b, actin), and a zona protein (c, ZP1). Notably, the problem with nonspecific binding of antibodies appears to be specific to rabbit antibodies, and even here the antibody to actin was affinity purified, and thus the staining to the zona seen in panel B is attributable to reagents of rabbit origin. Neither of the other antibodies demonstrates the zona staining, and precautions regarding antibody source should be coupled with appropriate controls of first antibody omission but preferably adsorption with epitope. The final panel in Fig. 1.4 shows the compatibility of DNA binding fluors such as Hoechst 33258 for counterstaining section materials processed for routine or confocal microscopy.

Confocal Microscopy of Ovarian Cells and Tissues

Proceeding from the era of wide-field fluorescence microscopy into that of single-photon confocal microscopy initiated in the 1980s, imaging as we know it today took a major step forward, and this has by no means evaded the interest of reproductive biologists studying the ovary, oocyte, and embryo. With central themes emerging from the use of transgenic mouse technology, many novel and fascinating phenotypes have been uncovered, and in some studies, the mechanistic basis for paracrine and hormonal regulation of ovarian function has been materially advanced [20–25]. This has been particularly helpful in evaluating the ovary as a developmental composite where the analysis of intact or sectioned tissues allows for the direct comparison of different stages of oogenesis and folliculogenesis [26–29].

For analysis of isolated oocytes, embryos, or follicles, we encourage the use of specialized fixation and processing techniques that have evolved over the years [1, 15, 18]. As described below, the MTSB-XF fixative was designed to optimally preserve components of the cytoskeleton, and we have now documented that this technique is well suited for preservation of nuclear epitopes, and it can be adapted for use with whole pieces of ovarian tissues or the extremely large cumulus-oocyte complexes found in the equine, ovine, and bovine species [30, 31]. While the advantages of MTSB-XF are based on the simultaneous extraction and fixation of cells under conditions of maximal cytoskeletal stability, it should be emphasized that all membrane-limited compartments including plasma membrane, mitochondria, and Golgi complexes are solubilized and not accessible for epitope localization after fixation [32]. For the experimentalist interested in retaining structure in membrane-limited structures, we recommend first fixing cells with 2 % paraformaldehyde for 5–10 min at room temperature before moving samples into the MTSB-XF fixative.

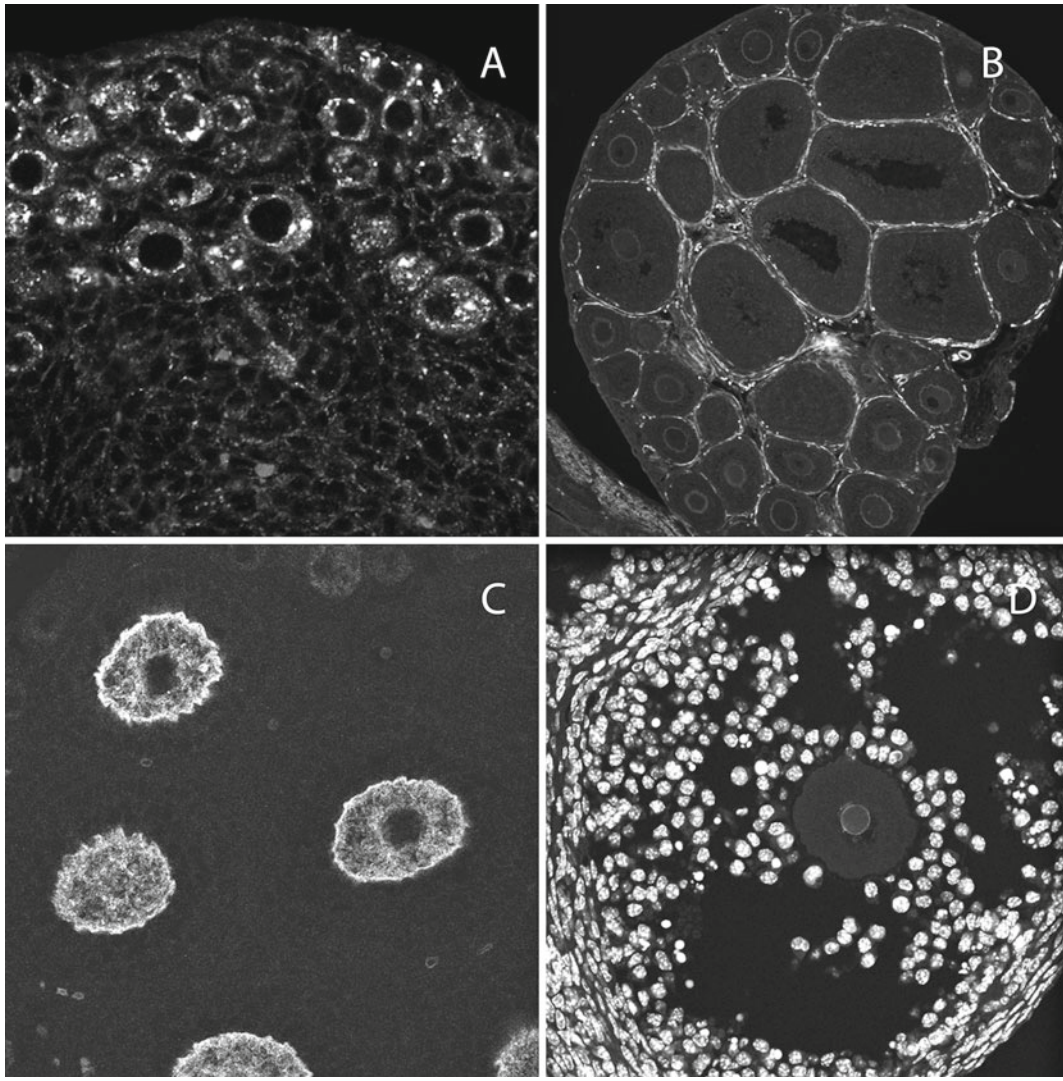

Fig. 1.4 Examples of the application of fluorescence immunohistochemical (**a**–**c**) and direct labeling (**d**) using conventional histological sections of mouse ovary. (**a**) LAMP1 mouse monoclonal; (**b**) rabbit anti-actin; (**c**) mouse antibody to ZP1; and (**d**) Hoechst 33258 staining of chromatin. Note in **b** that rabbit antibodies often yield a low level of background staining of the zona pellucida that is difficult to eliminate even with extensive washing and/or use of blocking reagents

MTSB-XF stands for microtubule stabilization buffer-extraction fixative and is compositionally based on the need to optimize elements of the cytoskeleton especially those having to do with microtubules. Toward this end, solution conditions (1) maintain low calcium (EGTA), (2) protect sulfhydryl groups by the addition of dithiothreitol, and (3) provide an environment that favors the stability of polymerized tubulin due to the presence of taxol, deuterium oxide, and slightly acidic pH. In our earlier studies, it was also found that the inclusion of a broad spectrum inhibitor of proteases was beneficial for epitope and structural preservation, hence the inclusion of aprotinin [32]. This concept of tailoring fixatives to maximize retention of epitope and structural disposition for proteins has gained widespread acceptance in the field of biochemistry but has been somewhat slow to appear in the context of biomedical imaging at the microscopic level.

Further, in analyzing the distribution of phosphoepitopes, it has become clear that due to the persistent activity of phosphatases during tissue solubilization and fixation, the addition of a phosphatase inhibitor cocktail greatly enhances the signal to noise ratio making quantitative assessments of protein phosphorylation possible in the context of fixed preparations of cells or tissues [30]. Addressing the question of epitope stability during and after fixation as it pertains to maintenance of phosphoepitopes emphasizes the need to extend this strategy to other posttranslational modifications in proteins given the enormous advances made in the area of histone modifications [33–36].

Considerations regarding fixation and sample conditioning are central to achieving optimal data when using confocal microscopy [16, 18]. Even though the ability to reduce noise above and below the optical plane being sampled is greatly reduced in a confocal modality, any and all efforts to assure antibody access and specificity are essential to producing reliable and repeatable results especially when semiquantitative measurements are anticipated [30]. We recommend that three factors assume priority when designing experiments that will require advanced imaging. First, once the samples have been fixed, they should be subjected to a conditioning step that assures both cessation of any residual fixation and includes a buffer whose composition is designed to maximize blocking of spurious antibody binding prior to exposure to primary antibody. Such solutions thus contain powdered milk, a protein source, and Tris buffer, all intended to reduce nonspecific binding. Second, using such solutions to prepare primary or secondary antibodies affords a great selective advantage for enlisting the reagents of highest affinity to meet stringency requirements satisfactory for optimal antigen-antibody complex formation. Besides reducing background, this step introduces a measure of signal to noise where stability of the immune complexes will also be likely to be maintained for prolonged periods of time (months) when samples are stored at 4 °C. The final factor that plays into optimizing specimen quality rests soundly on the issue of adequate washing between each of the labeling steps and prior to specimen mounting. In our hands, washing steps should not be trivialized. Isolated cells grown on coverslips and denuded oocytes or embryos are readily prepared with relatively short washing steps on the order of hours. However, when using large tissue samples, washing and labeling steps should be significantly extended over a period of days in order to obtain maximal antibody penetration and optimal washout of immune reagents. Typically, large samples such as whole rodent ovaries or 1 mm cortical strips of bovine ovary which are being prepared for confocal microscopy are both labeled and washed for a day at a time at 4 °C on a shaking platform. The results of this kind of approach are illustrated in Fig. 1.5.

Intact pieces of bovine ovarian cortex were fixed in MTSB-XF and processed by lengthening the times of washing and labeling as indicated above. Samples were prepared with two antibodies of mouse origin; one recognized a DNA damage marker known as gamma H2AX, and the other recognizes an acetylated form of alpha tubulin that is enriched in nerve fibers. The preparation was counterstained with rhodamine phalloidin to label f-actin and Hoechst 33258 to label chromatin. Image 1.5a shows the reconstruction of an intact follicle in which details of chromatin can be readily appreciated. When chromatin profiles are compared to the H2AX shown in the green channel (b), areas of overlap in the oocyte nuclei are readily apparent, and distinct patterns of innervations are demonstrated by the acetylated tubulin antibody. The overlay in Fig. 1.5c further attests to the conformity and clarity of each signal that is readily amplified from the confocal data set as shown in Fig. 1.5d. It is important to emphasize that this approach is a new extension of traditional technologies that combine the optical sectioning capability of the confocal microscope with optimal conditions for specimen labeling in an intact in vivo state. This preparation was sampled at 500 μm depth where all reagents were able to penetrate and retain significant signal. It should be possible to further develop this approach for future studies.

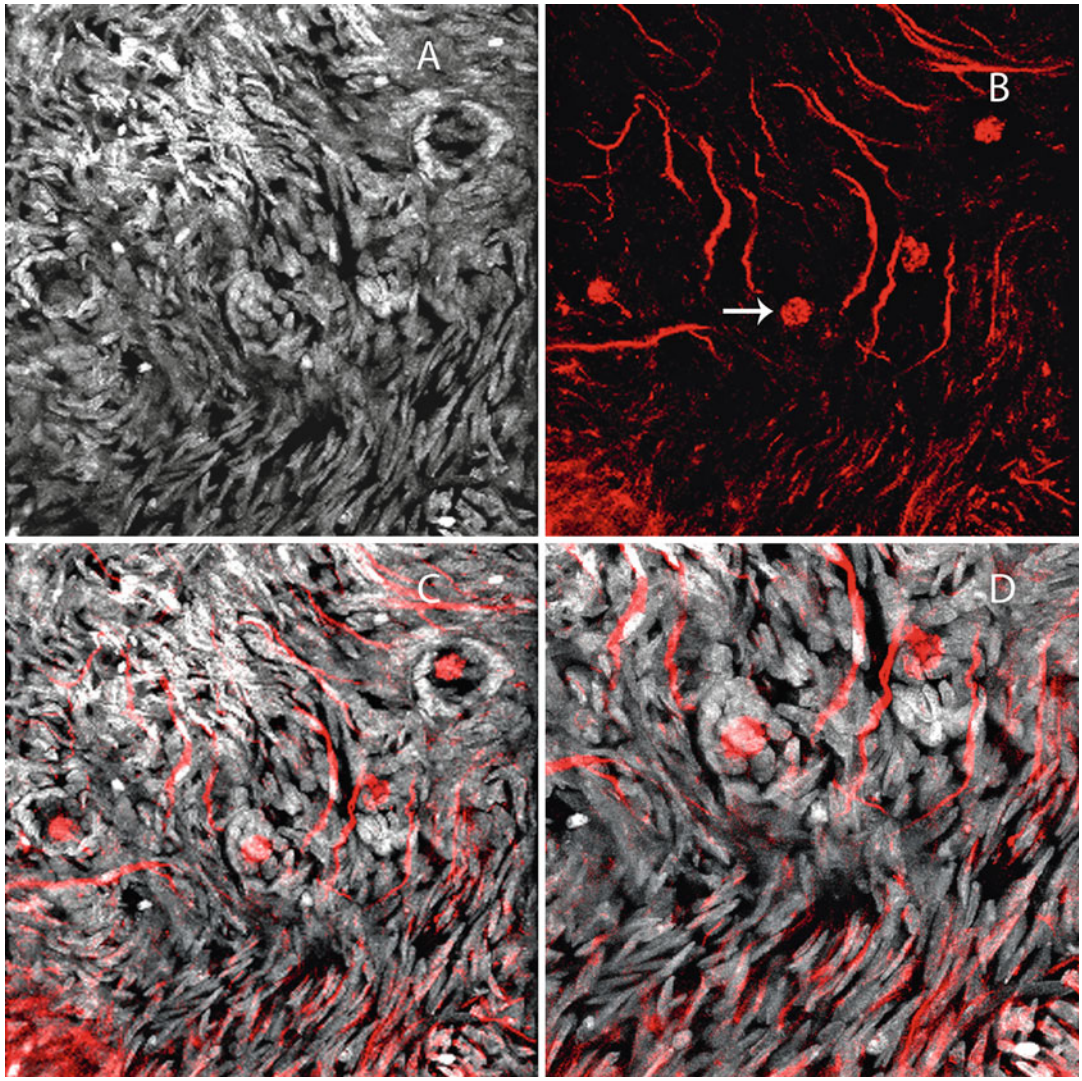

Fig. 1.5 Confocal fluorescence microscopy of intact bovine ovary processed for the demonstration of nerve fibers and histone H2AX within nuclei (*arrow*) of follicle-enclosed oocytes. (**a**) Projection of chromatin (6×1 μm stack of Hoechst 33258); (**b**) image of immunolabeled epitopes for acetylated tubulin (nerve fibers) throughout tissue and DNA damage detection in nuclei of oocytes; (**c**) combined image of **a** and **b**; and **d**, enlargement of 10-μm stack revealing proximity of nerve terminals to follicles

Confocal Microscopy of Living Ovarian Cells and Tissues

Extending the realm of confocal microscopy from that of experimentation with fixed samples to those that retain viability is a lofty and worthwhile goal for students of mammalian oogenesis [37–40]. This has been aided by the ability to control atmosphere and temperature within a variety of devices that are well suited to maintain tissue and cell viability over prolonged periods of time. It does come with a price to pay as most available vital imaging studies run the risk of introducing potentially compounding and damaging consequences due to the release of excited-state electrons from endogenous or exogenous fluorophores. Tailoring the right combination of fluors to answer specific questions is aided

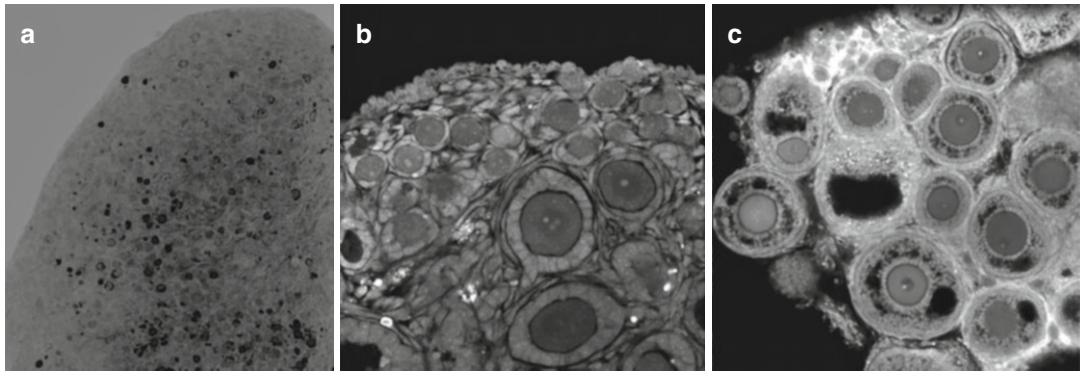

Fig. 1.6 Confocal images of lysotracker red-labeled mouse ovaries demonstrated in reverse contrast (**a**, whole ovary, 2-day postpartum) and single optical sections of ovaries from 6-day- (**b**) and 16-day-(**c**) old animals

presently by the ability to express fluorescent protein constructs that have successfully integrated into the genome of experimental animals such as mice. In addition, there have been great advances in the chemistry of fluor dyes that offer alternatives to genetic manipulation when the physiological status of oocytes or ovarian tissue is the subject of investigation. As an example of this strategy, we and others have adopted the use of lysotracker red as a vital indicator of acidic organelles in the developing ovaries of mice [1].

As illustrated in Fig. 1.6, lysotracker red (LTR) is a pH-sensitive fluor that readily penetrates living cells and tissues, and in the presence of an acidic environment, the dye becomes immobilized and emits a red fluorescence that is easily detected by microscopy. When living mouse ovaries are labeled with LTR for various periods of time and under routine cell culture conditions, the intact ovary is subjected to fixation, and following a clearing treatment to remove sources of light scattering within the tissue, the entire sample is mounted and viewed directly in the confocal microscope. The confocal is able to optically section the entire ovary, and when image stacks are rendered by contrast inversion (1.6a), each site of acidic staining is readily identified. In addition to offering a nonhistological means to evaluate all of the ovarian follicular contents, such an approach permits high-resolution evaluation at the single-cell level as revealed in Fig. 1.6b and c. LTR is merely one of a number of new fluorescent dyes being developed for use in vital staining microscopy, and using the approach we describe here, many applications for evaluating ovarian physiology and disease will be accessible [40–42]. Two final examples of this are demonstrated in Fig. 1.7.

Much attention has been paid to the configuration of chromatin in the germinal vesicle (GV) of mammalian oocytes [32, 40, 41]; Fig. 1.7 shows a three-dimensional projection of a mouse GV seen in anterior (a) and posterior (b) views and depicting the interaction of heterochromatin with the surface of the nucleolus. This image set was constructed from material fixed in MTSB-XF after vital staining with the DNA intercalating dye Hoechst 33342. By sampling the GV at 1 μm steps during confocal image acquisition, a complete rendering of heterochromatin is obtained that reveals details not previously appreciated with wide-field fluorescence microscopy. Finally, among the probes being developed for vital staining are those that carry fluorescent moieties linked to functional lipids. One such probe we have been experimenting with is a dextran-modified cholesterol derivative that partitions into membrane domains rich in cholesterol when it is applied to living cells. Figure 1.7c shows a single mouse oocyte that has been labeled as an intact cumulus-oocyte complex for 5 min at 37 °C. The oocyte was then mounted in culture medium and imaged directly using 488 nm excitation, and optical sections were taken at 1 μm intervals. The image reveals two distinct aspects of vital stain imaging. First,

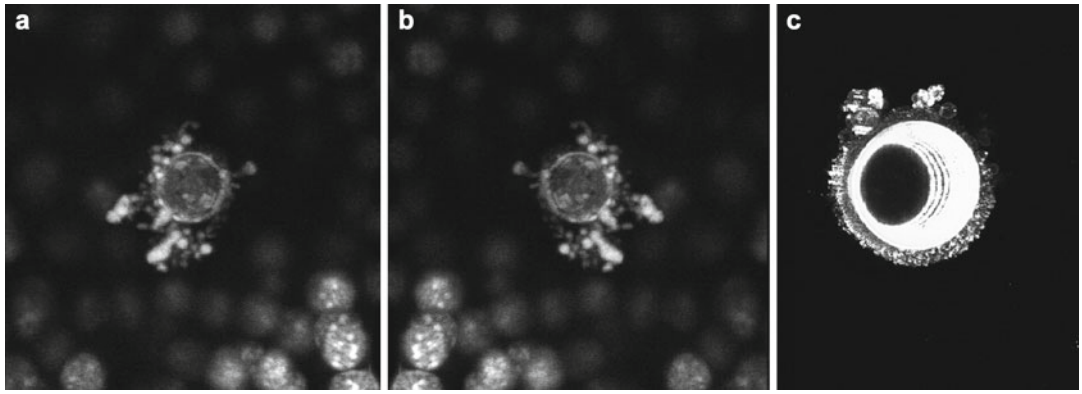

Fig. 1.7 High-resolution three-dimensional projections of living oocytes labeled with vital fluorescence dyes for chromatin (**a**, **b**, Hoechst 33342) or membrane lipids(**c**). Image rotation (**a**, **b**) permits definition of chromatin interaction with the nucleolus during active transcription. In (**c**), saturated ring signal in center corresponds to sequential sections of oocyte plasma membrane subtending the zona pellucida

it should be noted that the concentric superimposed rings representing the plasma membrane of the oocyte are saturated relative to outer structures that were the subject of interest. This is often the case as signal intensity will vary between different domains into which probes become intercalated. The second feature evident in this image is the interruption of continuity displayed by the staggered images representing sequential optical planes separated by a gap of 1 μm. This exemplifies the need to establish optimal sampling frequency in the z-axis that will depend directly on approximating 1 airy unit during sampling. Again, trial and error should prevail when setting up optimal conditions in the context of imaging living material.

Image Archiving and Analysis

Investigators already employing or planning to use contemporary imaging techniques to study oogenesis are well advised to consider the important topics of image analysis and archiving before embarking on experiments. It is neither within the purview of this chapter to cover these topics in depth nor is it reasonable given the many resources that are now available within imaging centers at research institutions. Instead, we will briefly review some applications for archiving and analysis that bear directly on the topic of oogenesis and recent efforts to recapitulate the ovarian development of oocytes using in vitro technologies of growing clinical interest [42–45].

Among the technologies receiving much attention for their clinical import are those aimed at performing oocyte in vitro maturation or follicle in vitro maturation. In both cases, confocal microscopy is aiding in the definition of oocyte quality, but sadly because of the dependence on fixation for most such studies, information on the viable oocyte and follicle remains lacking [40]. The essence for image archiving is to take heed to the notion that no amount of data storage space is ever enough. Once the power of confocal is truly appreciated and the key elements of a solid design embedded into experiments, gathering enormous quantities of data becomes the norm rather than the exception. Fortunately, the prepared stance for any investigator is to obtain archiving resources of an appropriate magnitude to fit with the current and future needs of a given laboratory.

Image analysis draws its relevance to the overall implementation of confocal microscopy in that data gathering becomes but a first step in the extraction and evaluation of image sets derived from experiments. Most confocal microscope companies by definition include image analysis tools to fit the immediate needs of the investigator, but in many cases, for example, volumetric rendering of three-dimensional data sets,

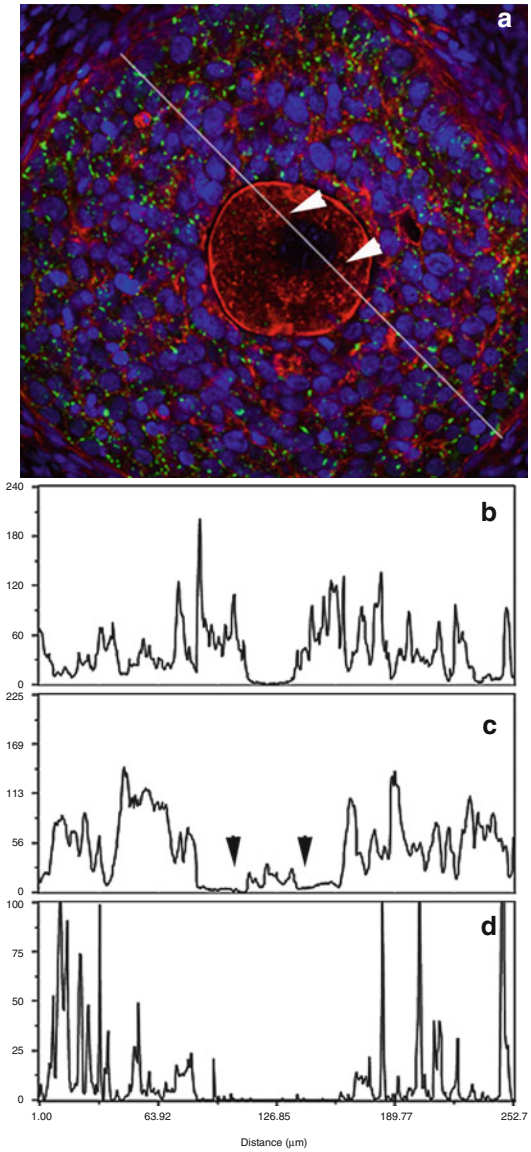

Fig. 1.8 Confocal micrograph of a single bovine ovarian follicle (**a**) labeled with Hoechst 33258 for DNA, Alexafluor-568 phalloidin for actin (*red*), and anti-connexin 43 for gap junctions (*green*). Optical density profiles are shown for each channel following the reference line that transects the follicle through the oocyte germinal vesicle as indicated by the *arrows* (see **a** and **c**). (**b** and **d**) show corresponding intensity plots for actin (**b**) and connexins (**d**)

specialized software needs are best met from a variety of commercial sources. An example of how image analysis is applied to in vitro follicle culture is shown in Fig. 1.8.

Balancing the needs of the oocyte with those of the follicle has become an interesting and important topic for studies attempting to link the processes of oogenesis and folliculogenesis [38, 39, 41]. At the heart of these studies are attempts to sort out the cell communication needs that, on the one hand, allow for the synchronous growth of the preantral follicle and yet maintain a communication system with the enclosed oocyte that must derive the majority of its metabolic reserves from the somatic granulosa cells [16, 18]. When evaluating this problem with cultured bovine follicles, composite sampling of three channels in entire z-stack data sets allowed for the identification of connexin expression patterns throughout the follicle in addition to a marker of cell adhesion. Separate plots of fluorescence intensity were propagated from line scan analysis tools, and the ratios of different signals and their geographic location were spatially mapped. These are shown in Fig. 1.8a–c that respectively define chromatin in the GV, actin at the oocyte periphery, and connexin 43 junctions that intersect the line profile. Measures such as these are useful when used under controlled conditions where background baseline values can be set at the lower limit of the typically 0–256 grayscale level. The discriminating "imager" will recall that establishing a useful signal to noise ratio for experiments of this kind begins with sample preparation and the strict control of reagent specificity for both primary and secondary reagents.

Conclusions

This chapter has attempted to highlight some of the major advances in imaging that can and will continue to play an important function in the analysis of mammalian oogenesis. It offers insights into the translation from single-cell experiments to those of complex tissues such as the ovary where the very earliest stages of oogenesis can be analyzed under native conditions and without damage to oocytes or follicles as a result of their isolation or culture. It is hoped that using the tools and guidelines reviewed here, the inertia to apply modern imaging techniques for the study of oogenesis will be lessened and that in the end, this

extraordinary process will be brought into view for all to appreciate.

Acknowledgments The authors express their gratitude to the many colleagues and friends who have worked in the lab to advance the perspectives of oocyte biology that we enjoy today. Special thanks go to Lynda McGinnis and Britta Mattson who at different ends of the spectrum contributed to the development of MTSB-XF. Finally, this chapter would not have been possible without the funding from the NIH, ESHE Fund, The March of Dimes, and the Hall Family Foundation.

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Part II

Folliculogenesis and Oogenesis

Origin, Migration, and Proliferation of Human Primordial Germ Cells

2

Massimo De Felici

Abstract

The first histological observations about the origin of the precursors of gametes termed primordial germ cells (PGCs) in extragonadal regions and their subsequent migration into the developing gonads in human embryos date back to the early twentieth century. Fuss (Anat Am 39:407–409, 1911, Anat EntwMech 81:1–23, 1912) and Felix (Die Entwicklung der Harn- und Geschlechtsorgane. In: Keibel-Mall Handbuch der 1qEntwicklungageschichte des Menshen, vol 2. Leipzig, Hirzel, pp 732–955, 1911) were apparently the first ones to describe the extragonadal location of PGCs in human embryos. In the youngest, 2.5 mm long, embryo examined (23–26 days postfertilization), These authors described PGCs in the endoderm of the yolk sac wall as cells identifiable by their large size and spherical shape. Subsequently, Politzer (Z Anat Entw Gesch 87: 766–80, 1928, Z Anat Entw Gesch 93:386–428, 1930, Z Anat EntwGesch 100:331–336, 1933) and Witschi (Contr Embryol Carnegie Inst 209:67–80, 1948) studied the distribution of PGCs in a considerable number of embryos from presomite stages (0.3–0.8 mm, about 3 weeks) to 8.5 mm (5 weeks). Both authors described the migration of PGCs from the yolk sac to the developing gonads. Following a hot debate, it is now generally accepted that after their arrival into the gonadal anlage, PGCs give rise to the oogonia/oocytes and gonocytes (or prespermatogonia) in the embryonic ovary and testis, respectively. These germ cells enter a complex series of events that in the

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adult end with the formation of fertilizable oocytes and sperm. Because of the inaccessibility of the human embryo to experimental investigations at these early stages, we still know little about cellular and molecular mechanisms controlling the formation, differentiation, and development of human PGCs. This chapter describes the life history of human PGCs combining old and new information and, where appropriate, making use of the most recent results obtained in the mouse.

Keywords
Primordial germ cells • Embryonic gametogenesis • Gonad development BMPs

Introduction

The differentiation and development of PGCs is an early event of the mammalian embryogenesis, crucial for assuring normal fertility of the individual and the correct transmission of the genome to the next generation. The basic principles governing these processes have been clarified and can be summarized as follows. Probably dictated by the necessity to protect the cells of the germ line from signals inducing somatic cell lineage differentiation, the precursors of PGCs are early committed and specified in the epiblast (one of the first two differentiated tissue of the embryo proper, the other being the hypoblast) before gastrulation and rapidly moved into an extraembryonic region where PGCs are determined. PGCs reenter into the embryo proper during early gastrulation to reach the developing gonads (gonadal ridges, GRs). During this journey, while undergoing proliferation, PGCs begin extensive nuclear reprogramming (activation of genes for pluripotency and epigenetic changes of the genome involving DNA demethylation and histone code) to regain differentiation totipotency and reset the genomic imprinting. These processes are completed after their arrival into the GRs. After some rounds of proliferation, PGCs finally differentiate into oogonia or gonocytes within ovaries and testes, respectively.

Despite the first morphological observations on the extragonadal formation and movements of PGCs in human embryos date back exactly 100 years ago [1–3] (Fig. 2.1 and Table 2.1), the inaccessibility of the human embryo to experimental investigations at these early stages made difficult, if not impossible, to obtain information on the complex temporal and spatial series of molecular events underlying these processes. Thanks to recent studies carried out in the mouse and the development of stem cell technologies, however, we are now obtaining precious information that can contribute to clarify crucial aspects of these processes. The present review is an attempt to summarize these results and the future perspectives.

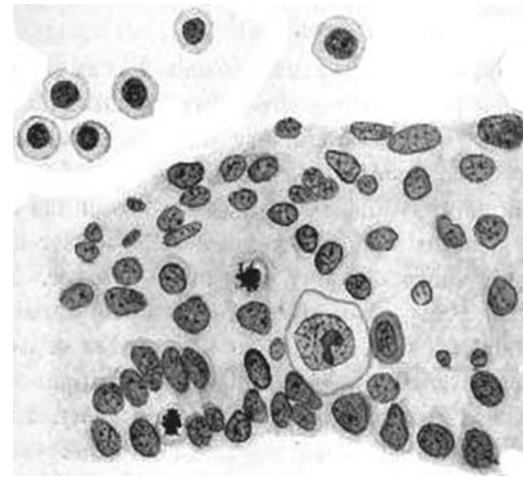
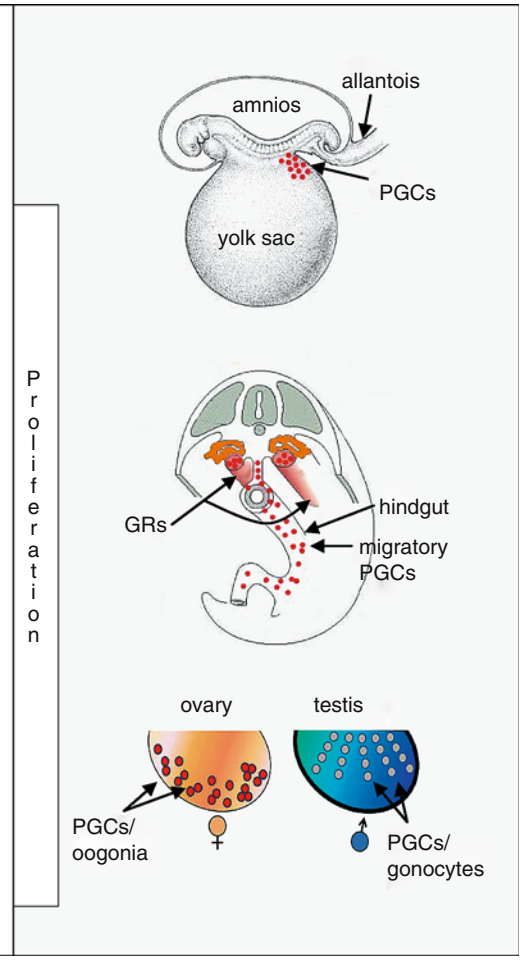


Fig. 2.1 Reproduction of the original drawing by A. Fuss [2]. The German scientist was likely the first to identify PGCs in the endoderm of the wall of the yolk sac in one human embryo 2.5 mm long (23–26 days postfertilization); in the center of the drawing, a PGC is clearly recognizable for its large size and spherical shape among several somatic cells

Table 2.1 Chronology of human primordial germ cell development

Age from conception (week)	
III	PGCs in the dorsal wall of the yolk sac near the developing allantois
IV	
V	PGCs into the midgut and hindgut endoderm
VI	
VII	PGCs into the midgut and hindgut endoderm; PGC migration through the dorsal mesentery into the gonadal ridges
VIII	
IX	Gonadal ridge colonization and sex differentiation (ovaries and testes)
X	
	PGC differentiation into oogonia (ovary) or gonocytes (testis)

Proliferation



Comparing Mouse and Human PGC Formation

In animals, the formation of PGCs basically occurs by one of two distinct mechanisms: inheritance of germ plasma or inductive signaling (for reviews, see [4, 5]). In most organisms, including invertebrate species such as *Drosophila* and *Caenorhabditis* and nonmammalian vertebrates such as frogs and fishes, germ cell arises through the former mechanism. Germ plasma is a maternally derived collection of cytoplasmic RNAs, RNA-binding proteins, and various organelles assembled within the mature oocyte and segregated during the first divisions of the embryo to the cells fated to become PGCs. In contrast, probably in all mammals, PGCs arise shortly before or during gastrulation through a process of inductive signaling. Specific signals secreted by neighboring cells induce the commitment and specification of PGC precursors among the epiblast cells before gastrulation. Shortly afterward, such precursors are determined as PGCs in an extraembryonic region. But when and where do these signals exactly take place, and what molecules are involved?

Recent elegant research carried out in the mouse (for a review, see [6]) has shown that in the pregastrulation period, interactions between

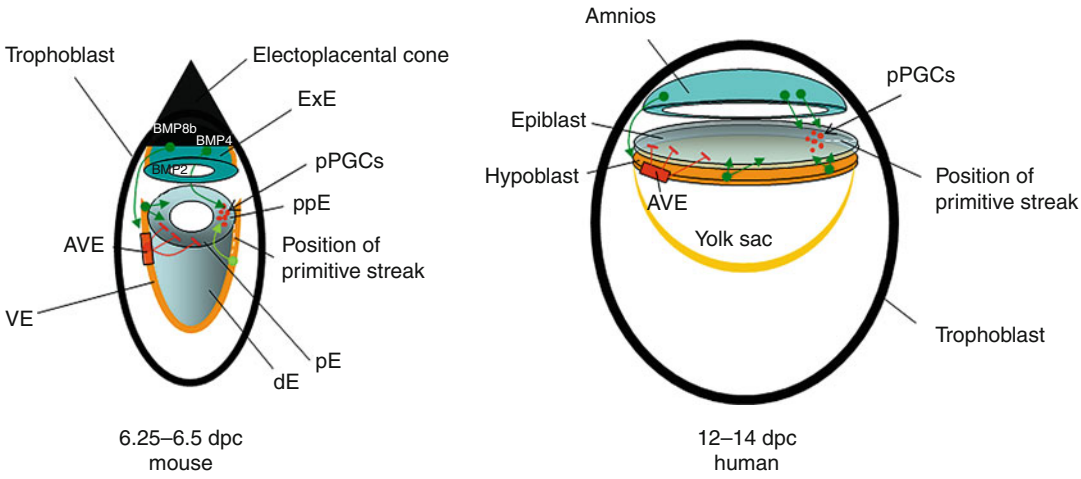


Fig. 2.2 Schematic drawings of the main tissues and BMPs involved in PGC specification in the mouse embryo. For comparison, a hypothetic scenario of PGC specification in the human embryo is drawn. For details, see text. *ExE* extraembryonic ectoderm, *ppE* posterior proximal ectoderm, *pE* proximal ectoderm, *dE* distal ectoderm, *VE* visceral endoderm, *AVE* anterior visceral endoderm, *pPGCs* primordial germ cell precursors

two extraembryonic tissues, the extraembryonic ectoderm (ExE), and visceral endoderm (VE) are crucial for the germ-line commitment and specification. In particular, in the mouse embryo around 6.25 *days post coitum* (dpc), six PGC precursors are set aside in the posterior proximal epiblast cells near the region where the primitive streak will form (Fig. 2.2). Members of the transforming growth factor β1(TGFβ1) super family, namely, bone morphogenetic protein 8a and 4 (BMP8a and BMP4) secreted by ExE and BMP2 produced by the VE, induce these early processes. The expression of the transcriptional repressor B-lymphocyte-induced maturation protein 1 (BLIMP1) (also known as PR domain-containing 1, PRDM1), closely followed by that of the companion PRDM14 and upregulation of fragilis (also known as interferon-induced transmembrane protein 3 or IFITM3), marks the emergence of PGC precursors. These BLIMP1-positive cells increase in number and begin to move out of the embryo through the forming primitive streak. During this period, PGCs are specified, and the expression of the putative RNA/DNA binding protein stella (also known as developmental pluripotency-associated 3, DPPA3, or primordial germ cell 7, PGC7) marks the event. Around 7.25 dpc, the PGC precursors are determined as PGCs in the extraembryonic mesoderm at the basis of allantois. PGCs form a cluster of about 40 cells held together by E-cadherin and expressing high levels of tissue nonspecific alkaline phosphatase (TNAP) and stella (for reviews about the formation of mouse PGCs, see [7–9]).

The place where PGCs were first identified in human embryos around the end of the third week is the same as in the mouse: the wall of the yolk sac at the angle with the allantois (Table 2.1). Exactly 100 years ago, Fuss [1, 2] and Felix [3] were apparently the first to describe the extragonadal location of PGCs (or *Urkeimzellen*) in human embryos. These cells were distinguished by their large size, spherical shape, and the presence of abundant glycogen granules in the cytoplasm (Figs. 2.1 and 2.3). In the youngest, 2.5 mm long, embryo examined (23–26 days postfertilization, 13–20 somites), Fuss described PGCs in the endoderm of the wall of the yolk sac. The extragonadal origin of PGCs was confirmed by the subsequent studies by Kohno [11] and Hamlett [12] and above all, by Politzer [13–15] and Witschi [16] who studied the distribution of PGCs in a considerable number of embryos from presomite stages (0.3–0.8 mm, about 3 weeks, 17 embryos) to 8.5 mm (5 weeks, 23 embryos). Both authors described the migration of PGCs from

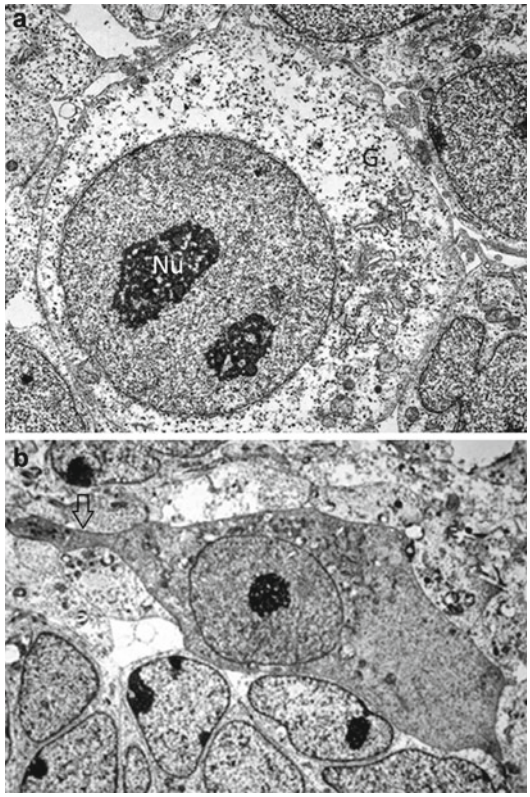


Fig. 2.3 Electron microphotographs of human PGCs between 5 and 6 weeks of gestation. (**a**) A stationary PGC in the hindgut around 5 weeks; note the presence of glycogen (*G*) aggregates and a few mitochondria (*M*) in the cytoplasm; *Nu* nucleolus. (**b**) An actively migrating PGC among mesenchymal somatic cells around 6 weeks; note its elongated shape and a pseudopodium (*arrow*) (Courtesy of Prof. Stefania Nottola, Department of Human Anatomy, University of Rome La Sapienza [10])

the yolk sac to the gonadal ridges. In an embryo 0.6 mm long (about 3 weeks), Politzer [13–15] counted 40 PGCs which increased to 600 in the 4 mm long embryo (4 weeks). Similarly, Witschi [16] counted 30–50 PGCs in the endoderm of the yolk sac around the end of the third week and 109 PGCs in an embryo a couple of days older. Histochemical methods for the identification of PGCs were first applied successfully to human PGCs for periodic acid- Schiff (PAS)-positive materials and mainly TNAP activity by Mc Kay and colleagues [17]. The presence in the cytoplasm of distinct morphological element called "nuage" was another characteristic later described in human PGCs [18]. This material is a conserved feature of germ cells in species across the animal kingdom. The "nuage" is distinct from germ plasma and under the electron microscope, appears as electron-dense granules localized to the cytoplasmic face of the nuclear envelope. There is currently a lack of information about the function of this material. In flies and mouse, an interesting possibility is that it might be involved in microRNA-pathways necessary for maintaining the germ cell lineage and for transposon repression [19, 20].

In humans, there is a lack of studies tracing back the germ-line origin to the earliest stage of development before gastrulation. In particular, no information is available about the inductive processes controlling PGC specification and determination. We can postulate that the basic principles governing human PGC origin are similar to those in mouse and other mammals as well. In humans, however, the timing and mode of formation of the extraembryonic tissues is significantly different from the mouse. This might have some implications for the formation of PGCs (Fig. 2.2).

Immediately before gastrulation (6.0 and 6.5 dpc), the mouse embryo can be visualized as a thick-walled cup of tissue (the epiblast or embryonic ectoderm), which will give rise to the entire fetus and some of the placental membranes. A second thick-walled cup of tissue (the extraembryonic ectoderm) placed overturned on the epiblast will give rise to the main part of the placenta. Both cups are enclosed in a thin bag of primitive endoderm. Taking into account only tissues involved in the formation of the germ line, in the mouse between 4.5 and 5.5 dpc, the primitive endoderm gives rise to the visceral endoderm (VE). In this epithelium, a specialized region termed anterior visceral endoderm (AVE), crucial for determining anterior-posterior embryo polarity, forms. During the same period, the extraembryonic ectoderm (ExE) arises from the polar trophectoderm and makes contact with the underlying epiblast. At 6.5 dpc, gastrulation starts with the formation at the posterior region of the embryo of the primitive streak. Epiblast cells migrating first through this structure include the PGC precursors expressing BLIMP1/stella and form

the extraembryonic mesoderm. In humans, at the beginning of the second week, the embryo consists of a bilaminar disc, the epiblast, and the primitive endoderm (or hypoblast). Polar trophectoderm above the epiblast differentiates into the syncytiotrophoblast that invades the uterine tissue and the cytotrophoblast contacting the epiblast. Within the latter, the amniotic cavity forms lined by the amnioblasts derived from the epiblast cells. The primitive endoderm forms the roof and the wall (Heuser's membrane) of the primary yolk sac. On day 10–11, extraembryonic mesoderm of uncertain origin appears between the cytotrophoblast and the yolk sac. Around the end of the second week, the definitive yolk sac is formed by a new wave of cells migrating from the primitive endoderm and displacing the Heuser's membrane. At the beginning of the third week, the primitive streak appears and the gastrulation begins. In humans, the primitive endoderm can be considered equivalent to the mouse VE, while no structure equivalent to the mouse ExE apparently exists (Fig. 2.2). Moreover, the formation of the extraembryonic mesoderm appears to precede gastrulation. However, reexamining the pregastrulation human embryos in the Carnegie collection, Lucket [21] observed that the caudal margin of the primitive streak develops precociously between 12 and 14 days and that this appears to be the source of all the extraembryonic mesoderm.

The significance of these differences for the formation of human PGCs will remain unclear until molecular markers for the human PGC precursors will be identified and a fate map of the human epiblast will become available.

Bona Fide Human PGCs from Stem Cells In Vitro

In order to compensate for the lack of information about the molecular mechanisms of PGC formation in the human embryo, in vitro culture systems able to reproduce some of these processes are now becoming available. These systems are based on the possibility to induce embryonic stem cells (ESCs) derived from blastocysts or induced pluripotent stem (iPS) cells produced from differentiated somatic cells of various origin and to develop into specific cell lineages including germ line. Identification of markers and genes expressed in cells of the germ line at the very early stages of their formation is indispensable to trace back their origin and the mechanisms underlying their formation. Beside TNAP and PAS positivity, molecular markers for human migratory PGCs now include the key pluripotency transcription factors octamer-binding transcription factor 4 (OCT4, [22–24]), Nanog [23, 24], and the tyrosine kinase receptor c-KIT [25] (Table 2.2). Moreover, the surface oligosaccharide, the stage-specific embryonic antigen 1 (SSEA1), and the RNA-binding protein dead box polypeptide 4 (DDX4, also called Vasa) have been reported to be expressed in migratory (5–6 weeks) PGCs by some authors [23, 55], but not by others [32, 56]. The chemokine receptor type 4 receptor (CXCR4) might be also expressed by human PGCs at this stage [60]. After penetrating into the GRs, PGCs continue to express these markers at least until differentiation into oogonia and prespermatogonia. In addition, they express SSEA4 and possibly the RNA-binding proteins, nanos homolog 2 and 3 (NANOS2 and NANOS3 [48]), deleted in the azoospermia (DAZ), and DAZ-like (DAZL) [23, 56]. While SOX2, another key pluripotency transcription factor expressed in early mouse PGCs and ESCs [63], is quite surprisingly not expressed in human PGCs [24], other transcription factors crucial for the formation and specification of mouse PGCs, such as BLIMP1, PRDM14, NANOS1, and stella (see above), have not yet been described in human PGCs in vivo (Table 2.2). They might represent suitable markers to trace back the PGC precursors in the human embryo. At the moment, they are being used to monitor the possible formation of PGCs from stem cell lines *in vitro*. Intriguingly, human ES and iPS cells express a panel of markers common to human and/or mouse PGCs such as TNAP, SSEA4, OCT4, Nanog, stellar (stella-related), BLIMP1, DAZ, DAZL, NANOS1, NANOS3 (in some but not all ESC lines), and c-KIT, but not SSEA1, CXCR4, and DDX4 or synaptonemal complex protein 1 and 3 (SCP1

Table 2.2 Main markers of human PGCs, EG, ES, and EC cells

Marker	PGCs (3–5 weeks)	PGCs (6–9 weeks)	EG cells	ES cells	EC cells
TNAP	+++ [17]	++ [17]	+++[26]	+++ [27]	+++ [28]
PAS histochemistry	+++ [17]	– [17]	++ [29]	++ [30]	ND
SSEA1	Variable [23, 31]	+++ [23]	+++ [26, 32–34]	– [27, 35]	– [35, 36]
SSEA3	ND	Variable [31]	+++ [26, 32–34]	+++[27, 35]	+++ [35, 36]
SSEA4	+++[23]	++[23]	+++ [26, 32–34]	+++[27, 35]	+++ [35, 36]
E-cadherin	ND	ND	ND	+++ [37]	+++ [38]
Fragilis	ND	ND	ND	++ [39]	ND
TRA1-60	– [23]	– [23]	+++ [26, 32–34]	+++ [27, 35]	+++ [40, 41]
TRA1-81	– [23]	– [23]	+++[26, 32–34]	+++ [27, 35]	+++ [40, 41]
OCT4	+++ [22–24]	++ [22–24]	+++ [26, 32–34]	+++[27, 35]	+++ [42, 43]
NANOG	+++ [23, 24]	++[23, 24]	++ [33]	++ [43–45]	++ [44, 46]
NANOS1	ND	ND	ND	+ [47]	ND
NANOS3	ND	ND	ND	++ [48]	ND
SOX2	– [24]	– [24]	ND	+++ [49, 50]	+++ [51]
Stella/stellar (DPPA3, PGC7)	ND	ND	ND	+/–[a] [39, 44, 45, 47, 52]	+ [52, 53]
BLIMP1 (PRDM1)	ND	++ [54]	±/+	±/+[a] [39, 44, 45, 47, 52]	– [54]
DDX4 (Vasa)	++/– [55, 56]	+++ [55, 56]	ND	– [39, 44, 45, 47, 52]	++ [57]
DAZ/DAZL	ND	++ [23, 56]	ND	+/–[a] [39, 44, 45, 47, 52]	– [54, 58]
c-KIT	++ [25]	++ [25]	ND	+ [59]	– [59]
CXCR4	++ [60]	ND	ND	–/+ [61]	++ [62]

[a]Depending on the ES cell lines

and SCP3) markers of pre- and meiotic germ cells [44, 48, 60, 64]. On the other hand, ES and iPS cells express some markers that human PGCs do not, such as SSEA3, tumor rejection antigen 1–60, 1–81 (TRA1-60, TRA1-81), and SOX2 [23, 24, 65] (Table 2.2). Using these in vitro systems, PGC differentiation has been diagnosed primarily by the analysis of germ cell gene and protein expression and more recently, by the use of reporter constructs with the expression of green fluorescent protein (GFP) under control of the DDX4 or OCT4 promoters. Progressively increasing numbers of studies show that human ES and iPS cells can spontaneously differentiate into PGC-like cells, albeit at a low frequency (around 5 %) [47, 66–68]. Most interesting, the efficiency of spontaneous differentiation to PGCs can be increased with the addition of BMP4, 7, and 8b [69, 70], the same growth factors governing the formation of mouse PGCs (see above). Small changes in stem cell culture conditions [60] or coculture with human fetal gonad stromal cells [71], or mouse embryonic fibroblasts (MEF) in the presence of basic fibroblast growth factor (bFGF) [72], have been also reported to favor the formation of putative human PGCs *in vitro*. In addition, silencing the *DAZ* family [70] and *NANOS3* [48] genes in human ESCs resulted in a marked reduction in the capability to give rise to PGC-like cells. These PGCs show ongoing removal of parental imprinting, erasure of global DNA methylation, and histone modifications typical of mouse PGCs [67, 70] supporting the PGC identity.

These data have provided the first experimental evidence that BMPs, and probably bFGF, are involved in the formation of human PGCs and that DAZ and NANOS3 proteins function at some stages of their development. Concerning DAZ and NANOS3, recent studies in the mouse have shown that disruption of *DAZL* gene resulted in postmigratory, premeiotic reduction in PGC number

accompanied by aberrant expression of pluripotency genes and failure to erase and reestablish genomic imprinting in germ cells [73]. Moreover, Gill and colleagues [74] found that in the absence of this gene, PGCs form and migrate to the GRs but do not develop either male or female features. Instead, they remain in a sexually undifferentiated status similar to that of migrating PGCs. Other studies have implicated NANOS3 in the maintenance of mouse PGCs during migration *via* suppression of apoptosis [75].

A Model for the Formation of Human PGCs In Vivo

Taking into account the knowledge reported in the previous sections, a hypothetical model for the human PGC formation can be drawn. It seems plausible that in the human embryo, the precursors of PGCs are set aside within the epiblast between day 10 and 11 following the action of BMP signals coming from the primitive endoderm and cytotrophoblast and/or amnioblasts lining the epiblast. On days 12–14, these precursors move together to the forming extraembryonic mesoderm out the embryo proper and reach the region of the wall of the definitive yolk sac where the allantois originates around day 16. Here they are specified as TNAP-expressing PGCs. Alternatively, these last processes might be delayed for a couple of days. In such a case, PGC precursors would leave the epiblast at the beginning of gastrulation (on day 14 and 15) together with the first wave of cells that replace the primitive endoderm and form the definitive endoderm.

Migration of Human PGCs

During the fourth week, when the embryonic disc undergoes a process of folding, PGCs are passively incorporated into the embryo together with the yolk sac wall. They become transiently segregated as single cells among the endodermal cells of the primitive hind- and midgut epithelium, near the aorta (Fig. 2.2a). The GRs are visible as a distinct structure at the beginning of the fifth week. At this time, PGCs are seen to penetrate the mesenchyme surrounding the gut epithelium through breaks in the basal lamina. In the 5-week-old embryo, PGCs reach the dorsal mesentery and continue to move laterally around both sides of the coelomic angle, pass beyond the primitive mesonephros bodies, and eventually enter the GRs [16, 17, 76–78] (Fig. 2.2b). PGCs colonize the GRs during the latter part of the fifth week or at the beginning of the sixth.

Electron microscopic studies describe human PGCs in vivo as having an irregular appearance and possessing pseudopodia during their journey toward the GRs. These features are interpreted as a manifestation of active migration [16, 77, 79] (Fig. 2.2b). An interpretation confirmed by several in vitro observations reporting that human PGCs, as those of mouse, show several features of motile cells and are able to move actively both on cellular and extracellular matrix substrates [80–83] (Fig. 2.4). The in vivo time-lapse experiments with confocal microscopy by Molyneaux and colleagues [84] in slices of mouse embryos appeared to definitively confirm that in mammals, PGCs reach the GRs by active migration. However, Freman [85], reinterpreting these and other observations reported above, concludes that morphogenetic movements and local cell divisions rather than active migration are mainly responsible for PGC displacement in the different regions of the embryo. Even Freman, however, admits that human PGCs might migrate actively to cover a distance of approximately 50 μm separating the preaortic region from the GRs.

The mechanisms by which not only human PGCs but also those of other mammals are finally delivered to and colonize specifically the GRs remain largely unknown. Contact guidance with somatic and/or extracellular molecules (ECM) and attractive (chemotaxis) and repulsive signals are two unmutually exclusive mechanisms suggested by evidence in the mouse and other vertebrate species (for a recent review, see [86]). In the human embryo, migratory PGCs appear to be surrounded by extracellular matrix components in which mesenchymal cells are immersed. PGCs seem to interact with the mesenchymal cells through different type of junctions, such as

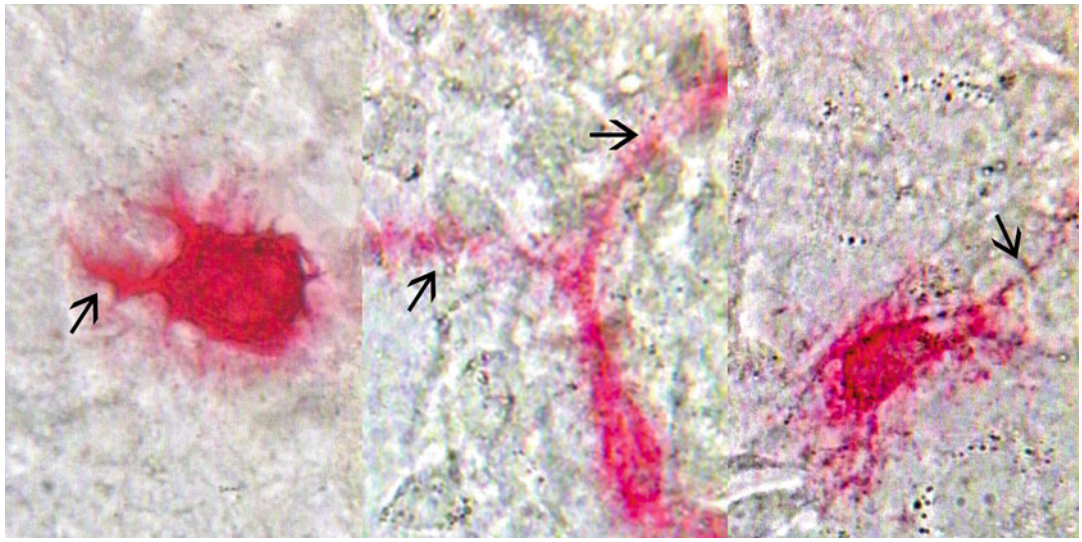


Fig. 2.4 Human PGCs isolated from 6–7 week embryo and cultured in vitro for 2 days onto STO fibroblasts identified by TNAP staining. PGCs usually appear elongate with pseudopodia and fine filopodial extensions (*arrows*)

desmosomes, gap junctions, and focal contacts [77, 87]. Glucosaminoglycans have been histochemically detected in the extracellular matrix surrounding migratory human PGCs ([87] and references here in). Moreover, several results demonstrated that mouse PGCs may use various types of integrins for dynamic adhesive interactions with extracellular matrix molecules such as fibronectin, laminin, and collagen IV (for a review, see [88]). In this regard, it is important to report that mouse PGCs lacking β1 integrins fail to migrate normally to the GRs [89].

In the opinion of Witschi [16], the coelomic epithelium in the region of the GRs releases specific molecules to attract PGCs. The use of migratory cell assays similar to those used for mouse PGCs [90, 91] could help to identify attractants for human PGCs. The tumor growth factor-β (TGFβ) [92], stromal-derived factor1 (SDF1) [93], and stem cell factor (SCF, or c-Kit ligand, KL) [91] have been proposed as chemoattractants for mouse PGCs. KL and SDF1 could be also implicated in directing the migration of human PGCs. The KL receptor c-KIT is expressed by human PGCs [25], while putative PGCs obtained from human ESCs express the SDF1 receptor CXR4 [60].

The difficulty in identifying specific chemoattractants in PGCs suggests that the underlying mechanisms of migration are complex and are likely to involve morphogenetic movements, interactions with ECM molecules and the surrounding cells, and attractive and repulsive signals as well. PGCs might recognize more than one attractive and repulsive signal at particular locations of their pathways and/or recognize different signals at different locations. The expression of transcripts for member of the olfactory receptor gene family in human PGCs from 10-week-old embryos makes this class of receptor additional candidates for PGC attractants [94]. Finally, in a recent study, Møllgard and colleagues [78] observed that human PGCs preferentially ascended from the mesentery of the hindgut to the gonadal anlage by migration along autonomic nerve fibers close to the Schwann cells and proposed that these nerve fibers and/or Schwann cells may release chemoattractants supporting PGC migration.

During migration, human PGCs contain a large PAS-positive cytoplasmic store of glycogen and several lipid droplets [16, 76, 77]. In other species, these cytoplasmic inclusions were not observed. Following their arrival in the GRs, the glycogen content is diminished. Round mitochondria with a pale matrix and small tubular vesicular cristae were observed near the nucleus.

They significantly increase in number during PGC migration and settlement in the GRs. Migratory PGCs have less than 10 mitochondria, while 100 mitochondria are present in ovarian PGCs and 200 in oogonia [95]. These observations suggest that PGCs might prevalently employ an anaerobic metabolism during migration and undergo a transition in their energy metabolism after reaching the GRs.

An interesting issue concerning PGC migration is the fate of the misallocated cells which fail to reach the gonads. While those remaining nearer the gonads were observed to enter meiosis irrespective of the sex [96–98], most of the other misallocated cells are believed to undergo apoptosis [98, 99] or to give rise to germ cell tumors (GCTs) after birth. Human GCTs are a heterogeneous group of tumors that may occur both in the gonads and at extragonadal midline sites such as the coccyx, the pineal gland, and the mediastinum [100, 101]. Runyan and colleagues [102] have recently identified in the caudal region of the mouse embryo a population of undifferentiated ectopic PGCs that might be the population of origin for sacrococcygeal tumors. The possible causes of the PGC transformation into tumorigenic cells will be discussed in the next section. Another possibility is that ectopic PGCs enter near the aorta and are distributed to various tissues throughout the embryo. In birds and reptiles, the vascular system is a normal way to deliver PGCs to the GRs [103]. In mouse [104] and bovine [105] embryos, electron microscope observations showed cells morphologically identifiable as PGCs entering or circulating in the bloodstream. Because of their intrinsic pluripotency, under certain circumstances, these cells might participate to normal tissue differentiation or enter a quiescent status to later give rise to tumors. In my view, such a possibility represents an intriguing working hypothesis for future investigations.

Proliferation of Human PGCs

Human PGCs proliferate during migration, mostly after reaching the GRs. Once relocated in the GRs, PGCs are rapidly surrounded by cords of somatic cells. The differentiation of PGCs into oogonia occurs apparent during the ninth week. Oogonia show a higher mitotic activity and possess a regular and smooth cellular profile. In the cytoplasm, lipid inclusion and glycogen granules are markedly reduced while the number of mitochondria is increased (see above) [106, 107]. In addition, oogonia tend to form clusters of dividing cells joined by rims of cytoplasm, termed intercellular bridges, originated by incomplete division of the cell body during cytodieresis [108, 109]. The mitotic proliferation of oogonia lasts several weeks and overlaps the period of their entry into meiosis (10–11 weeks). In fact, until the fifth month of fetal life, mitotic oogonia and primary oocytes in different stages of meiosis coexist [110, 111].

In the male, after reaching the developing testis, PGCs are usually termed gonocytes. While being enclosed within seminiferous sex cords, gonocytes assume some distinct morphological features, such as a large nucleus and a prominent nucleolus [112]. Male sex determination is marked by the expression of sex determining region Y (SRY) and SRY-box 9 (SOX9) genes at 5–6 weeks [113]. Thereafter, sex cords, formed from Sertoli cells and gonocytes, become increasingly evident within the testis from the seventh week during late embryonic life. During the first trimester, gonocytes are mitotically active (they correspond to the M-prospermatogonia of Wartenberg's classification [114]). They appear to form a quite homogenous single, round cell population both morphologically and histochemically [115]. Of relevance, gonocytes continue to express markers typical of pluripotent cells and PGCs such as OCT4, SSEA1, DDX4 (Vasa), Nanog, and c-KIT [23, 114]. This means that despite some morphological differences, gonocytes are basically equivalent to PGCs. During the second trimester, most but not all gonocytes progressively lose mitotic activity together with the pluripotency and PGC markers. At this time, two new types of germ cells have been described, intermediate germ cells still able to proliferate and mitotic quiescent prespermatogonia or T (transition)-spermatogonia. The former are mostly in pairs, while the latter form groups

of cells interconnected by cytoplasmic bridges [114–116]. Interestingly, a few cells maintaining gonocyte characteristics seem to remain among the prespermatogonia [23, 115]. Similar results have been recently reported in the mouse [117]. The fate of these undifferentiated cells could be relevant in establishing the spermatogonia stem cell population in the postnatal testis and for the development of testis tumors.

Considering the observations and the results reported above, we can estimate that the period of human PGC proliferation in the female lasts from the beginning of the fourth week to about the ninth week, when oogonia become clearly recognizable within the fetal ovary. In the male, if we consider the gonocytes equivalent to PGCs, proliferation continues probably for a little longer period until about the end of the first trimester (10–12 weeks), when the most part of gonocytes differentiate into prespermatogonia. Significantly, in both sexes, the occurrence of intercellular bridges appears to mark the differentiation of PGCs into oogonia and gonocytes into intermediate germ cells and prespermatogonia. In the ovaries, oogonia continue to proliferate until the fifth month. During the same time in the testes, intermediate germ cells proliferate while prespermatogonia become progressively mitotically quiescent.

Counting the number of PGCs at different stages of development provides estimation of their proliferation capability and possible sex differences. As reported in a previous section, in an embryo of 3 weeks, Politzer counted 40 PGCs that increased to 600 in an embryo of 4 weeks. Similarly, Witschi [16] counted 30–50 PGCs around the end of the third week and 109 in an embryo a couple of days older. The same authors also counted about 450 and 1,400 PGCs in two embryos of 4 weeks. In his classical study, Baker [110] estimated the number of female germ cells in a total of 12 ovaries covering 2–7 months postconception. He estimated a mean of a total 600,000 oogonia in two 9-week-old ovaries and a peak of about 6,000,000 at the fifth month. Six recent publications presenting stereological estimations of the number of germ cells in much higher numbers of ovaries and testes (overall 103) for the first two trimesters have been recently analyzed by Mamsen and colleagues [118–124]. Extrapolating the old and the new data covering the 4- to 9-week period, it results that the total number of PGCs increases from about 1,000 to about 450,000 in female and 150,000 in male (Fig. 2.5).

The regulation of human PGC proliferation in both sexes is poorly understood. The scant available information comes mainly from in vitro studies of human PGCs cultured on cell monolayers. These studies indicate that human PGCs appear to respond to the same compounds (forskolin, retinoic acid) and growth factors (SCF, bFGF, leukemia inhibitor factor, LIF) reported to stimulate the survival and/or proliferation of mouse PGCs ([26], our unpublished observations). Most importantly, like mouse PGCs, human PGCs give rise to pluripotent embryonic germ cells (EGCs) (see also next section), when cultured in vitro in the presence of a cocktail of compounds and growth factors [26], suggesting that the mechanisms controlling PGC growth in mammals are largely conserved. Hiller and colleagues [125] recently reported that the addition of recombinant BMP4 increased the number of human PGCs after 1 week of in vitro culture in a dose-responsive manner. The efficiency of EGC derivation and maintenance in culture was also enhanced.

Analysis of gene expression in human PGCs and the study of spontaneous mutations resulting in reduction or absence of fertility may help to confirm or disprove such similarities. For example, as reported above, c-KIT is expressed by both male and female PGCs. Mutations in the *c-KIT* gene affect both hematopoietic and melanocyte lineages in humans, but to date, no association of mutations in this gene with human infertility has been documented, contrary to the mouse model. However, while no mutations have been detected in codon encoding Y721 (analogous to Y719 in the murine *c-kit* gene, a residue known to be essential for a normal mouse spermatogonial proliferation) of the human *c-KIT* gene of infertile idiopathic patients [126], other results indicate that genetic variants within the genomic sequences of the *c-KIT* and *KITLG* genes are associated with idiopathic male infertility [127]. On the other

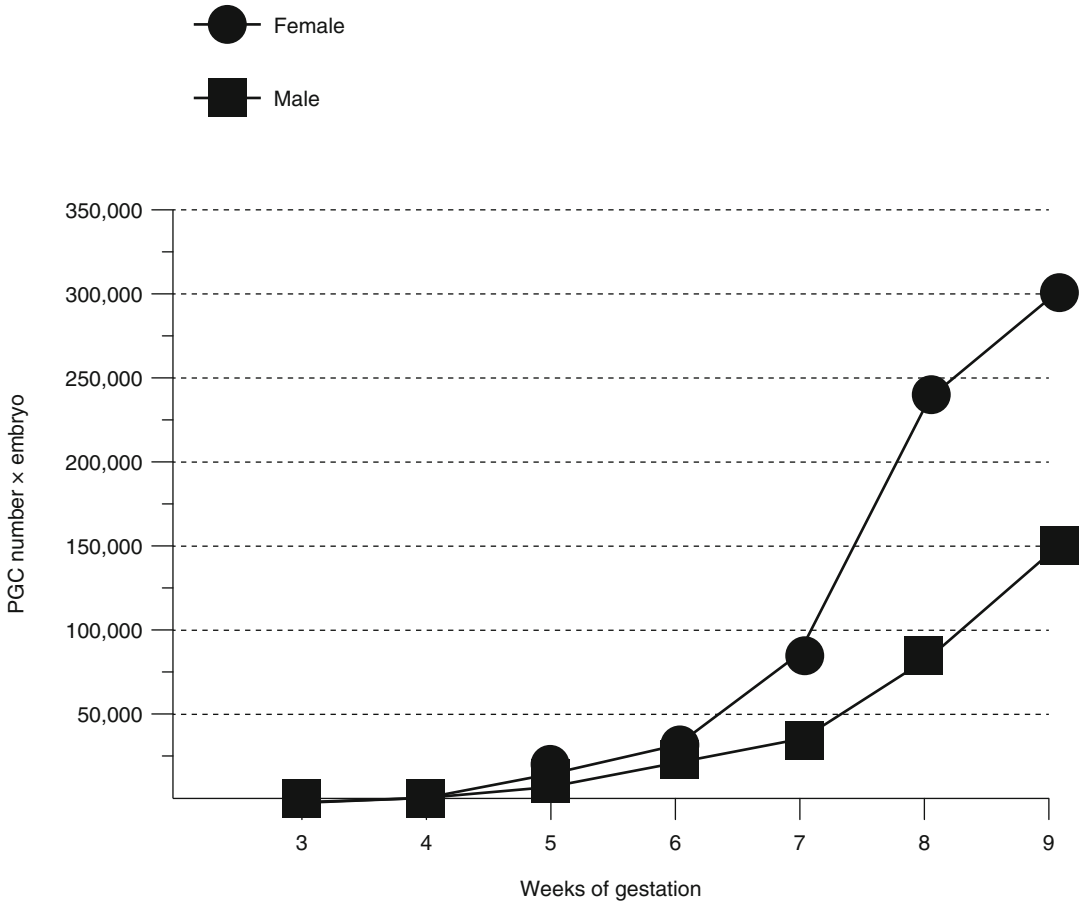


Fig. 2.5 The number of human PGCs in female and male embryos from 3 to 9 weeks of gestation extrapolated by the data of papers [14–16, 110, 118–122]. Note that from 5 weeks onward, the number of PGCs is much higher in female than in male embryos. This difference continues also when PGCs differentiate into oogonia or gonocytes/prespermatogonia. At the end of the proliferation period (around fifth month), the estimated number of oogonia is around 10,000,000 and of prespermatogonia between 3,000,000 and 4,000,000 per embryo [110, 118]

hand, *c-KIT* is strongly upregulated in some types of germ cell testicular tumor that are believed to originate from PGCs (for a review, see [128]), suggesting that it plays a crucial role in the control of human PGC survival/proliferation as in mice. In this regard, *OCT4* was found to be expressed in all human germ cell tumors containing undifferentiated cells [129]. In Fanconi's anemia (FA), individuals are characterized by several congenital abnormalities including decreased fertility (for a review, see [130]). Interestingly, targeted mutation of *Francc* in mice results in significantly slower proliferation of PGC [131], suggesting again shared control mechanisms of PGC proliferation in these species. Finally, in Trisomy 16 of mouse, an animal model of the human Down's syndrome leading frequently to sub- or infertility, a delay in migration and reduction of PGC number was observed [132]. Gene expression studies on single human PGC obtained from 10-week-old embryos have been reported [133, 134]. The preparation of cDNA libraries and microarrays from human PGC should be valuable resources for researchers in this field.

Human EGCs and Their Potential Uses

Derivation of human embryonic germ cell (EGCs) has been reported by several groups from PGCs obtained from 5- to 9-week-old embryos [26, 32–34]. The differentiation capability both in vitro and

in vivo of these cells into several types of tissues has been reported, and important studies toward therapeutic use of human EGCs are in progress ([135] and references therein). An important difference with mouse is that human EGCs maintain the methylated status of imprinted genes, those genes that are expressed from either the maternal or paternal allele, without undergoing the erasure of these epigenetic marks that normally occurs in PGCs [136]. Genomic imprinting is an epigenetic process that involves DNA methylation in order to achieve monoallelic gene expression without altering the genetic sequence. These epigenetic marks are established in the germ line and are maintained throughout all somatic cells of an organism. Appropriate expression of imprinted genes is important for normal development, with numerous genetic diseases associated with imprinting defects. Imprinting is erased during the PGC development and reestablished in germ cells during gametogenesis according to the sex of individuals (for a review, see [137]). In mouse, gene imprinting is progressively erased in migratory PGCs and lost almost completely by the time they complete the colonization of the GRs (for a review, see [138]). The timing of this erasure is not known in humans. In the mouse, deregulation of imprinted genes in EGCs has been reported to cause imprinting-related developmental abnormalities [139]. Similarly, mouse ESCs fail to properly control the expression of imprinted genes [140]. In humans ESCs, gene-specific differences in the stability of imprinted loci have been reported [141]. The stability of imprinting in human EGCs suggests that there may be no significant epigenetic barrier to human EGC-derived tissue transplantation. The difference between human and mouse EGCs in the imprinting status also suggests that the timing of the crucial epigenetic changes involving gene imprinting erasure might be different in human and mouse PGCs. In the latter, as reported above, gene imprinting is progressively erased and lost almost completely by the time of GR colonization by PGC (for a review, see [138]). This pattern is reflected in the variability of the imprinting status reported in mouse EGCs derived from PGCs at different developmental stages [139, 142, 143]. Deregulation of imprinted genes can be associated with tumorigenesis and altered cell differentiation capacity. Noteworthy, human EGCs have a normal karyotype and do not form tumors at least when transplanted into immunocompromised mouse hosts ([33] and references therein).

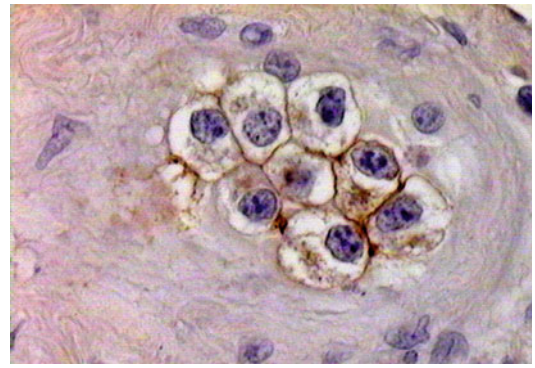


Fig. 2.6 A group of carcinoma in situ (CIS) cells inside a seminiferous tubule of a human testis. The derivation of these tumorigenic cells from PGCs or gonocytes or spermatogonia and the molecular mechanisms of their formation are still debated

Besides the use in tissue therapy, studies on human EGCs can offer clues about important aspects of cell stemness and on the mechanisms underlying the transformation of PGCs into tumorigenic cells. For a long time, as reported above, human PGCs have been believed to give rise to germ cell tumors (GCTs) both in the testis and extragonadal sites, but an experimental model was lacking. GCTs can be classified in three categories. The first group includes teratomas and teratocarcinoma and yolk sac, which occur in fetus and infants. Teratocarcinomas are malignant tumors containing undifferentiated cells known as embryonal carcinoma (EC) cells, able to propagate the tumors after host transplantation. The second group consists of adult tumors and includes both seminomas and nonseminomas. The third class characterizes spermatocytic tumors which occur in elderly men. In the mouse, early studies have demonstrated that teratomas and EC cells originate directly from PGCs [144] so that the same origin is plausible for these type of tumor cells in humans. Seminomas and nonseminomas derive from a common precursor cell, called carcinoma *in situ* (CIS) cells. It has been hypothesized that CIS cells originate from fetal germ cells [145] (Fig. 2.6). Comparative microarray studies have shown that CIS cells show a high degree of pluripotency and

are indeed very similar to PGCs and gonocytes. CIS cells are consistently aneuploidy. Moreover, CIS cells possess an abnormal chromosome described as isochromosome 12p, or i(12p), formed from two fused short arms (p arms) of chromosome 12 [146]. The arrest of PGC differentiation and their nuclear reprogramming that in mouse have been convincingly reported to occur during PGC transformation into EGCs (for a review, see [138]), might be the key first events, that may be followed by malignant transformation into EC or CIS cells associated to the acquisition of an abnormal karyotype and overt germ cell cancer later in life.

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Damage Control in the Female Germline: Protecting Primordial Follicles

3

Michelle Myers and Karla J. Hutt

Abstract

One of the major challenges faced by the oocytes of primordial follicles during their genesis and throughout their prolonged stasis is staying alive while maintaining high cytoplasmic and genomic quality, such that fertility is ensured and offspring are healthy. One way to manage this predicament is to employ a host of machinery to repair damage as it transpires. Furthermore, the primordial follicle must also be endowed with proapoptotic proteins so that if repair cannot occur, the oocyte is eliminated from the ovarian pool. This chapter focuses on the mechanisms by which primordial follicles maintain a balancing act between apoptotic elimination, repair, and tolerance to ensure quality control within the female germline. Additionally, as our understanding of the specific pathways involved in oocyte quality control improves, new opportunities are emerging to manipulate the size of the primordial follicle reserve to extend the natural reproductive lifespan and to preserve fertility during DNA-damaging anticancer treatment.

Keywords

Apoptosis • DNA damage • DNA repair • Oocyte • Primordial follicle Female germ cell • Oocyte quality

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Introduction

The female germline is stored within the ovary in the form of primordial follicles, which are comprised of non-growing, meiotically arrested oocytes surrounded by a single layer of flattened pre-granulosa cells [1]. Although recently challenged [2, 3], it remains widely accepted that the population of primordial follicles established during fetal life (humans) or the perinatal period (rodents) is finite. Throughout life, the number of primordial follicles progressively declines, and eventually, the supply becomes so low that the menopause or reproductive senescence begins [4]. Primordial follicles have one of two fates: (1) recruitment into the growth phase, with the possibility of ovulation or (2) death. Although there is clear evidence to suggest that apoptotic mechanisms are involved in germ cell loss [5], other mechanisms of oocyte death are certainly feasible and are beginning to be explored [6–8]. What mechanisms control the initial size of the primordial follicle pool, and how are they selected to grow? Why are certain primordial follicles recruited into the growth phase, while others remain dormant for decades, or even die? These questions are essential endeavors in women's health because the length of a female's reproductive lifespan, her ability to bear healthy offspring, and the quality of her overall health are determined by both the number and quality of oocytes in her ovaries. Similarly, factors that reduce primordial follicle number and quality, such as increasing maternal age and gonadal insults (i.e., systemic chemotherapy and/or radiotherapy), severely compromise female fertility. The following sections review our current knowledge of DNA repair and cell death pathways utilized by primordial follicle oocytes during their genesis and lifetime. This is followed by a discussion of the potential consequences of manipulating these quality control processes in order to preserve fertility during normal aging or following DNA-damaging anticancer treatments.

Establishing a High-Quality Reserve: Quality Control of Pre-diplotene Germ Cells

Perhaps one of the most perplexing aspects of oogenesis is the disappearance of large numbers of germ cells from the ovary around the time of primordial follicle formation [9]. Recent evidence suggests that these pre-diplotene oocytes are eliminated through multiple processes, including apoptosis, autophagy, and expulsion [6–8, 10, 11]. However, the reason behind this massive wastage remains unknown; why expend significant energy to generate a large pool of oocytes only to eliminate them shortly after they are made?

Accumulating data supports the notion that at least some of this loss is related to oocyte quality. Adult mice bearing mutations in genes essential for chromosome pairing, homologous recombination, DNA repair, and synapsis during the early stages of the first meiotic prophase invariably have ovaries devoid of oocytes and are infertile [12–16]. For example, oocytes from mice deficient in the *Atm* gene exhibit defective chromosome crossover and as a consequence undergo apoptosis before the diplotene arrest [12, 13]. Similarly, oocytes in mice null for *Dmc1* are unable to effectively synapse and are quickly eliminated from the ovarian reserve [14, 15]. Furthermore, *Trip13* deficient mice exhibit oocytes with unrepaired DNA damage resulting from failed homologous recombination and consequently undergo apoptosis [16]. Therefore, strategic mechanisms (presumably apoptotic) are employed by the ovary to ensure the removal of low-quality oocytes that arise as a consequence of inherently error prone early stages of meiosis.

Directly responsible for the execution of oocytes during apoptotic-mediated cell death is the mammalian family of caspases (reviewed in [17]). Targeted deletion of a variety of caspases has revealed how different family members are important in mediating separate apoptotic pathways in germ cells. For example, in both the human and mouse, germ cell death during the meiotic prophase stages of development appears

to involve caspase-2 [18, 19] but not caspase-3 [20], while caspase-9 plays an important role in cytokine deprivation-induced death [17]. Collectively, these data indicate that surveillance and effecter systems exist in pre-diplotene oocytes to ensure that defective oocytes are not permitted to contribute to the germline.

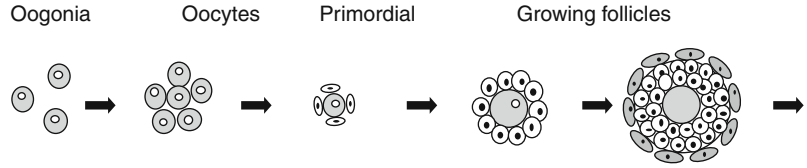
Quality Control During Diplotene Arrest

The great majority of oocytes that survive the initial culling that occurs during the early stages of meiotic prophase I in fetal or early neonatal development subsequently die in postnatal life [21]. Again, one reason behind this loss might be quality control. It is known from various data that primordial follicles are sensitive to DNA damage-inducing agents and undergo rapid apoptosis [22]. In fact, oocytes exhibit a stage-specific sensitivity to γ (gamma)-irradiation-induced DNA damage, with oocytes in the earlier stages of the meiotic prophase I (leptotene, zygotene, and pachytene), together with oocytes recruited into the growing pool, being much more resistant than those in diplotene arrest [23] (Fig. 3.1). Though the basis of this sensitivity is not clear, it may be that diplotene DNA is more vulnerable to damage, or it is possible that repair mechanisms are lacking or inadequate in primordial follicles. Alternatively, special surveillance systems may exist in primordial follicles to rapidly detect and eliminate damaged oocytes. In this regard, TAp63, a homologue of the p53 tumor suppressor protein, plays a pivotal role in protecting the germline from DNA damage [24, 25]. TAp63 is an isoform of p63 that is specifically expressed by oocytes of primordial follicles and its rapid activation, by phosphorylation, in response to DNA damage results in apoptosis [24, 25]. Interestingly, McKeon and colleagues showed that tolerance for γ-irradiation-induced DNA damage by diplotene oocytes is very low, with as few as three to ten DNA breaks required to induce a cell death response by TAp63. Moreover, increased damage (i.e., the greater the irradiation dose) results in a faster apoptotic response. These authors concluded that TAp63 is the mechanism by which the postnatal ovarian reserve surveys DNA damage and ensures the fidelity of the genome for the next generation. The availability of TAp63 may also be partly responsible for the stage sensitivity of germ cells to DNA damage-induced apoptosis: TAp63 is not expressed in female germ cells until the post-pachytene stage and then expression levels decrease following primordial follicle activation and oocyte growth [25, 26]. It is also possible that p53 and/or p73 (the third member of the p53 family) play important roles in the management of quality control in the female germline in pre-diplotene and growing oocytes, when TAp63 is absent or only expressed at low levels (Fig. 3.1).

Quality Control During Aging

As women age, their chance of successfully conceiving decreases, while their likelihood of miscarriage and bearing children with compromised health increases [27–29]. Chromosomal abnormalities, resulting in spontaneous abortion and genetic disorders such as Down's syndrome (i.e., trisomy 21), are strongly correlated with maternal age, but a significant number of chromosomally normal pregnancies also fail in older women for unknown reasons, suggesting that multiple factors are involved [30]. The primary causes of this age-associated decline in female reproductive capacity are both a reduction in the number of oocytes in the ovary and the deterioration in the quality of those oocytes that remain [31]. In particular, oocytes from older females show defects in their ability to successfully complete meiosis (meiotic competence) and in their ability to promote healthy embryonic development (developmental competence) [32, 33]. However, the underlying cellular and molecular mechanisms that reduce the quality of an aging oocyte remain largely unknown but are continually being explored [34, 35].

Given that the ovarian reserve is established before birth in humans, a primordial follicle may remain in the ovary for a period of 40 years or more before resuming meiosis and undertaking



Cell cycle	Mitotic	Mitotic	Arrested in diplotene/dictyate →	
Response to exogenous DNA damage	Sensitive	Not sensitive	Sensitive	Less sensitive →
Detector of DNA damage	?	?	TAp63 ——— ----------→	
			p73? ——— ----------→	
Presence of DNA repair enzymes	?	Homologous recombination	Yes	Yes

Fig. 3.1 The quality control mechanisms employed by the female germline to protect the primordial follicle population are dependent upon how the oocyte detects and subsequently repairs damaged DNA. Germ cells harboring unrepairable DNA are disposed of via apoptotic mechanisms. Germ cells from different developmental stages have varying levels of sensitivity, possibly reflecting the differential expression of p53 family members and their potential downstream targets. Oocytes are capable of repairing endogenous and exogenous DNA damage by homologous recombination (during meiosis) and via enzymatic mechanisms during their genesis. However, the role of DNA repair in maintaining oocyte quality prior to and during the meiotic prophase remains largely uncharacterized

a developmental program that eventually results in the ovulation of a mature oocyte. Although all stages of folliculogenesis are potentially susceptible to age-related damage, it has been hypothesized that defects may first arise within primordial follicles during their highly unusual period of stasis within the ovary [36–39]. It has been proposed that this long interval between protracted meiotic arrest (dictyate) and its reinitiation may result in the oocytes being unable to detect and correct recombination errors generated during fetal life [39]. However, there is a dearth of information on the effects of aging on primordial follicle quality and the possibility that age-related changes in the primordial follicle oocyte might underlie the observed reduction in meiotic and developmental competence has not been fully explored.

During its prolonged diplotene arrest, the oocyte is subjected to endogenous (i.e., reactive oxygen species from metabolism) and exogenous (i.e., environmental toxicants, chemotherapy, radiotherapy) sources of damage-inducing factors, which may lead to a progressive deterioration of oocyte quality. Reactive oxygen species in particular have been shown to cause damage to proteins, lipids, and DNA (genomic and mitochondrial) in aging periovulatory oocytes [40]. It has also been hypothesized that the diffuse state of the dictyate chromosome makes the DNA more susceptible to intercalating mutagenesis [22, 41]. Interestingly, age-related changes in the dimensions and ultrastructure of primordial follicles have been documented [42, 43]. For example, the ooplasm of primordial follicles from older women has more vacuoles and less mitochondria [43]. While these data suggest that starting the point of follicular development is altered with age, it is unclear how these morphological changes relate to oocyte quality.

In addition to the increased risk of damage to primordial follicles due to their longevity, the mechanisms in place to detect and repair the damage may also deteriorate with age [34]. Such an outcome might allow defective oocytes to slip through less stringent quality control measures and thus contribute to the increased incidence of infertility, aneuploidy, miscarriage, and other birth defects associated with age. For example, high levels of ATP are required for DNA repair to occur [44], but as mitochondrial number and function have been shown to become impaired with age [45, 46], efficient repair might be inhibited.

Guli and Smyth (1989) showed that UV-irradiated oocytes from both young and old mice exhibit similar levels of unscheduled DNA

synthesis, indicative of DNA repair [47]. While this work suggests that the oocytes of female mice retain the capacity to repair double-strand DNA breaks throughout reproductive life, many different types of damage can occur (including of x-rays, γ (gamma)-rays, hydrolysis, mutagenic chemicals, chemo- and radiotherapeutic compounds, viruses) and more work is required to determine if other systems of repair are also affected by age. Alternatively, the ability of oocytes to initiate apoptosis might become altered with age. Increased levels of the proapoptotic protein Bax have been observed in aged oocytes [48]. Microarray analysis has also demonstrated that numerous apoptosis-related genes were misregulated in ovulatory oocytes from old mice [49]. In this and similar studies, changes in the expression levels of genes involved in mitochondrial function, stress responses, and spindle assembly were also dramatically affected in old oocytes [49–51]. The application of microarray analysis to young and old primordial follicles has not yet been reported, but this approach would be an ideal starting point from which to characterize age-related changes in primordial follicle quality.

As discussed earlier, the roles of the transcription factor p53, and family member's p63 and p73, as guardians of maternal reproduction have recently been explored [24, 25, 52, 53]. Each of these proteins exists as a number of different isoforms, with unique localization and potential roles for each isoform now emerging. Intriguingly, p73 has been linked to reduced fertility and the high incidence of chromosomal abnormalities in newborns from older women. In particular, mature oocytes harvested from older women and mice displayed a reduction in the TAp73 isoform [52]. However, it is not yet known if TAp73 expression levels are reduced in the oocytes of aged primordial follicle, or if reduced expression is characteristic of ovulatory oocytes only.

DNA Repair Processes in Oocytes

Unrepaired DNA damage can lead to either apoptosis or tolerance, but both of these circumstances come at a severe cost to fertility; apoptotic elimination of defective oocytes may lead to a shortened reproductive lifespan, whereas tolerance of DNA damage is a source of potential mutations, which can result in failed pregnancies or birth defects. Therefore, the repair of damaged DNA is an important aspect of quality control within the ovary because it ensures oocyte quality while maintaining oocyte number.

Oocytes have the capacity to repair DNA double-strand breaks that occur during meiotic recombination, as well as DNA damage induced by chemical exposure and irradiation. Early studies showed that diplotene oocytes undergo repair following UV-induced DNA damage, but the repair response in primordial follicles was substantially reduced when compared to that of growing follicles [47, 54]. Inadequate repair systems in primordial follicles may be one reason that primordial follicles are more sensitive to DNA damage-inducing agents than growing follicles. Numerous studies have demonstrated the expression of γH2AX, in diplotene oocytes in response to meiosis and exogenously induced DNA damage, which is indicative of the initiation of repair [24, 25]. Though direct evidence of the specific repair mechanisms employed by primordial follicles is very limited, microarray analysis of human germinal vesicle stage oocytes has shown that growing oocytes express many pathways for DNA repair including one-step repair procedure, base excision repair, mismatch repair, and nucleotide excision repair [55]. This study indicates that oocytes do utilize multiple mechanisms to avoid transmitting mutations into the next generation.

While the functional importance of few specific repair proteins has been directly studied in primordial follicle oocytes, expression of Rad51 has been observed [34]. Rad51 is part of the homologous recombination repair pathway for double-strand (ds) DNA breaks. Interestingly, it was detected at higher levels in primordial follicle oocytes from young mice compared to old mice, in an inverse relationship with the proapoptotic protein Bax [34]. Thus, primordial follicle oocytes may lose the capacity to undertake efficient repair of dsDNA breaks as they age, making them more susceptible to Bax-mediated

apoptosis. This interaction may be partly responsible for the accelerated loss of primordial follicles observed toward of the end of reproductive life in mice.

An intriguing question remains: Are there differential repair capacities available to germ cells/oocytes at different stages of development, and is this responsible for the stage-specific sensitivity of oocytes to DNA damage-induced apoptosis (Fig. 3.1)? During meiotic recombination, germ cells naturally have the capacity to repair DNA double-strand breaks by homologous recombination and are somewhat resistant to apoptosis [23]. However, unlike early-meiotic oocytes, mitotically active germ cells (oogonia) and primordial follicles oocytes are much more sensitive to DNA-damaging agents [23, 56]. Similarly, growing follicles do not appear to have the same sensitivity threshold as diplotene oocytes, displaying a much slower repair response to UV-induced DNA damage oocytes [47, 54]. Clearly, future studies are required to determine how oocytes at all developmental stages (1) detect DNA damage and (2) repair the damage or, (3) initiate apoptosis.

Oocyte Quality and the Balance Between Apoptosis, Repair, and Tolerance

Is oocyte quality compromised if apoptosis is inhibited? This question has gained considerable importance with the advent of technologies and therapies designed to preserve primordial follicle numbers by inhibiting apoptosis. In particular, anti-apoptotic measures might be useful for protecting the primordial follicle reserve during anticancer treatment and during aging. Mouse models bearing deficiencies in proapoptotic genes (acid sphingomyelinase and Bax) and selective caspases (caspases −2 and −11) have provided surprising insights into the role of apoptosis in germ cell quality control [19, 57–59]. These models have provided proof of principle for the concept that fertility can be prolonged and preserved by inhibiting apoptosis.

Inhibition of oocyte apoptosis by interfering with the sphingomyelin pathway of cell death and caspases −2 and −12 have also been investigated as a means of preserving the ovarian germ cell population following anticancer therapy [57, 60]. Mouse models deficient in each of the aforementioned genes suppressed the normal apoptotic program, leading to an over endowment of primordial follicles as germ cells were largely resistant to radiation and chemotherapy. Furthermore, *in vivo* treatment of female mice with sphingosine-1-phosphate protected oocytes from radiation-induced apoptosis [57]. The authors went on to test the viability of the oocytes rescued from radiation-induced apoptosis by examining *in vitro* fertilization rates and preimplantation embryonic development. While the results were very favorable, the ovulated oocytes examined in this study were collected only 2 weeks after exposure to ionizing radiation. Given that it is estimated to take approximately 20 days for a follicle to develop from primordial through to ovulatory, it is likely that the oocytes tested were part of the radioresistant growing follicle pool at the time of treatment, and not the primordial follicle pool. Furthermore, the 0.1 Gy dose of radiation used in this study did not destroy all primordial follicles and so it is not possible to know if the oocytes tested for quality were the rescued cohort or part of the naturally resistant pool.

Bax is a proapoptotic Bcl-2 family member shown to play a role in oocyte apoptosis [7, 24, 58, 61, 62]. Adult female *Bax*$^{-/-}$ mice have a surfeit of primordial follicles, presumably due to the survival of oocytes that would have undergone apoptosis due to some deficit [58]. Interestingly, the population of primordial follicles within the ovaries of *Bax*$^{-/-}$ mice persists well after the ovarian reserve has been exhausted in their wild-type counterparts [58]. But, if the surfeit of follicles in *Bax*$^{-/-}$ mice is the result of retaining defective oocytes that should have undergone apoptosis, it follows that the ovary might then be populated with a cohort of low-quality oocytes. One would predict that the rescue of low-quality oocytes in *Bax*$^{-/-}$ mice might result in reduced developmental competence and low litter number, or loss of offspring viability. However, it was reported that *Bax*$^{-/-}$ mice have an extended ovarian lifespan and produced viable offspring for a prolonged

period of time [61]. One explanation for this apparent incongruity might be that under normal circumstances, tolerance of damage is very low because the consequences of certain deficits are severe (i.e., infertility, miscarriage, or birth defect). Low tolerance might mean that the first line of defense for protecting germline quality is to induce apoptosis. However, in situations where apoptosis is prevented, as is the case with *Bax*$^{-/-}$ ovaries, the oocyte has the opportunity to restore quality by deploying DNA repair processes. Unfortunately, it is impossible to determine if the offspring reported in this study arose from defective oocytes rescued by the absence of *Bax* or if they were derived from the "normal" primordial follicle pool. It is also worth noting that there was an initial delay in litter bearing in young adult *Bax*$^{-/-}$ females. It is not known what dictates the order in which primordial follicles are selected to enter the growing follicle pool, but this study raises the possibility that low-quality oocytes are among the first to grow, mature, and ovulate.

Models of primordial follicle over-endowment can also provide interesting clues as to the relative contribution of oocyte number versus oocyte quality to the decline in fertility associated with maternal age. Wild-type mice experience a reduction in fertility after 8 months of age, but *Bax*$^{-/-}$ mice continue to have viable litters for some time. These data suggest that the normal drop in fertility at 8 months is an issue of low follicle numbers resulting in inadequate endocrine support, rather that reduced quality due to aging-related damage *per se*. Surprisingly, very aged (581–640 days) *Bax*$^{-/-}$ mice responded to superovulation (5 cumulus oocyte complexes were retrieved from 3 mice), and a single embryo was generated following *in vitro* fertilization and embryo culture. Additionally, ovarian grafting experiments demonstrated that very old oocytes can give rise to viable offspring [61]. These data hint that very old primordial follicles can undergo folliculogenesis, complete meiosis, and sustain early embryogenesis, but the very low numbers of oocytes, embryos, and offspring obtained suggest that this is the exception rather than the rule. A larger cohort of animals is required to thoroughly investigate the effect of chronological aging on oocyte quality. For example, a study of *Bax*$^{-/-}$ mice at an intermediate age (12–18 months), when primordial follicles numbers are still high enough to yield sufficient ovulatory oocytes, would allow a more accurate assessment of the meiotic and developmental competence.

Final Remarks

The evolution of multiple and diverse processes to eliminate unwanted oocytes from the primordial follicle pool suggests that tolerance for damage within the germline is low and stringent quality control mechanisms are in place. However, tantalizing data from models of apoptosis inhibition suggest fertility can be artificially prolonged and the primordial follicle reserve can be protected during anticancer therapies by preventing apoptosis. While these studies are certainly promising, the quality of the rescued follicles has not yet been adequately investigated. If future experimental evidence bears out the hypothesis that apoptosis is induced for good reason (i.e., the elimination of poor-quality oocytes), then alternative routes of primordial follicle preservation are preferable. In this regard, therapies that simultaneously prevent apoptosis while encouraging repair would be an advantage. On the other hand, it would seem that preventing damage rather than preventing death is an important avenue of future research.

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Regulation of Quiescence and Activation of Oocyte Growth in Primordial Follicles

4

Deepak Adhikari and Kui Liu

Abstract

Once formed, the pool of dormant primordial follicles serves as the source of developing follicles and fertilizable ova for the duration of a female's reproductive life. Depending upon the species, primordial follicles can remain quiescent for months, years, or even decades, and the highly regulated process of primordial follicle activation ensures the availability of growing follicles throughout the reproductive period. We have recently begun to elucidate the molecular mechanisms underlying the maintenance of follicular quiescence and the activation of primordial follicles, mainly through the use of genetically modified mouse models. Both overactivation as well as the failure of activation of primordial follicles can lead to pathological conditions such as premature ovarian failure (POF) in the experimental models. A thorough understanding of the underlying mechanisms that regulate quiescence and activation of oocyte growth in primordial follicles will have important biological and clinical implications.

Keywords

Activation • Quiescence • Primordial follicle

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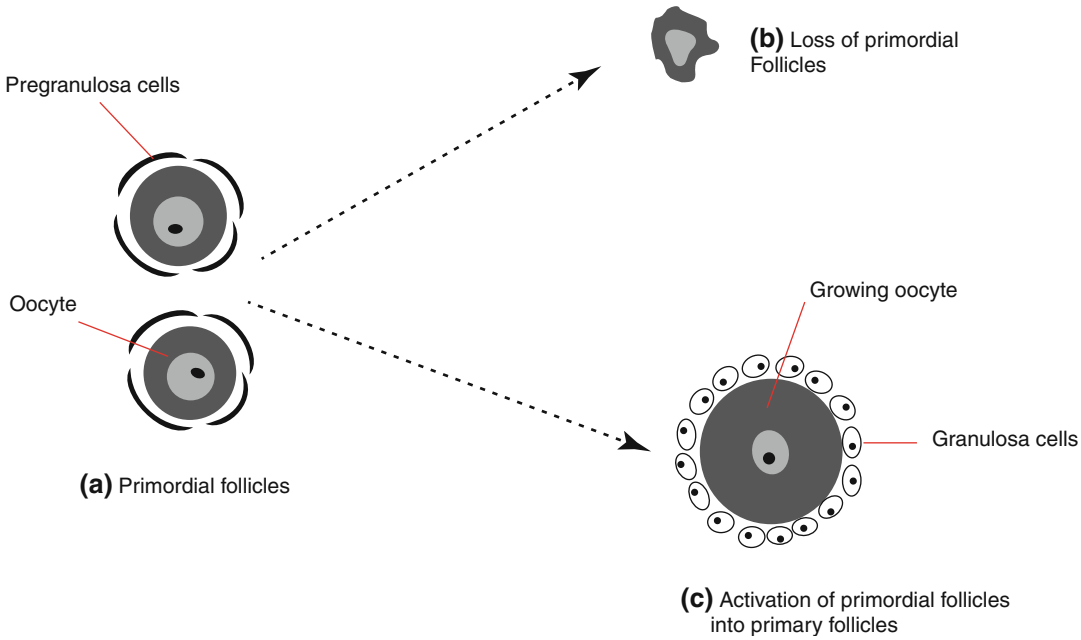

Fig. 4.1 Schematic illustration of the possible fates of primordial follicles. In the mammalian ovary, the pool of dormant primordial follicles serves as the source of developing follicles and fertilizable ova for the entire duration of reproductive life. As shown in (**a**), the immature oocyte in a dormant primordial follicle is surrounded by a few flattened pregranulosa cells. There are three different courses of development of primordial follicles: (**a**) they can remain dormant (not growing but surviving), (**b**) they can die out progressively and be cleared from the ovary directly from the dormant state, and (**c**) limited numbers of primordial follicles are continuously activated into the growing follicle pool. During follicular activation, the oocyte grows aggressively and undergoes an approximately 300-fold increase in volume by the end of its growth. Pregranulosa cells also differentiate and proliferate from a few flattened cells to multiple layers of cuboidal granulosa cells. The reproductive lifespan and timing of menopause of a woman are decided by the size and persistence of her primordial follicle pool

Introduction

Primordial follicles are the first small follicles to appear in the mammalian ovary. A primordial follicle consists of an immature oocyte arrested at the diplotene stage of meiosis I, which is surrounded by several flattened somatic cells, termed pregranulosa cells [1, 2]. There is a fixed number of primordial follicles in the ovaries during the early life of a mammal. Once formed, the pool of primordial follicles serves as a source of developing follicles and oocytes [3, 4]. Although an alternative view, promoted in recent studies, proposes that new oocytes are formed during adult life [5, 6], it is generally accepted that females are born with a finite number of oocytes that declines with age [3, 4].

To produce a mature oocyte, a primordial follicle emerges from its pool to become a primary follicle, and this transition is known as primordial follicle activation. It is characterized by dramatic growth of the oocyte itself, accompanied by proliferation and differentiation of the surrounding pregranulosa cells [7] (Fig. 4.1). The activated follicle then develops through a secondary stage before acquiring an antral cavity. The numbers of primordial follicles that start to develop and the numbers of eggs that are finally ovulated vary depending on the species of mammal [7–11]. However, not all of these endowed primordial follicles give rise to a mature fertilizable egg; the total number of eggs ovulated throughout the reproductive period is far less than the total number of primordial follicles initially endowed. For example, a woman ovulates less than 500 eggs in total throughout her reproductive life, but a young girl has about 300,000–400,000 oocytes per ovary at birth [4, 12, 13]. So, roughly speaking, only 1 in 1,600 primordial follicles undergoes ovulation. Most of the others die out directly without starting to grow [14]. As a result of continuous activation and death, the pool of primordial follicles

gradually dwindles with age. However, it is interesting to note that among the follicles at various stages of development, primordial follicles are the most predominant type of follicle at any age of an individual. Since follicle activation is essentially an irreversible process and no new oocytes are formed after the initial endowment, these primordial follicles have been surviving without activation. Such persistent primordial follicles are called quiescent follicles, and they can remain so for months, years, or even decades, depending on the species [7, 15]. In this way, each primordial follicle can have three possible fates: (1) to remain quiescent; (2) to become activated, to mature, and to undergo ovulation; (3) or to undergo atresia—either directly from quiescence or after activation [16, 17] (Fig. 4.1).

To maintain the normal length of female reproductive life, the majority of primordial follicles must be kept in a quiescent state for later use. When the available pool of primordial follicles has become depleted, ovulation ceases and women enter menopause [3, 4, 15, 18]. During quiescence, the appearance and size of primordial follicles does not change significantly with advancing age; a primordial follicle in a young girl looks similar to one in a woman almost approaching menopause [19]. Despite the prime importance of maintaining quiescence of primordial follicles for fertility, the underlying mechanisms are poorly understood. More than 15 years ago, it was noticed during in vitro culture of medulla-free but primordial follicle-rich ovarian cortical tissues from cows and baboons that most follicles became activated by day 2 of culture [20–22]. However, when intact neonatal mouse ovaries that also contained only primordial follicles were organ-cultured for 8 days, only a few follicles were activated [23]. This in vitro development was similar to the follicular development seen in vivo in 8-day-old mice [23]. These disparities in the patterns of primordial follicle activation between intact mouse ovary and cortical pieces of bovine and baboon ovaries led to the hypothesis that the primordial follicles may be subject to an inhibition of growth initiation, possibly secreted from the medullary region of the ovary [24].

Our understanding of the molecular mechanisms behind the activation of primordial follicles from their quiescence has been equally intriguing. Mouse models have shown that failure of primordial follicle activation leads to unavailability of fertilizable eggs, whereas overactivation leads to pathological conditions due to premature exhaustion of the ovarian reserve [reviewed in: 25]. Very little is currently known about the regulators of primordial follicle activation [17]. In the past, major efforts have been made to understand how the gonadotropins regulate follicular development. However, gonadotropins do not act directly on primordial follicles because functional gonadotropin receptors are not developed in them [7]. So, activation of primordial follicles remains unaffected in mice lacking the β (beta)-subunit of follicle-stimulating hormone (FSH) [26] or lacking the expression of FSH receptor [27, 28].

For obvious reasons, studies using human ovarian tissues are extremely limited. As one of the major approaches to identification of the activators of primordial follicles, neonatal rodent ovaries [reviewed in: 29] and cortical pieces of bovine ovaries [reviewed in: 30] have been cultured in vitro either in the presence or absence of a putative regulator of primordial follicle activation. In such experimental settings, growth of an almost uniform population of primordial follicles present in newborn mouse ovary or bovine cortical pieces can be followed after a certain period of culture in defined culture medium. Another approach has been the identification of differentially expressed genes (in primordial or primary follicles) that may have important roles in maintenance of the quiescence of primordial follicles and/or in their activation [31–35]. These studies have found significant differences in the expression patterns of various genes between primordial follicles and primary follicles. However, the functional roles of these candidate genes during the transition from primordial to primary follicle are yet to be elucidated. In recent years, significant progress has been made toward our understanding of molecules and signaling pathways involved in regulation of the quiescence of primordial follicles and in their activation, mostly by using genetically modified mouse models. The following parts of this chapter (1) provide an up-to-date account of such molecules and signaling pathways, (2) provide evidence of the pathological consequences of deregulation of the signaling pathways implicated, and finally (3) raise some unresolved issues that might serve as guidelines for future research.

Inhibitors of Primordial Follicle Activation

Anti-Müllerian Hormone (AMH)

AMH (also called Müllerian inhibitory substance, MIS) is a strong candidate as an inhibitor of activation of primordial follicles because the ovaries of *Amh*$^{-/-}$ mice show significant depletion of the pool of primordial follicles due to loss of quiescence [36]. In vitro, about 40–50 % more primordial follicles remain quiescent when neonatal mouse ovaries are cultured in the presence of AMH than in its absence [37, 38]. Similarly, more follicles remain quiescent when pieces of human ovarian cortex [39] or neonatal rat ovaries [40] are cultured in the presence of recombinant rat AMH. Alternatively, when pieces of ovarian cortex from bovine or baboon fetuses [24, 41] or newborn mouse ovaries [42] are grafted beneath the developing chorioallantoic membrane (CAM) of chick embryos (*in ovo*), primordial follicle quiescence is maintained. However, when ovaries from AMH receptor-null mice are grafted beneath the CAM [42], quiescence of primordial follicles is no longer maintained. These data show that AMH inhibits the activation of primordial follicles and thus maintains their quiescence.

Phosphatase and Tensin Homolog Deleted on Chromosome Ten (PTEN)

When the gene for the tumor suppressor PTEN, a negative regulator of the phosphatidylinositol 3 kinases (PI3Ks) [43, 44], is deleted specifically from oocytes of primordial follicles, premature activation of the entire pool of primordial follicles occurs [45, 46]. It is worth noting, however, that *Pten* in oocytes has stage-specific roles in controlling follicular development. When *Pten* is deleted from the oocytes of primary and further developed follicles, the female mice show normal folliculogenesis and fertility, indicating that PTEN is specifically required for the maintenance of primordial follicle quiescence but that it is dispensable for the further stages of follicular development [47].

Foxo3a

FOXO3a is a member of the FOXO family of forkhead transcription factors [48] and a substrate of Akt, which acts downstream of PI3K/PTEN signaling. In *Foxo3a*$^{-/-}$ mice, primordial follicles undergo spontaneous global activation so that the primordial follicle reserve is totally depleted by 2 weeks after birth [49]. This shows that Foxo3a maintains the quiescence of primordial follicles. It is noteworthy that in mice, Foxo3a is mainly expressed in the nuclei of oocytes of primordial follicles and early primary follicles, but its expression is dramatically downregulated in oocytes of larger primary follicles and further developed follicles [50]. These observations correlate well with the functional roles of Foxo3a in the maintenance of quiescence of primordial follicles. However, the downstream targets of this transcription factor in oocytes have not yet been described.

Tuberous Sclerosis Complex (TSC)

Heterodimeric complex of tuberous sclerosis complex 1 (TSC1 or hamartin) and TSC2 (or tuberin) regulate mammalian target of rapamycin complex 1 (mTORC1) activity [51, 52] (Fig. 4.2). TSC1 stabilizes TSC2 and protects it from ubiquitination and degradation [55]. Lack of either *Tsc1* [56] or *Tsc2* [57] from oocytes of primordial follicles causes global activation of all primordial follicles around the time of puberty. Mutant females lose their ovarian reserve during early adulthood. Thus, these results provide functional evidence that TSC in the oocyte maintains the quiescence of primordial follicles [58].

Cyclin-Dependent Kinase (Cdk) Inhibitor p27Kip1

$p27^{Kip1}$ (p27) is a member of the Cip/Kip family of Cdk inhibitors and is a negative regulator of the mammalian cell cycle and cell growth [59, 60]. In *p27*$^{-/-}$ mice, the primordial follicle pool is prematurely activated, and the overactivated follicular pool is largely depleted, leading to premature ovar-

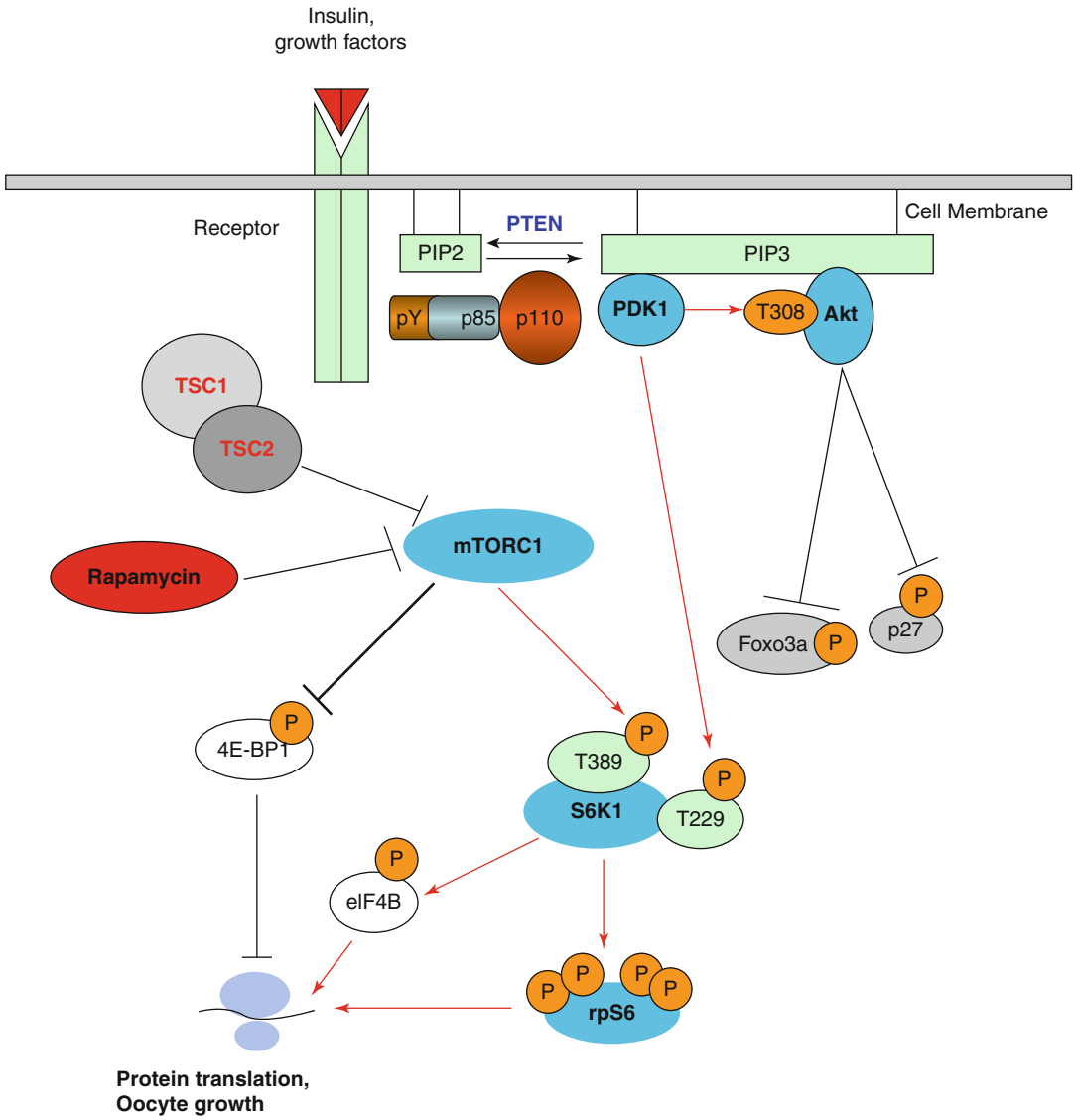

Fig. 4.2 Pathway illustrating PDK1-mediated activation of S6K1-rpS6 by PI3K and mTORC1. Upon binding of ligand, in most cases, the RPTK molecules are dimerized and autophosphorylated and present one or several phosphorylated tyrosine residues that are capable of binding to the SH2 domain of the regulatory subunit of PI3K (p85). The catalytic subunit of PI3K (p110) phosphorylates the 3′-OH group of the inositol ring of inositol phospholipids. The recruitment of PI3K from the cytoplasm to the inner membrane area of the cell leads to the production of PIP_3 from PIP_2. As a negative regulator of PI3K, the phosphatase PTEN converts PIP_3 to PIP_2. Kinases containing PH domains (such as PDK1 and Akt) are recruited through binding of their PH domains to PIP_3, and Akt is subsequently phosphorylated by PDK1. The activated Akt is a serine/threonine kinase with many substrates. Akt substrates Foxo3a and p27 are shown in this figure. PTEN in oocytes suppresses follicular activation through negative regulation of PI3K signaling and of the function of PDK1, which leads to subsequent inhibition of phosphorylation of S6K1 at T229 by PDK1 [53]. On the other hand, Tsc1 in oocytes suppresses follicular activation by negative regulation of mTORC1 signaling, leading to suppressed phosphorylation of S6K1 at T389. Thus, both PTEN and Tsc suppress the phosphorylation/activation of rpS6, but by regulating the phosphorylation of distinct threonine residues in S6K1. PDK1 is required to mediate overactivation of S6K1-rpS6 caused by the loss of Tsc1 or Pten. It is also shown in the figure that mTORC1 integrates cellular energy levels, growth factors, and other signals to regulate protein translation through phosphorylation of S6K1 and 4E-BP1. S6K1 phosphorylates and activates rpS6, which enhances protein translation. Rapamycin inhibits the activity of mTORC1. Note that this illustration is a simplified version of the PI3K and mTORC1 pathways [for details see: 43, 44, 54]

ian failure (POF) [61]. p27 is strongly expressed in the pregranulosa cells and oocyte nuclei of primordial follicles, but its expression is gradually reduced as the follicles develop [61]. These results show that p27 is required for the maintenance of quiescence of primordial follicles.

Forkhead Box L2 (FOXL2)

In mice, Foxl2 is mainly expressed in somatic cells of the fetal mouse ovary, but it is absent from germ cells [62–65]. In *Foxl2*$^{-/-}$ mice, the pregranulosa cells do not complete their squamous to cuboidal transition, a characteristic of primordial follicle activation. However, oocytes of almost all of these follicles start to grow on expression of growth differentiation factor 9 (GDF-9) [63, 64], which normally starts to be expressed from the primary follicle stage [66, 67]. These findings show that Foxl2 in pregranulosa cells is required for the maintenance of quiescence of oocytes in primordial follicles. In its absence, the whole pool of primordial oocytes becomes prematurely activated without synchronizing with differentiation and division of the surrounding pregranulosa cells.

Stromal-Derived Factor-1 (SDF-1)

When neonatal mouse ovaries are cultured with recombinant stromal-derived factor-1 (SDF-1), a significantly higher proportion of primordial follicles is inhibited from activation compared to culture without it. However, an inhibitor AMD3100 counteracts this inhibitory effect of SDF-1 [68]. SDF-1 has been shown to act through its chemokine (C-X-C motif) receptor 4 (CXCR4) in maintaining the size and longevity of the primordial follicle pool [68].

Steroid Hormones

Very little is currently understood about the possible functions of steroid hormones in the maintenance of primordial follicle quiescence. However, in aromatase knockout ($Ar^{-/-}$) mice, the numbers of primordial follicles are less, and the diameters of oocytes in primordial follicles are increased. Since aromatase is a key enzyme in the estrogen biosynthesis pathway, treatment of $Ar^{-/-}$ mice with estradiol leads to a reduction in oocyte diameter [69]. Similarly, estradiol and progesterone inhibit follicular activation in rodent ovaries [69, 70] and bovine ovaries [71]. Together, these results show the inhibitory effects of these hormones on activation of primordial follicles.

It was proposed more than a decade ago that large follicles in the ovary may produce a substance that inhibits the activation of smaller follicles [21]. AMH and estrogen, produced by large follicles, have now joined the list of such possible substances. More importantly, several recent studies using novel mouse models have broadened our understanding of the mechanisms behind maintenance of primordial follicle quiescence by showing that molecules in pregranulosa cells and in oocytes of primordial follicles also have decisive roles in the maintenance of their own quiescence. Accordingly, primordial follicles can no longer maintain their quiescence if any of these inhibitors loses its function.

Promoters of Primordial Follicle Activation

Kit/KL

The receptor protein tyrosine kinase Kit, expressed on the surface of oocytes of mice, rats, and humans and its ligand, Kit ligand (KL, also called stem cell factor, SCF), produced by the surrounding granulosa cells, are known to facilitate the development of primordial follicles in mouse ovaries [9, 11, 72–80] and rat ovaries [79]. Addition of KL to the culture medium can also enhance primordial follicle activation in pieces of bovine cortex [30]. KL has been shown to activate the PI3K signaling pathway in cultured oocytes from mice and rats [81, 82], indicating that the PI3K pathway in oocytes may be important for their activation and development [83, 84].

PI3K Signaling

The functional role of enhanced PI3K signaling in the activation of oocytes was confirmed after it was

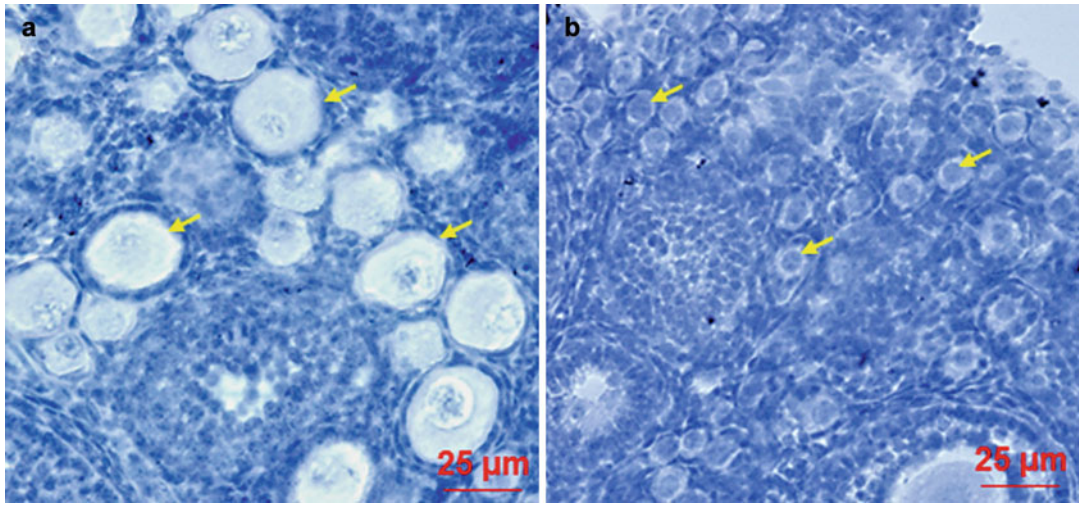

Fig. 4.3 Rapamycin reverses the overactivation of primordial follicles caused by oocyte-specific loss of *Tsc1*. The overactivation of primordial follicles in mice caused by oocyte-specific deletion of *Tsc1* can be reversed by treatment with rapamycin. Rapamycin (5 mg per kg body weight) was injected once a day into mutant female mice from PD4 to PD22, and the ovaries were collected for morphological analysis at PD23. As a control, mutant mice were treated similarly but with vehicle only. In the ovaries of mutant mice that were treated with vehicle only, all the primordial follicles were activated by PD23 (**a**, *arrows*), whereas rapamycin treatment largely prevented the overactivation of follicles and clusters of primordial follicles were present in the mutant ovaries (**b**, *arrows*)

found that all the primordial follicles start to develop simultaneously in mice when *Pten* is deleted specifically from oocytes [45]. In *Pten*-null oocytes, PI3K becomes constitutively activated, which can be efficiently abolished by its specific inhibitor LY294002 [45]. A later study has also confirmed that PI3K signaling in oocytes has a role in activating primordial follicles [46]. Moreover, when ovaries from neonatal mice or cortical pieces from human ovary are cultured in vitro with a PTEN inhibitor and a PI3K-activating peptide, activation of primordial follicles is significantly enhanced [85].

mTORC1 Signaling

When either *Tsc1* [56] or *Tsc2* [57] is deleted specifically from oocytes of primordial follicles, it leads to a global activation of all primordial follicles around the time of puberty. It has been found that the driving force underlying the overactivation of primordial follicles in ovaries of these mutant mice is elevated mTORC1 activity, which promotes protein translation and ribosomal biogenesis in oocytes [56, 57] (Fig. 4.2). However, when the mutant mice lacking Tsc1 from their oocytes are injected with the mTORC1-specific inhibitor rapamycin, the excessive activation of primordial follicles is halted (Fig. 4.3). This confirms that mTORC1 signaling enhances primordial follicle activation.

For follicular activation, it has been observed that PI3K signaling and mTORC1 signaling act synergistically. This notion is supported by the finding that double deletion of *Tsc1* and *Pten* leads to synergistically enhanced oocyte growth relative to singly mutated mice [56]. (See later sections for discussion of this.)

Insulin

Although in vitro culture of pieces of bovine cortex leads to spontaneous activation of primordial follicles [21], this activation is halted if the culture medium lacks insulin [30]. Insulin has also been found to promote the activation of primordial follicles in cultured fetal hamster ovary [86] and neonatal rat ovaries [87]. Thus, insulin appears to be essential for activation of primordial follicles, at least in vitro. Insulin might act through its receptor on the oocytes of primordial follicles [87], but removal of insulin receptor from oocytes does not affect primordial follicle activation in

mice [88]. Since primordial follicles are present in the nonvascular cortex of the ovary, it is possible that insulin might not reach there to produce its effects in vivo.

Other Growth Factors

In order to determine the growth factors that may have positive roles in primordial follicle activation, ovarian tissues rich in primordial follicles have been cultured with or without a range of such putative factors in the culture medium. Accordingly, basic fibroblast growth factor (bFGF) [89, 90], keratinocyte growth factor (KGF) [91], platelet-derived growth factor (PDGF) [92], glial-derived neurotrophic factor (GDNF) [93], leukemia inhibitory factor (LIF) [94], bone morphogenic protein 7 (BMP-7) [95, 96], BMP-4 [97], and GDF-9 [98–101] have been found to enhance the activation of primordial follicles in vitro. Similarly, Smad3 [102] and growth hormone [103] have also been found to enhance follicular activation in mice.

Oocyte-Specific Transcription Factors

Results obtained from genetically modified mouse models have revealed the requirement for certain oocyte-specific transcription factors for the transition from primordial follicle to primary follicle. These include: spermatogenesis- and oogenesis-specific basic helix-loop-helix 1 (Sohlh1), LIM homeobox protein 8 (Lhx8) [104], and Sohlh2 [105, 106]. Although primordial follicles are formed in mutant mice lacking the above factors, their transition to primary follicles does not happen. Most of the follicles appear empty due to oocyte degeneration.

Pathological Conditions Caused by Abnormalities in Follicular Activation

Due To Overactivation of Follicles

Once a primordial follicle is activated, it either ovulates or dies by atresia [107]. Two main features of primordial follicle activation are worth noting. First, once a follicle is activated, there is no pause in growth; if it does not undergo atresia, it grows continuously toward ovulation. Second, primordial follicle activation is essentially an irreversible process. This means that no other types of activated follicles can survive in the ovary for as long a period of time as quiescent primordial follicles do. Thus, the only mechanism for the preservation of fertility over an extended period of time is by maintaining the pool of primordial follicles and regulating their activation. This model has been verified by results obtained from several novel mouse models in which all the primordial follicles activate prematurely. Such a general activation of primordial follicles leads to premature exhaustion of the ovarian reserve [reviewed in: 17].

Due To Lack of Follicular Activation

A fraction of primordial follicles that are endowed during the early life of females remain quiescent but survive until menopause, which is essential for maintenance of the usual time span of female fertility [7]. Data accumulated using a number of mouse models indicate that a range of inhibitory molecules and signaling pathways keep the primordial follicle quiescent, most likely by maintaining the activating signal in a suppressed state [reviewed in: 17]. For instance, loss of function of PTEN, a negative regulator of the PI3K pathway, leads to overactivation of this pathway. Due to the heightened PI3K activity in oocytes, primordial follicles lose their quiescence [45]. In such *Pten*-deleted oocytes, Akt-p70 S6 kinase 1 (S6K1)-ribosomal protein (rp)S6 signaling is highly activated, which is mediated through 3-phosphoinositide-dependent protein kinase-1 (PDK1) [45, 53]. However, it is still imperative that a basal level of PI3K activity be maintained for the survival of quiescent primordial follicles [108]. Due to the inability to maintain basal PI3K activation in *Pdk1*-null oocytes, the majority of primordial follicles die directly from the dormant state around the onset of sexual maturity. This leads to POF during early adulthood [53]. In such *Pdk1*-null oocytes, the Akt-S6K1-rpS6 pathway cannot be activated. In addition, similar accelerated loss of primordial follicles occurs in mice when *rpS6* is deleted from the oocyte [53].

Thus, it is clear that premature exhaustion of primordial follicles can occur either from their failure to survive and activate, or due to their overactivation—both of which end up in POF and infertility. In addition to the infertility, early ovarian aging leads to long-term estrogen deprivation, which has serious implications for female health in general, for bone density, and for integrity of the cardiovascular and neurological systems [109].

The Balance Kept by Signaling Cascades

Overactivation of primordial follicles—due to the deletion of either *Pten* or *Tsc1* from their oocytes—is mainly caused by an overactivated S6K1-rpS6 cascade that enhances protein translation and ribosomal biogenesis in oocytes [45, 56]. In contrast, the S6K1-rpS6 cascade remains inactive in *Pdk1*-null oocytes, and such primordial follicles cannot survive, which leads to their death directly from dormancy [53]. Although Pten and Tsc both control the activation of primordial follicles through suppression of the S6K1-rpS6 cascade, Pten suppresses the phosphorylation of S6K1 at its T229 residue whereas Tsc1 suppresses S6K1 phosphorylation at T389 [56] (Fig. 4.2). Accordingly, simultaneous removal of *Tsc1* and *Pten* leads to more synergistically enhanced primordial follicle activation than deletion of only *Pten* or only *Tsc1* from oocytes [56].

Interestingly, overactivation of primordial follicles due either to deletion of *Pten* or *Tsc1* from oocytes is largely prevented by simultaneous deletion of *Pdk1*. In other words, death of primordial follicles due to deletion of *Pdk1* from oocytes is largely prevented if *Pten* or *Tsc1* is deleted at the same time [53, 56]. PI3K-mediated signaling, which mainly converges at PDK1, is important for the phosphorylation and activation of both Akt (at T308) and S6K1 (at T229) [110] (Fig. 4.2). Signaling studies with oocytes lacking Pdk1, Tsc1, or Pten—or with double mutants lacking either Pdk1 and Pten or Pdk1 and Tsc1—have revealed that phosphorylation and activation of S6K1-rpS6 in oocytes is one of the key downstream events that plays a decisive role in the various developmental cues of primordial follicles. Thus, a fine balance between the various signaling pathways is essential for regulation of the survival, quiescence, and activation of primordial follicles in mice. A net resultant signaling force generated by interaction between multiplex signaling pathways directs the developmental fate of a primordial follicle.

Specific Order Versus Random Selection of Primordial Follicles

Only a certain number of primordial follicles become periodically activated before puberty and throughout the reproductive period. Whether there exist subclasses of primordial follicles within the pool that have predetermined developmental fates, or whether they take various developmental cues randomly, remains unknown. According to the so-called production-line hypothesis [111], it has been proposed that the order in which oocytes become activated, grow, and ovulate during a woman's reproductive life may be determined by the order in which they were produced during embryonic life. In this regard, very recently, it has been shown in mice that primordial follicles of the medullary and cortical regions can be classified into two separate populations, depending on the origin of their pregranulosa cells [112]. Accordingly, primordial follicles in the medullary region of the ovary that are activated immediately after birth and reach the antral stage before puberty have had their pregranulosa cells specified during a very early stage of embryonic development. On the other hand, primordial follicles in the cortex that are periodically activated in adult life have had their pregranulosa cells specified only during the final stages of embryonic development, which continues up to the end of the postnatal follicle assembly period [112].

As discussed earlier and as shown in Fig. 4.2, differential interactions between the PI3K and mTORC1 pathways across the oocytes of various primordial follicles must end up in providing different levels of S6K1-rpS6 activation. Depending on the net status of S6K1-rpS6 activation, oocytes might follow any one of the developmental fates shown in Fig. 4.1. As exemplified by S6K1-rpS6 here, there may exist other as yet unidentified signaling molecules that act as similar regulatory switch in oocytes. With the mouse models

Table 4.1 Molecules/pathways involved in the various developmental cues of primordial follicles

Function	Molecule/pathway (source)	Experimental approach and references
Inhibition of activation	AMH (granulosa cells of large follicles)	In vivo [36], *in ovo* [24, 41, 42], in vitro [37–40]
	Pten (oocyte)	In vivo [45, 46]
	Foxo3a (oocyte)	In vivo [49, 113]
	Tsc1 (oocyte)	In vivo [56]
	Tsc2 (oocyte)	In vivo [57]
	p27 (oocyte, pregranulosa cells)	In vivo [61]
	Foxl2 (pregranulosa cells)	In vivo [63, 64]
	SDF-1	In vitro [68]
	Estrogen and progesterone	In vivo [69], in vitro [69–71]
Promotion of activation	Kit/KL (oocyte/granulosa)	In vivo [75, 76, 80], in vitro [30, 78, 90]
	mTORC1 (oocyte)	In vivo [56, 57]
	PI3K (oocyte)	In vivo [45, 46, 53], in vitro [85]
	Insulin	In vitro [30, 86, 87]
	Growth factors: bFGF, KGF, PDGF, GDNF, LIF, BMP-7, BMP-4 and GDF-9	In vitro [89–97] and [98–101], respectively
	Oocyte-specific transcription factors: Lhx8, Sohlh1/2	In vivo [104–106]
Survival	Pdk1	In vivo [53]
	rpS6	In vivo [53]

available, it has only been possible to eliminate, decrease, or enhance the activation signals to the same extent in all the primordial follicles. For technical reasons, it has not been possible to examine an individual follicle for possible differential regulation by various signaling pathways. Thus, it remains to be seen how the various pathways are regulated within individual follicles under physiological conditions. Despite the possibility of various subsets of primordial follicles with predetermined developmental fates already existing, the general assumption is that the selection of a particular set of primordial follicles for activation happens randomly.

Conclusion

Recently, the mechanisms that control the survival, quiescence, and activation of primordial follicles have become clearer due to results obtained mainly from studies on genetically modified mice and also from in vitro ovary culture and other approaches (Table 4.1). Results from genetically modified mouse models have shown that the synergistic and coordinated action of several inhibitory molecules keeps the primordial follicle quiescent. Lack of function of any of the members of this inhibitory machinery leads to premature activation of primordial follicles. Research results obtained over several years have led to the general view that primordial follicles are mainly regulated by being held constitutively in a quiescent state. Positive activators might be needed to trigger primordial follicle activation by suppressing the activities of one or more of these inhibitors. Close communication between oocytes and the surrounding somatic cells is crucial for the activation and growth of follicles. Very little is currently known about the differential regulation of

various signaling pathways within individual primordial follicles, which dictates whether they should become activated, remain quiescent, or die.

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The Structural Basis for Coordinating Oogenesis and Folliculogenesis

5

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Abstract

Oogenesis is a complex process that leads to the ovulation of developmentally competent oocytes. The process whereby the oocyte acquires meiotic competence involves the transcription and translation of key regulatory enzymes and signaling molecules, whose balance between production and degradation determinates arrest or oocyte meiotic progression. In mammals, ovarian follicular development and atresia are regulated by gonadotropins and intraovarian regulators that interact to promote primordial follicle activation, proliferation, survival and cellular differentiation. Changes in interactions between oocytes and somatic cells are associated with distinct modulations of gene expression at various stages of follicle development. In fact mural and cumulus cells, together with the oocyte, form a gap junction-mediated syncytium, allowing a paracrine bidirectional communication able to coordinate oocyte growth and maturation with differentiation of surrounding granulosa cells. This chapter will illustrate the role and the regulation of transzonal projections (TZPs) as a structural basis of the communication between granulosa cells and oocytes. Particular emphasis will be given to the involvement of TZPs in aspects that are relevant to the field of human assisted reproduction.

Keywords

Oogenesis • Folliculogenesis • Transzonal Projections • Paracrin Regulation

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Section 1: Introduction

Oogenesis is the complex process that leads to the ovulation of developmentally proficient oocytes. In mammals, much attention has been paid to the gene networks that participate in the process of primordial follicle formation, survival, and recently factors regulating the transition from follicle activation into the subsequent phases of folliculogenesis [1]. Uncovering these genetic interactions has been aided by the introduction of technologies in mice where the ability to eradicate cell-specific or systemic function of individual genes and has led to the identification of factors in either germ cell or somatic cell lineages of the ovary. Despite this new knowledge base, there remain many aspects of oogenesis and folliculogenesis, often viewed as independent processes, which are enigmatic especially when viewed in the context of human reproductive medicine. Among these are the substantial wastage of follicles that occurs during ovarian development in nearly all mammals and the subsequent loss of follicles that attends the selection of dominant follicles during periods of normal reproductive cyclicity [2]. In fact, cohort selection of follicles and determination of which follicle in the cohort will proceed to ovulation stands singularly as the most pressing challenge yet to be solved in the physiology of the mammalian ovary. Dominant follicle selection is tightly linked to reproductive success since both the emergence of a functional corpus luteum and the liberation of developmentally proficient oocytes, are the natural and essential by-products of successful folliculogenesis.

Coordinating oogenesis and folliculogenesis has long been appreciated to depend on the interactions between oocytes and somatic cells that comprise the ovarian follicle [3]. Changes in transcellular interactions are associated with distinct alterations in gene expression at the time of follicle activation and the transition from primordial to growing follicles [4, 5]. In highlighting the importance of cellular interactions at the various stages of follicle development that coincide with milestone events occurring within the oocyte, two significant paradigms have been proposed that have changed the way that we think about the fundamental objectives for achieving and sustaining ovarian function. The first of these, advanced primarily by the work of Eppig and colleagues, emphasizes the prominent impact that the oocyte has on the growth and differentiation of the follicle [6]. This oocentric perspective recognizes and substantiates the facts that oocytes process and secrete factors that directly influence the viability and proliferation of granulosa cells. Among the many oocyte factors that are now recognized to impose regulatory influences on the follicle are GDF9 and BMP15 whose function has been explained through the deployment of negative feedback loops that are active at discrete and successive stages of follicular development [7].

Because the basic framework for folliculogenesis and oogenesis is similar between rodents and humans [8], it is generally assumed that the mechanisms involving cell interactions at critical junctures of follicle development would bear similarity between various mammalian species and this prospect appears to be validated by studies emphasizing the role of paracrine interactions between the oocyte and granulosa cells (see below). What remains less well studied, however, are the detailed cell biological principles that govern cell adhesion, communication, and polarity at the interface between germ and somatic cells. This chapter will emphasize the role of transzonal projections (TZPs) as a dimension of the symbiotic relationship between granulosa cells and oocytes that is essential in achieving reproductive fitness and is likely the target of action in human-assisted reproduction technologies (ARTs) as practiced today in the treatment of infertility.

Section 2: Paracrine Regulation

As mentioned above, many factors are now recognized to meet the criteria of originating from the oocyte and influencing the behavior of surrounding granulosa cells [6, 7]. And the discovery of feedback loops in this form of cell communication appear to at least initially involve reciprocal relationships between GDF9 and the Kit ligand pathway that has been previously

appreciated to be involved in the process of follicle activation [3]. Kit ligand (KL) is a prominent growth-regulating factor in many cell types and has a central role during the earliest stages of follicle development [1, 9]. With expression of c-kit, the cognate receptor tyrosine kinase for KL, being confined to the oocyte cell surface, the downstream role of KL production in granulosa cells on oocyte growth was an immediate and likely course of action during primordial follicle activation. With the demonstration of follicle arrest at the primary stage in GDF9 knockout mice [7], it became apparent that GDF9 expression patterns at this stage could impart a positive regulatory loop such that oocyte-derived GDF9 would modulate the secretion of KL and hence stimulate oocyte growth through activation of c-kit [10, 11]. Many growth factors may also participate in such a regulatory loop, including keratinocyte growth factor [12], but there is little doubt that the use of oocyte-specific transgenic mice will continue to contribute to the dissection of the multiple paracrine pathways involved in the initial stages of follicle development [13]. Moreover, elements of these pathways have been documented in human materials [14], suggesting conservation of core signaling pathways and the documentation of posttranscriptional and post-translational modes of processing, especially with regard to the secreted ligands, will add to the much-needed resolution of mechanisms that underlie these seminal and essential events at the onset of folliculogenesis [15]. What remains less clear with respect to elucidating paracrine interactions between the oocyte and granulosa cell is the extent to which cell contact is involved in the processing and/or delivery of these factors. That TZPs may participate in this process is addressed in a subsequent section.

Section 3: Oocyte Growth and Maturation

The original work on GDF9 knockout mice revealed two compelling pieces of data that reinforced the notion of how this and other paracrine factors regulate oocyte growth and maturation during the course of oogenesis [7]. First, oocytes of animals deficient in GDF9 were abnormally large in size and failed to attain the normal constitution of organelles. Second, when assayed for their ability to initiate and resume meiosis in vitro, GDF9 null oocytes lacked the ability to proceed through in vitro maturation and exhibited both a lack of TZPs and an advanced state of chromatin condensation. Remarkably, ovaries from GDF9 null animals lacked any signs of apoptosis, and rather than demonstrating atretic loss of follicles, follicle remnants were transformed into steroidogenic structures resembling corpora lutea. Collectively, these findings raise important questions regarding the mechanisms underlying oocyte survival and death and the extent to which programmed cell death (apoptosis) plays a role especially in the earliest stages of follicle development [15–18]. This remains a much-discussed issue in ovarian physiology and has immediate bearing on the subject of ovarian aging and the loss of oocyte quality in older women seeking treatments for infertility [19]. Some promise for understanding the actions of growth factors on oogenesis derives from the use of in vitro systems for follicle culture where it is possible to correlate events in early folliculogenesis with oocyte maturation, fertilization, and embryo outcomes, but to date, these systems have been only applied widely with the murine models [20].

Section 4: Paracrine Signaling and TZPs

Most paracrine signaling systems involve physical approximation of a ligand-producing cell with an adjacent responding cell. This is no different at the interface between the oocyte and granulosa cells that envelope the oocyte and must traverse the zona pellucida in order to make direct physical contact with the oolemma. What distinguishes cell communication interactions between germ and somatic cell compartments in the ovarian follicle from most other mammalian systems involving heterocellular compartments is the fact that oocyte is encased within the zona pellucida during both the growth phase of oogenesis and

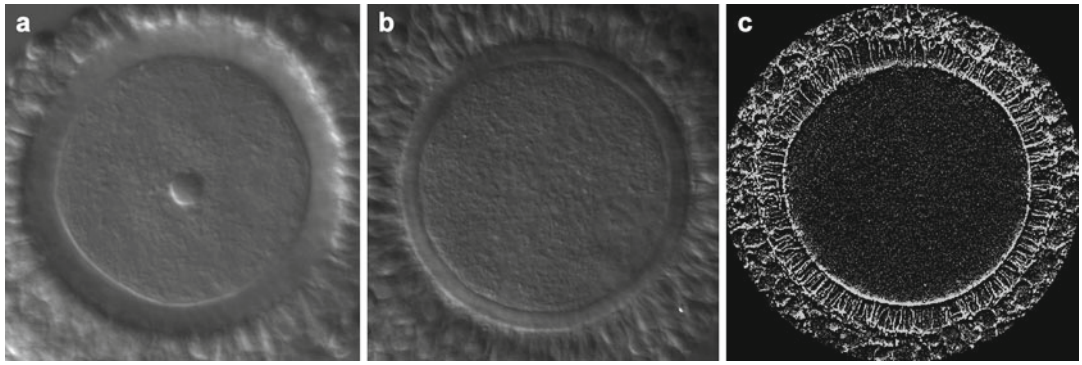

Fig. 5.1 Illustrations of transzonal projections (TZPs) under conventional bright field Nomarski optics (**a**, **b**) and after labeling with rhodamine phalloidin to accentuate details using confocal and image sharpening software. (**c**) All fixed samples represent GV stage oocytes

throughout and beyond the processes of oocyte maturation and fertilization. This confers upon the zona pellucida a unique problem that bears on both the means by which physical contact is established between two dissimilar cell types and the role, if any, that the zona may subserve as a storage site for the many factors that traverse this specialized extracellular matrix in a bidirectional manner. In some of our earlier work summarizing the structural complexity of TZPs, we confronted the issue of exchange for typically basic charged growth factors in the context of the highly acidic chemical properties that mammalian zonae exhibit due to the high content of terminal sialic residues on the zona glycoproteins [21]. Based primarily on the transmission electron microscopy literature, it had been apparent that TZPs are ubiquitous among mammalian oocytes and follicles and that variations in structure clearly exist as a function of the stage of follicle development and/or the mammalian species under study. We now demonstrate in this chapter, as a result of many recent studies, that TZPs are indeed dynamic structures that respond to the paracrine signaling pathways resident within the follicle but in addition are sensitive to gonadotropin interactions with receptors on the granulosa cells.

TZPs are formed from the surface of granulosa cells that is apposed to and anchored at the external surface of the zona pellucida [22]. As previous models had proposed, there are at least two distinct types of TZPs. One type is actin rich sending projections along a tangential course into the outer third of the zona pellucida and rarely do these terminate at the oocyte surface [23]. In addition, most species exhibit TZPs that are oriented perpendicular to the zona pellucida that typically form coiled projections terminating at the oolemma as distal dilatations of expanded adhesion zones indenting the oocyte cell surface. With the advent of confocal microscopy, a higher-resolution definition of TZP organization and density at the oolemma has been obtained.

As shown in Fig. 5.1, intact cumulus masses from the cow reveal little structure by conventional bright field microscopy and yet, when probed with the actin-labeling reagent rhodamine phalloidin, the high density of actin-rich TZPs can be readily appreciated. Moreover, using image enhancement technology that uncovers the density, asymmetry, and terminations of TZPs, it has become readily appreciated that much of the mass of the zona pellucida is occupied by these structures of somatic cell origin.

For example, in cumulus corona cells attached to the outer zona surface, dense accumulations of polymerized actin mediate firm attachments required to maintain polarity and physical interactions with the oocyte (Fig. 5.2). Using phalloidin as an actin probe also reveals details of the actin cytoskeleton in the oocyte cytoplasm and at the sites of TZP contact in the oolemma. This is demonstrated in rat oocytes and other species (Figs. 5.2 and 5.3),

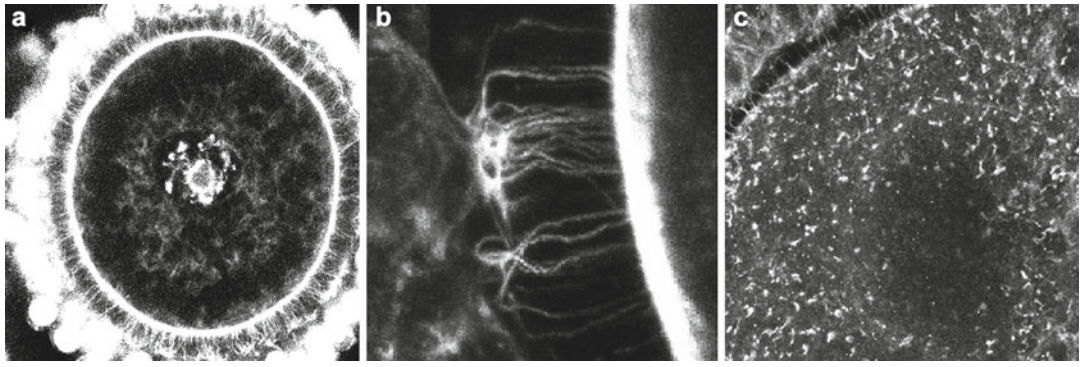

Fig. 5.2 Examples of TZPs from rat (**a**) and bovine (**b**, **c**). GV stage oocytes processed for confocal microscopy after fixation and labeling with rhodamine phalloidin to enhance detection of F-actin. Note in A that nuclear chromatin has been labeled with Hoechst 33258 and a system of actin filaments extends from the perinuclear region to the intensely stained oocyte cortex. (**b**) Illustrates TZPs emanating from two corona cells that penetrate the zona pellucida at right angles to the oocyte surface. Terminations of TZPs at the oolemma are characterized by the presence of numerous actin-filled plaques (**c**)

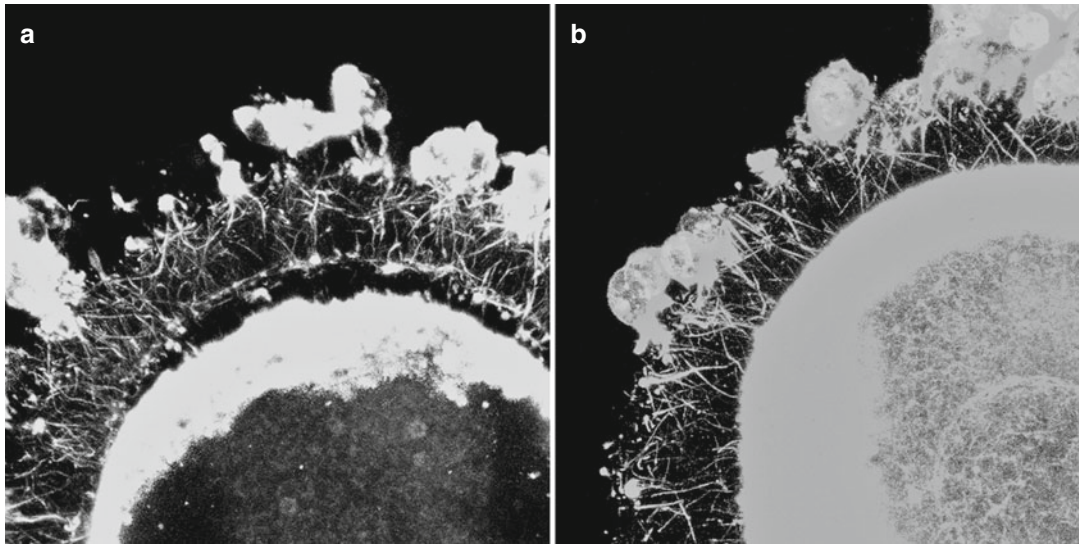

Fig. 5.3 Confocal microscopy images of intact human cumulus-oocyte complexes. In (**a**), a cumulus-oocyte complex labeled to detect actin is illustrated. It is important to note the presence of a dense network of TZPs from some corona cells that extends into the perivitelline space. In (**b**), a projection of Z stacks from another human GV stage oocyte fixed and labeled with rhodamine phalloidin is shown. The image was modified to enhance the system of actin filaments that extends through the zona pellucida up to oocyte's cortex. The images are derived from a collaborative study between Biogenesi and the laboratory of Prof. D. F. Albertini

where a perinuclear network of actin filaments extends to the oocyte cortex and assembles focal adhesion plaques at the termini of TZPs. Moreover, under appropriate conditions, three-dimensional reconstruction of image Z stack illustrates the full extent of TZP organization for individual corona/cumulus cells. In such cases, the elaboration of 7–9 TZPs from a single cell is confirmed, each taking a parallel course of penetrance through the structure of the zona pellucid before ending within the zona or approximating the oolemma.

These findings have now been extended to other mammalian species, and in the case of equines,

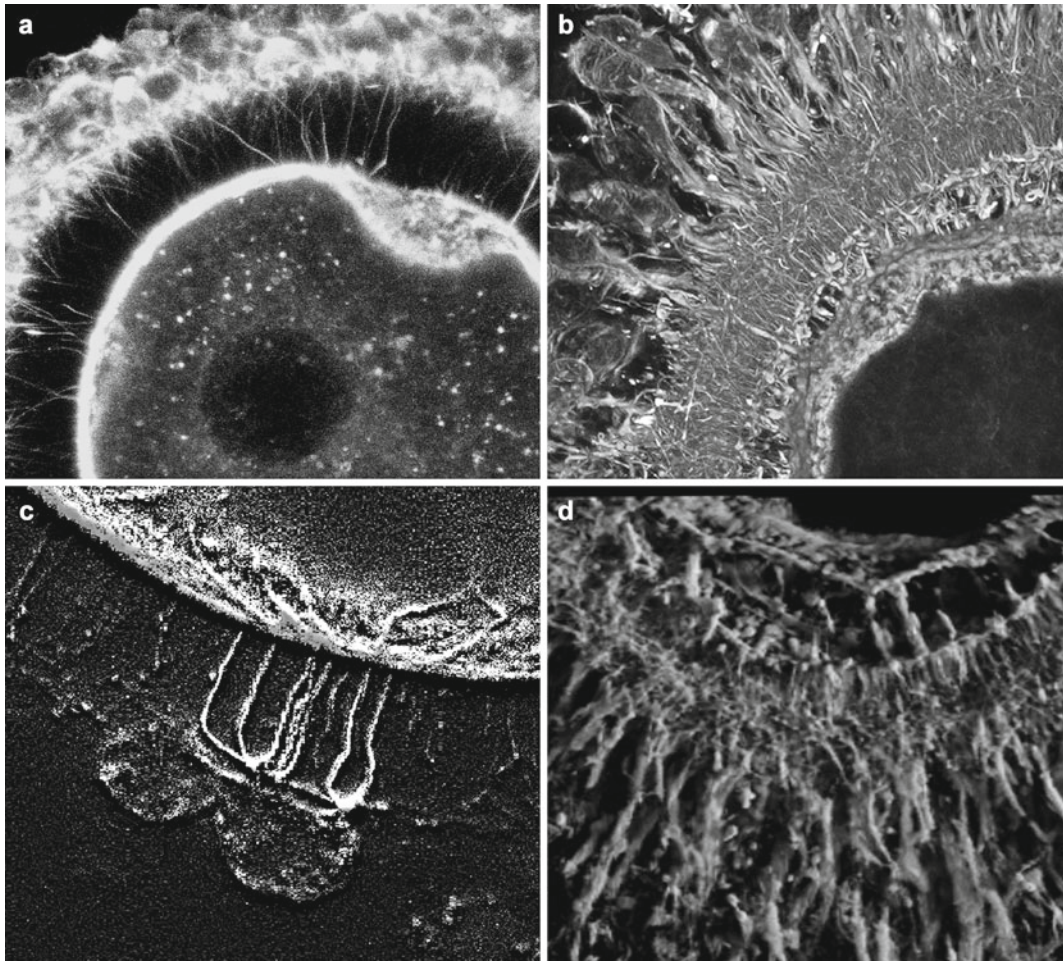

Fig. 5.4 Projections of Z stacks from bovine GV stage (**a**, **c**) and equine metaphase-2 (**b**, **d**) oocytes fixed and labeled with rhodamine phalloidin. Note oolemmal infolding at sites of TZP retraction (**a**) and dense network of TZPs from corona cells that are dilated in the perivitelline space (**b**). In (**c**), a computer-enhanced image details branching of TZPs from corona cell anchoring site at the outer edge of the zona pellucida; also at zones of attachment to the oolemma, subcortical actin filaments converge near the site of TZP terminations. (**d**) Illustrates a high-magnification view of the terminations of TZPs in the perivitelline space that are firmly anchored to the outer oolemma (*bottom* to *top*)

remarkable modifications of TZPs have been documented during the course of oocyte maturation (Fig. 5.4). By conventional confocal microscopy, it is apparent that horse oocytes have firmly anchored corona/cumulus cells along the entire extent of the zona and the internal dilatations of TZPs located at the oocyte surface occupy the thin perivitelline space typical of immature oocytes. Once oocyte maturation has proceeded to the metaphase-2 stage, the distal ends of TZPs become dilated and expanded, thereby increasing the surface area of contact at the oolemma, and the cell bodies of cumulus/corona cells are now displaced from the outer limits of the zona. Three-dimensional reconstructions of Z stack in the equine uncover several features of TZPs that had not been previously recognized. For example, each corona cell appears as a solitary entity and acquires a polarized character such that TZPs are branched on the oocyte side and a single cell extension from the opposite side extends into the expanded cumulus mass. In addition, within the

enlarged perivitelline space, the distal expansions of TZPs further bifurcate to form a dense network of cytoplasmic extensions that are absent in immature GV stage oocytes. These new findings raise important questions with regard to the effect of gonadotropin stimulation prior to and during ovulation and the impact of controlled ovarian hyperstimulation in the treatment of human infertility.

Previous studies using FSH beta knockout mice demonstrated that the elaboration and maintenance of TZPs was enhanced under conditions resulting from the genetic ablation of FSH and that upon stimulation of FSH null mice with PMSG, a rapid retraction and remodeling of TZPs took place [24, 25]. These studies also demonstrated that in the absence of functional FSH, the centrosome of corona cells migrated to the zona-apposed surface where prominent microtubule-based TZPs were observed. Coupled with the findings summarized above for the horse, it would appear that ovulation triggered by LH in this species has an equivalent effect in causing disengagement of cumulus cells and yet wholesale penetration and expansion of those TZPs that had previously established contact with the oolemma. Whether these changes will be reflective of direct responses to gonadotropins is unlikely given the fact that the signaling pathways activated in response to FSH or LH have now been shown to be far more complex than previously thought. There is, however, a growing interest in defining markers of oocyte quality based on the identification of gene products within the cumulus cells, and linking these approaches to structural rearrangements in TZPs may be a profitable direction to pursue. In fact, many laboratories have made progress defining the appropriate ligand composition for media used in in vitro maturation of oocytes, and these results will be interesting to compare at the level of TZP organization described above [26].

The most telling advances in paracrine signaling have emerged from a series of reports that now emphasize the importance of cell contact between oocyte and granulosa and the acknowledged contribution of gap junctions within the cumulus-oocyte complex. Several laboratories have combined to materially advance our understanding of oocyte-granulosa interplay with respect to the regulation of meiotic maturation and the integral nature of metabolic cooperation during the growth phase of oogenesis. Most compelling has been the recognition of post-LH-elicited events in the ovulating follicle with the identification of cGMP and EGF-like factors mediating the switch to meiotic reinitiation and progression [27–29]. While gap junctions had long been thought to control meiotic arrest via delivery of meiosis-arresting substances like cAMP to the oocyte, gap junctional closure has been uncovered as a key regulatory component of this pathway [27] and is detailed in the chapter by Mehlmann in this volume. Central to this new paradigm is the role of cGMP that appears to decrease in the oocyte and therefore releases the oocyte from meiotic arrest, as a consequence of EGF-mediated closure of connexin 43 gap junctions that may be manifest at both the level of communication between the oocyte and granulosa as well as the lateral integration of metabolism between the cumulus cells themselves [28, 29]. Yet another example of the importance of cell contact relationships mediated by TZPs is recent work on the control of cholesterol metabolism in oocytes by paracrine factors.

That the oocyte is “metabolically” challenged and subject to the whims and metabolism of the surrounding granulosa cells has been appreciated in various contexts, such as maintaining meiotic arrest. Only recently has the structural integration of oogenesis and folliculogenesis been viewed as a major determinant in establishing oocyte quality. This growing body of evidence strongly suggests that the physical interaction of oocyte and granulosa cells establishes a persistent symbiotic relationship that mediates many aspects of oocyte metabolism [30, 31]. Gene expression profiling in mouse oocytes has confirmed that many key enzymes in metabolic pathways like that used in the biosynthesis of cholesterol are either underrepresented or non-detectable in the oocyte transcriptome, whereas transcripts for this pathway are well represented in granulosa cell transcriptomes. The elegant studies on cholesterol synthesis in the mouse oocyte have now provided

evidence to support the notion that oocyte-derived BMPs convey to granulosa the stimulus to initiate cholesterol synthesis that will be needed to support the demands of sterol precursors in the growing oocyte and most likely in the embryo later [30]. Most interestingly, the actual transfer of cholesterol from granulosa to oocytes seems to require physical contact between these two discrete cell types suggesting further that the TZP is a likely conduit for direct relay of membrane-associated molecules. These findings raise important questions that in the least imply that whenever cell contact relationships are compromised at the oocyte-granulosa interface, serious compromises in metabolic capacity and cell cycle regulation would be experienced by the oocyte [32, 33].

Section 5: TZPs and Meiotic Competence

At or around the time follicles become responsive to FSH, oocytes acquire the property of meiotic competence. This property refers to the fact that when isolated from follicles prior to antrum formation, most mammalian oocytes remain in the dictyate stage arrested in G2 of the meiotic cell cycle and are unable to initiate the metabolic events that normally lead to the resumption of meiosis and progression to the metaphase state of meiosis-2 [6, 24]. The metabolism of the oocyte up to the acquisition of meiotic competence is mainly centered on the hypertrophy or growth of the oocyte to nearly the full-grown size that in rodents approaches 70–80 μm in diameter, whereas in other species such as the human, a diameter of greater than 100 μm is attained. In metabolic terms, the pre-antral growth phase of oogenesis must be at a maximum, and it is not surprising that in the absence of FSH sensitivity, nearly all of the granulosa cells maintain a connection to the oocyte through TZPs [24]. This tight coordination and integration of metabolism thus circumvents the later shift in metabolism of the mural granulosa cells that will become steroidogenic and results, upon stimulation with FSH, in the formation of the specialized lineage of cumulus granulosa cells that will retain metabolic properties consistent with nurturing the oocyte and assuring that it does not undergo precocious entry back into the meiotic cell cycle [6]. The fact that acquiring meiotic competence is tightly coordinated with maintenance of TZPs is likely to underscore the importance of diet and nutrition in oocyte production in large animals [32, 33] and links elements of lifestyle many weeks or months before ovulation to the final quality of oocytes that were affected at these earlier stages of folliculogenesis [34, 35].

A well-studied and important aspect of metabolism centers on the availability of ATP to support the many alterations in protein phosphorylation that take place prior to and after the resumption of meiosis [36]. Adequate reserves and sources of ATP are required to support chromatin remodeling, meiotic competence acquisition, and cell cycle arrest in order to synchronize the pace of oogenesis with that of folliculogenesis [37–39]. Phosphorylation of histones in response to the activation of cell cycle kinases drive the condensation of oocyte chromatin and elicit changes in the cytoskeleton that mediate progression through the meiotic cell cycle [39–41]. Finally, it is becoming increasingly clear that these metabolic demands both prior to and during the maturation of oocytes at ovulation impart significant developmental advantages and capabilities that are manifest at the time of fertilization and during subsequent cleavage divisions in the preimplantation embryo [36, 42, 43]. Recently, the involvement of natriuretic peptide signaling has been discovered as yet another key regulator in the control of meiotic competence and the implications that both estradiol and WNT signaling pathways may converge on the cumulus oocyte complex during ovulation reinforces the belief that modifications in TZPs and cell interactions may be targeted by specific elements of the repertoire of signaling systems associated with this pivotal event in the reproductive life cycle [44–47].

Section 6: Clinical Implications and Oocyte Quality

In the end, our understanding of how oogenesis and folliculogenesis are integrated is key to clinical and agricultural areas that directly depend on artificial forms of reproduction. For humans

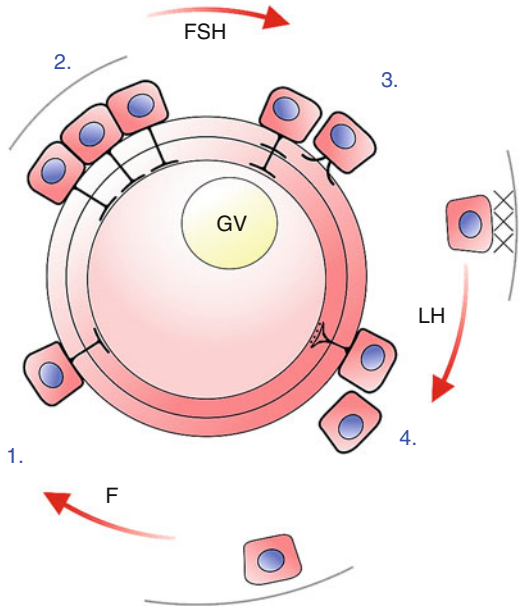

Fig. 5.5 Schematic illustrating patterns of TZP organization in response to *FSH* or *LH*. Prior to FSH stimulation, it is proposed that TZPs are oriented and stable around the oocyte, but after FSH stimulation, a reorganization takes place as a result of granulosa cells entering the cell cycle. TZPs are reestablished in the cumulus subset of granulosa cells, and at the time of ovulation, individual corona cells maintain TZPs, whereas the outer cells of the cumulus mass lose gap junctions and migrate away from the oocyte

undergoing clinical treatment for infertility, the routine use of controlled ovarian stimulation to retrieve oocytes raises questions about the poor quality of oocytes obtained in this way which have often been ascribed to the pharmacological doses of gonadotropins employed. And as noted above, the importance of maintaining the correct level of integration between the oocyte and its follicle is crucial for establishing the timing of meiosis resumption and the events that will take place in the embryo following fertilization [48]. As clinical practices move away from controlled ovarian stimulation to more natural cycle retrievals, or those involving minimal gonadotropin doses, the possibility exists that excessive stimulation abrogates the network of physical interactions between oocyte and granulosa and thus compromises in quality and quantity the dependence of the oocyte on the metabolism of the follicle in which it resides (Fig. 5.5). With in vitro technologies currently in use clinically, or in development, the impact of these manipulations will need to be thoroughly investigated in order to ascertain the degree to which cell interactions are modified as a result of being in an in vitro environment [49, 50]. Interestingly, studies on the oocytes that fail to exhibit maturation during traditional infertility procedures support the idea that both at the level of chromatin organization and meiotic competence, these oocytes may very well be the by-product of the desynchronization of oogenesis and folliculogenesis [50].

In conclusion, as our knowledge about the delicate balance of metabolic integration between oocyte and granulosa grows, it will be important to maintain focus on the role of TZPs or other modes of interaction that serve to coordinate these vital processes during the reproductive lifespan of mammals.

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How the Oocyte Influences Follicular Cell Function and Why

6

Martin M. Matzuk and Qinglei Li

Abstract

During ovarian follicular development, bidirectional communication between oocytes and their companion somatic cells is indispensable. The oocyte plays a leading role in regulating follicular cell development and function including growth and proliferation, apoptosis, differentiation, steroidogenesis, metabolism, and cumulus expansion. By modulating these critical functions, the oocyte orchestrates the rate of follicular development and creates a favorable microenvironment essential for its own development and destiny. A better understanding of the oocyte-somatic cell regulatory loop is essential for unraveling the myths surrounding oocyte developmental competence and may provide novel therapeutic strategies for female reproductive disorders resulting from defects in oocyte-follicle cell interactions.

Keywords

Oocyte • Folliculogenesis • Granulosa cells • Transforming growth factor β Growth differentiation factor 9 (GDF9) • Bone morphogenetic protein 15

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Introduction

A primordial follicle, which comprises an oocyte arrested in the diplotene stage of prophase I and a single layer of squamous granulosa cells (i.e., pregranulosa cells), belongs to the earliest class of follicles that appear in the ovary [1]. In mice, primordial follicles are formed shortly after birth, usually within a few days, while in humans, formation of primordial follicles takes place approximately between 15 and 22 weeks [2, 3]. It is generally accepted that mammalian primordial follicles are the resting pool of a finite set of germ cells during the postnatal life, although there are controversial debates on whether female germline stem cells exist and whether oocytes could be dynamically replenished in females after birth [4–7]. Recent studies, especially those using genetic mouse models, have achieved significant insights into the regulation of primordial follicle activation, whereby a complex interplay among hormones, growth factors, and inhibitory machineries intrinsic to oocytes emerges as fundamental regulatory mechanisms [8]. Once primordial follicles are recruited from the resting pool, they can progress to primary, secondary, antral, and eventually preovulatory follicle stages, the classification of which has been well established [9]. Of note, upon antral formation, the somatic cells in the follicles are separated into two populations (i.e., mural granulosa cells lining the follicle antrum and cumulus cells in close proximity to the oocyte), which have distinct gene expression patterns and functions [10, 11].

During development, ovarian follicles are influenced by endocrine, paracrine, and autocrine factors [12]. The role of endocrine regulation of follicular development has been well documented. For example, follicle-stimulating hormone (FSH) and luteinizing hormone (LH), heterodimeric glycoprotein hormones secreted by the pituitary, are essential stage-dependent regulators of follicular development. FSH and LH comprise a distinct β-subunit and a common α-subunit. Genetic ablation of FSHβ or FSH receptor renders follicles unable to progress to the antral stage without affecting preantral folliculogenesis [13, 14]. In contrast, LH plays a critical role in antral follicle development, ovulation, and luteinization [15–17]. Thus, a gonadotropin-independent mechanism governs follicular development before antrum formation, whereas antral follicle development requires gonadotropin support.

One prominent feature of ovarian folliculogenesis is that the intercellular communications are bidirectional and established via gap junctions and paracrine signaling between the two cellular compartments: oocytes and their companion somatic cells [18]. The oocyte-somatic cell cross talk is indispensable and will benefit both cell types [11, 19, 20]. The oocyte plays a leading role during folliculogenesis [11, 19, 21], and via secretion of paracrine factors, it regulates follicular cell development and function including, but not limited to, apoptosis, differentiation and steroidogenesis, and metabolism [22–28]. Consequently, a favorable microenvironment is established to facilitate the development of oocyte competence, which is progressively acquired during follicular development [11, 20, 21, 29, 30]. The term oocyte-granulosa cell regulatory loop has been used in the literature to describe the complex interplay between oocytes and their associated somatic cells [11]. In the following sections, we will introduce the role of intraovarian factors, particularly transforming growth factor β (TGF-β) superfamily proteins, in the regulation of ovarian function because of the importance of these factors in mediating oocyte regulation of follicular cell functions. Then we will focus on the paracrine regulation of various follicular functions by oocytes. Potential implications of the studies in this field will be highlighted in the end. It needs to be pointed out that although data from mice will serve as a main source for this chapter, they can be potentially extrapolated to other species and humans under many conditions.

TGF-β Family Proteins and Ovarian Function

Intraovarian growth factor signaling, particularly TGF-β signaling, is involved in the regulation of a wide spectrum of female reproductive processes and required for normal ovarian folliculogenesis and function [10, 22, 31–41]. Dysregulation of TGF-β signaling pathway may lead to pathological conditions such as premature ovarian failure

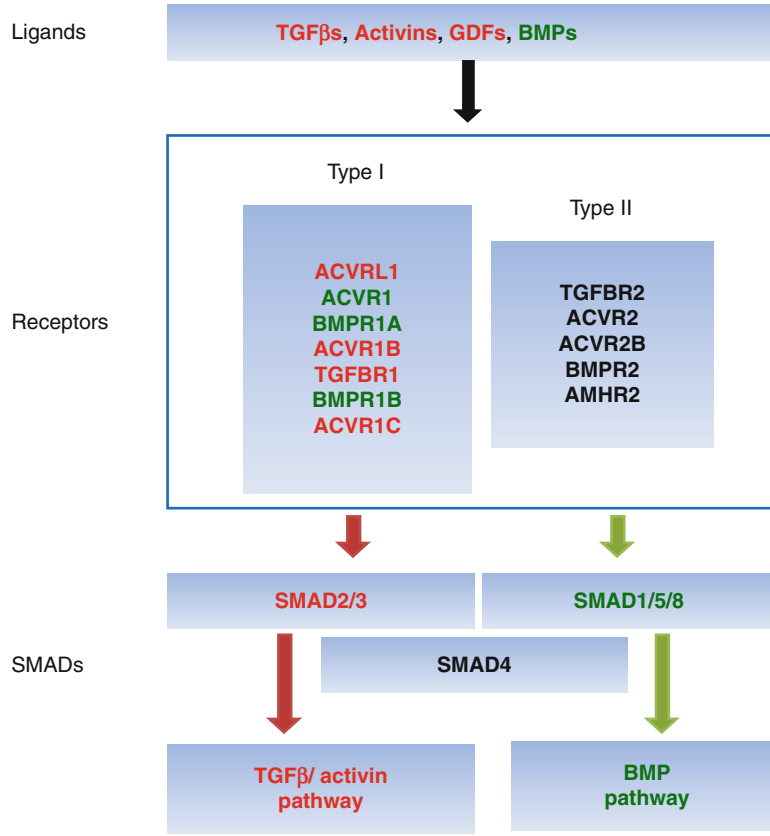

Fig. 6.1 Major components of TGF-β signaling pathway. The TGF-β signaling pathway consists of ligands, receptors, and SMAD proteins. TGF-β ligands can selectively bind to the type II and type I receptors and activate the corresponding SMAD proteins to induce signal transduction. The type I receptors ACVRL1, ACVR1B, TGFBR1, and ACVR1C and SMAD2/3 are generally associated with TGF-β/activin signaling, whereas ACVR1, BMPR1A, and BMPR1B and SMAD1/5/8 are related to BMP pathway. Activated (i.e., phosphorylated) SMADs can translocate to the nucleus with SMAD4 to regulate gene transcription in concert with coactivators and corepressors

(POF) and cancer development [42–46], thereby limiting reproductive success. Major components of the TGF-β signaling pathway comprise ligands, receptors, and intracellular SMAD proteins. TGF-β ligands signal through the type II and type I receptors and SMAD proteins which include receptor-regulated SMADs and a common SMAD (SMAD4) to elicit cellular responses. The heteromeric receptor complex is formed by a combination of type I (i.e., ACVRL1, ACVR1, BMPR1A, ACVR1B, TGFBR1, BMPR1B, and ACVR1C) and type II receptors (i.e., TGFBR2, ACVR2, ACVR2B, BMPR2, and AMHR2) [47–49]. Receptor-regulated SMADs (R-SMADs) consist of SMADs 1, 2, 3, 5, and 8, which can interact with TGF-β family receptors to mediate ligand signaling. In general, SMAD2/3 mediate TGF-β and activin signaling, whereas SMAD1/5/8 mediate BMP signaling (Fig. 6.1). SMADs can regulate gene transcription in the nucleus in concert with coactivators and corepressors. Within a cell, TGF-β can signal through both SMAD-dependent [48, 50] and/or SMAD-independent [51, 52] (i.e., non-SMAD) pathways depending on the cellular context. Regulation of TGF-β signaling occurs at multiple levels by a plethora of factors including ligand traps (e.g., follistatin) and inhibitory SMADs (i.e., SMAD6 and SMAD7) as well as inputs from other independent pathways that influence the TGF-β signaling intensity and outcome [50, 53–56].

Among known TGF-β ligands, growth differentiation factor 9 (GDF9) and bone morphogenetic protein 15 (BMP15) are well-characterized oocyte-derived factors whose functions in follicular development have been revealed by loss-of-function/mutation studies [31, 57, 58], animal immunization with peptides [59–62], and the in vitro culture experiments using recombinant proteins [23, 26–28, 63–72]. *Gdf9* is expressed in oocytes from primary stage follicles in mice and humans [73, 74]. *Gdf9* knockout mice are infertile and develop striking ovarian defects where follicular development is blocked at the primary stage [31]. As a result of GDF9 loss of function, ovarian kit ligand (*Kitl*) is upregulated in granulosa cells [75] due to the loss of GDF9 inhibition on *Kitl* expression [76], leading to the development of overgrown

oocytes in *Gdf9* null ovaries [77, 78]. Moreover, the defect in thecal layer development in *Gdf9* null mice [75] suggests that GDF9 plays a direct or indirect role in thecal cell development besides its primary action in granulosa cell compartment. Subsequent to the initial identification of an obligatory role for GDF9 in primary to secondary follicle transition, additional studies extended the GDF9 function to early antral and preovulatory follicle development [63, 79]. Unlike GDF9, loss of mouse BMP15, an oocyte homolog of GDF9, leads to cumulus cell defects with minimal abnormalities on early follicular development [57]. However, transgenic mice with oocyte overexpression of BMP15 (a chimeric protein of human BMP15 proregion-mouse BMP15 mature region) show the growth-promoting effect of BMP15 on ovarian follicles [80]. GDF9 and BMP15 are fundamental regulators of fertility in multiple species including mice, sheep, and humans [31, 32, 57, 81–87]. Both in vitro and in vivo data support that these two oocyte factors can act synergistically in the regulation of ovarian cell function [57, 88–91]. In contrast to mice, mutations in ovine *Bmp15* or *Gdf9* gene enhance fertility in heterozygotes but cause infertility in homozygotes [32, 81]. In women, mutations or polymorphisms of the two genes are associated with ovarian failure or other reproductive abnormalities [82–85, 87, 92–94]. Of note, the involvement of additional TGF-β family members [e.g., activin, inhibin, BMP4, BMP7, anti-Müllerian hormone (AMH)], as well as other pathways (e.g., notch signaling, hedgehog signaling, and Wnt signaling) or growth factors [insulin-like growth factors, epidermal growth factors (EGFs), and vascular endothelial growth factors (VEGFs)], in ovarian development and function has also been documented [95–103]. However, this is beyond the focus of this chapter.

Oocytes Regulate Follicular Cell Function

Oocytes Regulate Follicular Cell Function: What and How

Within each ovarian follicle, the functional unit of the ovary, an oocyte is surrounded by somatic cells. The oocyte communicates with somatic cells through gap junctions and paracrine signaling, and this bidirectional signaling is critical for folliculogenesis, oocyte growth, and acquisition of oocyte developmental competence [11, 18–21, 104]. It is now known that the oocyte is the "driver" instead of a "passenger" in the journey of folliculogenesis. Importantly, oocytes specify the phenotype and lineage of the adjacent cumulus cells and maintain their appropriate differentiation status [10]. Within a follicle, the oocyte actively regulates somatic cell functions essential for follicular development and normal female reproduction. Below is a summary of several key follicular functions that are subject to the regulation by oocytes, and an emphasis is given on the recent advances in the understanding of these regulatory processes (Fig. 6.2).

Growth and Proliferation

Cell growth and proliferation are common features of follicular development, the rate of which is controlled by oocytes via an intrinsic developmental program [11, 18–20, 29, 31, 104, 105]. It is known that oocytes influence granulosa cell proliferation [22, 26, 65, 106–108]. However, how do oocytes regulate the granulosa cell proliferation? Recent studies demonstrated that oocyte-produced paracrine factors, such as GDF9, play a prominent role in this process [23]. GDF9 has been proposed to signal through TGF-β type 1 receptor [(TGFBR1), also known as activin-like kinase 5 (ALK5)] and BMP receptor type II (BMPR2) complex, and impinge on SMAD2/3 in ovarian granulosa cells [20, 109–112], although our recent study has shown that ALK5 might not be the functional GDF9 type 1 receptor [40]. GDF9, TGF-β1, and activin can enhance DNA synthesis of mouse granulosa cells [23]. The effect of oocytes on granulosa cell proliferation seems to be mediated by ALK4/5/7, because SB431542, an ALK4/5/7 inhibitor, attenuates the effects of both oocytes and GDF9 on granulosa cell proliferation [23]. Furthermore, the oocyte-promoted granulosa cell proliferation can be antagonized by the BMPR2 ectodomain which competes with ligands that bind to BMPR2, but not those of TGFBR2, ACVR2, or ACVR2B, suggesting that GDF9 is able to bind to BMPR2 [23].

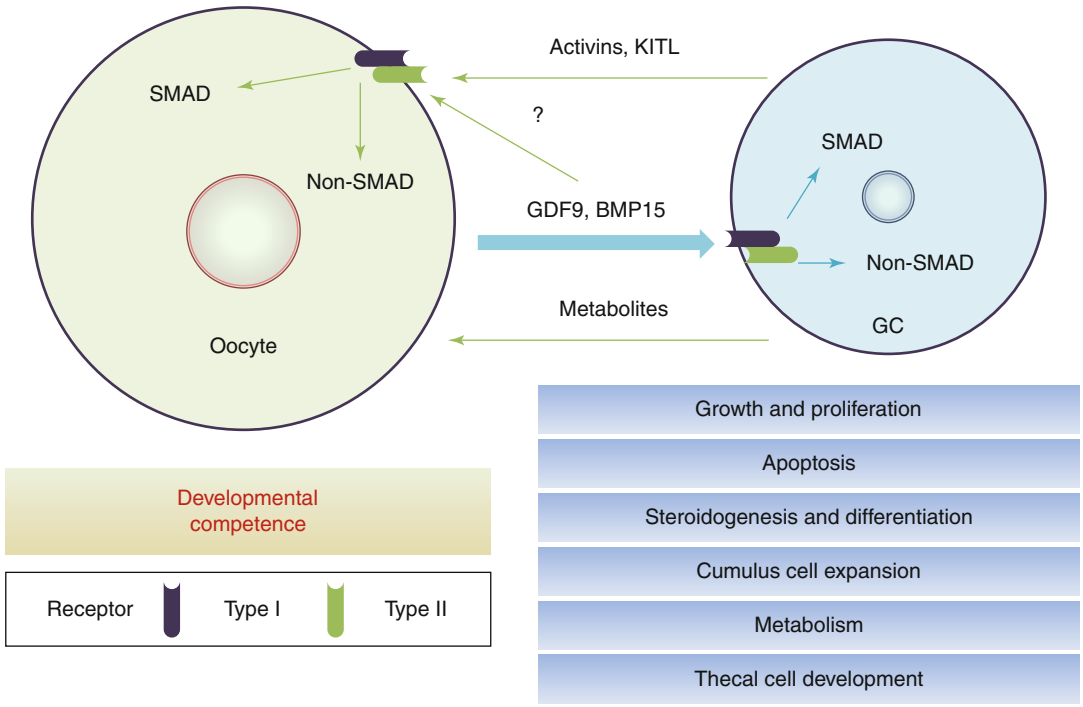

Fig. 6.2 TGF-β signaling and oocyte developmental competence. During follicular development, the oocyte produces TGF-β family proteins (e.g., BMP15 and GDF9) to regulate granulosa cell functions such as growth and proliferation, apoptosis, differentiation and steroidogenesis, cumulus cell expansion, metabolism, and thecal cell development. Meanwhile, granulosa cells secrete regulatory signals (e.g., activins and KITL) via paracrine pathway or provide nutrients (e.g., metabolites and L-alanine) through gap junctions to regulate oocyte development and function. It is unknown if oocyte-derived factors can signal through autocrine pathway to regulate its own development

It was recently found that epidermal growth factor receptor (EGFR)-extracellular signal-regulated kinases 1 and 2 (ERK1/2) signaling-induced phosphorylation on the SMAD3 linker region is an essential element for oocyte paracrine factors such as GDF9 to induce granulosa cell proliferation [113]. This finding highlights the importance of the signal input received from pathways that cross talk with TGF-β signaling in the regulation of somatic cell function by oocytes. Intriguingly, oocytes cannot activate BMP signaling in granulosa cells [23], which raises the question as to whether mouse BMP15 per se is biologically active or whether secretion of bioactive mouse BMP15 is a time-controlled event during follicular development [71]. It has been suggested that mouse BMP15 cannot be processed appropriately in cell lines [34], and biologically active recombinant mouse BMP15 by itself has not been available. However, studies using human recombinant BMP15 suggest that BMP15 signals via a BMPRIB (ALK6)/BMPR2 receptor complex and downstream SMAD1/5/8 to evoke paracrine actions in target cells (i.e., granulosa cells) [114]. The above evidence suggests that oocytes, via secretion of paracrine factors, regulate ovarian somatic cell proliferation.

Apoptosis

Apoptosis, also called programmed cell death, is a genetically controlled cellular death that widely occurs in numerous pathological and physiological events including ovarian folliculogenesis. After leaving the primordial follicle pool, follicles undergo continuous growth and become preovulatory follicles and ovulate if they are selected to do so. However, the actual scenario is that the growth and proliferation of granulosa cells are often accompanied by apoptosis during folliculogenesis, where the vast majority of follicles undergo atresia, a process mediated by apoptosis [115–117].

It is plausible that healthy cumulus cells are beneficial to oocyte development, while extensive apoptosis in cumulus cells does not contribute to a favorable microenvironment. The evidence supporting that oocytes prevent cumulus cell apoptosis comes from studies by Gilchrist's group in which they showed that the apoptosis rate of cumulus cells increases after oocyte removal from the cumulus-oocyte complex [24]. Furthermore, oocyte paracrine factors, mainly BMP15 and BMP6, but not GDF9, play a major role in suppressing cumulus cell apoptosis, which is associated with altered BCL-2/BAX ratio [24]. The antiapoptotic effects of these BMPs can be dramatically attenuated by their antagonists such as follistatin (for BMP15) or the neutralizing antibody. Most interestingly, the general pattern of apoptosis present in the outer layer of cumulus-oocyte complex could be reversed (i.e., the apoptosis occurs mainly in the inner layer instead of the outer layer) when oocytectomized cumulus complexes were cultured in the presence of oocytes [24]. The apoptotic pattern of cumulus cells and the effect of BMPs on cumulus cell apoptosis thus suggest that oocyte-generated morphogenetic gradients suppress cumulus cell apoptosis. In the rat, intraoocyte injection of GDF9 antisense oligos blocks preantral follicle growth and induces apoptosis associated with upregulation of caspase-3, the effect of which can be reduced by exogenous GDF9 protein [79]. Furthermore, the antiapoptotic effect of GDF9 during follicular development is mediated via the phosphatidylinositol 3-kinase (PI3K)/Akt pathway [79]. The different observations for the GDF9 actions in granulosa/cumulus cell apoptosis in the two aforementioned studies may imply species-specific or stage-specific roles for GDF9 in the regulation of ovarian somatic cell apoptosis, a widespread phenomenon during folliculogenesis.

Differentiation and Steroidogenesis

Granulosa cells are relatively homogeneous before antral formation. However, when follicles develop to the antral stage, two subpopulations of granulosa cells are formed within a follicle: mural granulosa cells and cumulus cells. The mural granulosa cells and cumulus cells are distinct in the proximity to the oocytes, morphology, gene expression patterns, and cellular functions. For example, mural granulosa cells express higher levels of luteinizing hormone/choriogonadotropin receptor (*Lhcgr*), steroidogenic enzyme P450 side-chain cleavage enzyme (*Cyp11a1*), and immune marker CD34 antigen (*Cd34*), whereas cumulus cells express more abundant anti-Müllerian hormone (*Amh*) and androgen receptor (*Ar*) [10]. The oocyte has been recognized as an important player in establishing this unique gene expression pattern of cumulus cells versus mural granulosa cells [10].

Production of progesterone is a common indicator of granulosa cell luteinization. In antral follicles, there is an opposing gradient generated by FSH and oocyte-initiated SMAD2/3 signaling, and the latter is important in preventing granulosa cell luteinization [10] associated with enhanced steroidogenic activities. Granulosa cells are the sites for the synthesis of estradiol and progesterone. Upon the LH surge, mural granulosa cells which express LHCGR respond to LH by exiting the cell cycle and undergoing terminal differentiation/luteinization to form the corpus luteum, which is an endocrine structure composed of both granulosa cells and thecal cells and is important for progesterone production during pregnancy [118]. It has long been postulated that oocytes can regulate steroidogenesis and luteinization of somatic cells based on the observation that spontaneous luteinization and enhanced production of progesterone occurred in follicles where oocytes were removed [119]. Supporting the role of the oocyte in regulating granulosa cell differentiation/luteinization, oocyte-secreted factors prevent *Lhcgr* expression in cumulus cells and restrict its expression to mural granulosa cells [25]. Consistently, *Lhcgr* transcript levels were increased, and the production of progesterone was enhanced by cumulus cells when oocytes were removed from the cumulus-oocyte complexes by oocytectomy [25, 120, 121]. Furthermore, the oocyte-secreted factor seems to

inhibit progesterone but stimulate estradiol production in cumulus granulosa cells [120]. The oocyte factors can affect 3β-hydroxysteroid dehydrogenase (3β-HSD), an enzyme involved in the synthesis of progesterone as well as progesterone metabolism [26, 63, 65, 67, 122]. During luteinization, the steroid-regulating activity of the oocytes is increased [121]. In mouse granulosa cells, recombinant GDF9 promotes progesterone production [63]. In the rat, GDF9 stimulates basal progesterone and estradiol production in granulosa cells but suppresses FSH-stimulated steroidogenesis [26]. In bovine granulosa cells, GDF9 inhibits the production of progesterone and estradiol induced by insulin-like growth factor 1 (IGF1) [122]. Similar to GDF9, BMP15 inhibits FSH-induced progesterone synthesis and related enzymes such as steroidogenic acute regulatory protein (STAR) and CYP11A1; however, BMP15 does not affect FSH-stimulated estradiol production [65, 67].

As mentioned above, the oocyte-activated SMAD2/3 signaling is important in preventing somatic cell differentiation or luteinization [10]. Conditional deletion of the common *Smad*, *Smad4*, in mouse granulosa cells causes premature luteinization of follicles, accompanied by increased expression of genes including *Lhcgr*, *Star*, secreted frizzled-related protein (*Sfrp4*), *Cyp11a1*, and hydroxysteroid (17-β) dehydrogenase 7 (*Hsd17b7*) [123]. These findings thus suggest that TGF-β family paracrine and/or autocrine signaling is required to prevent granulosa cell luteinization.

Cumulus Cell Expansion

In response to the LH surge, cumulus cells synthesize cumulus matrix enriched in hyaluronan (HA), the backbone of the cumulus matrix [124]. To initiate successful ovulation, cumulus cells surrounding the oocyte must undergo expansion, or mucification, whereby cumulus cells are embedded in a mucified matrix [125–127]. In response to the LH surge, a number of genes, particularly pentraxin 3 (*Ptx3*), hyaluronan synthase 2 (*Has2*), tumor necrosis factor α-induced protein 6 (*Tnfaip6*), and prostaglandin-endoperoxide synthase 2 (*Ptgs2*), are induced to form cumulus matrix [127, 128]. Mice with deletion or dysregulation of *Ptx3* [129], *Ptgs2* [130], or *Tnfaip6* [131, 132] show defects in cumulus expansion. Moreover, PTGS2 is a critical enzyme involved in the biosynthesis of prostaglandins essential for follicle rupture and ovulation, and *Ptgs2* null mice exhibit ovulation defects [133, 134]. The aforementioned cumulus expansion-related transcripts are regulated by oocyte-produced factors GDF9 and BMP15 [40, 63, 71, 135, 136]. Thus, by secretion of paracrine factors, oocytes promote cumulus cell expansion and ovulation via induction of critical genes involved in these processes.

Since cumulus cells do not express LHCGR [137], how the LH surge induces cumulus expansion has intrigued scientists until the myth was uncovered by the Conti laboratory [16]. An intricate signaling network has been identified in the preovulatory follicles, whereby LH binds to its receptor in mural granulosa cells to induce a number of EGF-like family members including amphiregulin (AREG), epiregulin (EREG), and betacellulin (BTC), which are putative physiological ligands of EGF receptor (EGFR) and can act as key paracrine mediators of LH action on cumulus cells [16]. These growth factors can mimic the effect of LH on cumulus expansion and interact with EGFR in cumulus cells to induce the expression of cumulus expansion-related transcripts (*Has2*, *Ptx3*, *Tnfaip6*, and *Ptgs2*) [16]. The role of AREG and EREG has been validated using knockout mouse models in which cumulus expansion is compromised in the mutant mice [138]. The EGF-like growth factors may activate mitogen-activated protein kinases 3 and 1 (MAPK3/1) [i.e., extracellular signal-regulated kinases 1 and 2 (ERK1/2)] [139]. Conditional deletion of MAPK3 and MAPK1 in mouse granulosa cells leads to defective cumulus expansion and ovarian functions related to ovulation and luteinization [17].

It is well known that the oocyte is required for cumulus expansion in vitro, at least in mice [140], since oocytectomy does not seem to prevent cumulus cell expansion in the rat [141] and

other domestic animals [141–144]. In mice, the cumulus cells are unable to expand in the presence of FSH or EGF when the oocyte is surgically removed from the cumulus-oocyte complex [140, 141]. Therefore, the oocyte may secrete certain factors to promote cumulus cell expansion. Although the true identities of the presumptive cumulus expansion-enabling factors (CEEFs) remain unclear [145], it is generally accepted that fully grown oocytes are capable of producing CEEFs and TGF-β family proteins (e.g., GDF9 and BMP15) are the candidates that induce the cumulus expansion and the expression of cumulus expansion-related transcripts [36, 63, 71, 135, 146–148]. GDF9 and BMP15 are synthesized as prepropeptides (signal peptide-prodomain-mature domain), the activation of which requires the cleavage of proregion by proprotein convertase subtilisin/kexin (PCSK) [49, 149]. Most knowledge on GDF9 function in late follicular development is derived from studies using GDF9 recombinant proteins and in vitro granulosa cell/follicle culture experiments. Recombinant GDF9 is capable of inducing the expression of cumulus expansion-related genes in mouse granulose cells and stimulating cumulus cell expansion in vitro [63]. Consistent with GDF9 signaling through SMAD2/3 in granulosa cells [111, 150] and the involvement of SMAD2/3 in cumulus expansion, inhibition of SMAD2/3 signaling using an ALK4/5/7 inhibitor abolishes cumulus expansion in vitro [10, 36]. Furthermore, when both *Smad2* and *Smad3* were conditionally deleted in mouse granulosa cells, cumulus expansion was compromised, and meanwhile, the induction of GDF9-promoted cumulus expansion-related transcripts was impaired [38]. The cumulus cell phenotype of *Smad2* and *Smad3* conditional knockout mice partially mimics that of granulosa cell deletion of *Smad4* [123]. BMP15 is also associated with cumulus cell development, as evidenced by cumulus cell defects in *Bmp15* null mice [57]. Recombinant human BMP15 containing a C-terminal epitope tag was reported to promote cumulus expansion [71]. In mouse granulosa cells, the paracrine action of BMP15 on cumulus expansion is mediated by BMPRIB, as *Bmpr1b* null female mice exhibit defects in cumulus expansion [151], and their granulosa cells do not respond to recombinant BMP15 stimulation but are fully responsive to recombinant GDF9 treatment to express cumulus expansion-related genes [40]. In vivo, GDF9 and BMP15 act synergistically [57]. Therefore, by modulating cumulus cell expansion, the oocyte paracrine signaling serves as an integral regulator of ovulation and oocyte maturation during ovarian folliculogenesis.

Metabolism

Granulosa cells are known to nurture oocytes. As an example, mouse oocytes can utilize pyruvate produced by cumulus cells as an energy source, but cannot directly use glucose for their growth and development [27, 152, 153]. Oocyte deletion of pyruvate dehydrogenase E1-alpha 1 (*Pdha1*), a gene encoding a subunit of pyruvate dehydrogenase complex, causes defects in mouse oocyte maturation [154]. Although the above evidence strongly suggests the cooperativity between oocytes and somatic cells in utilizing glucose, the underlying molecular mechanism remains unknown until oocyte-secreted factors were found to coordinately regulate cumulus cell glycolysis [27]. It was revealed that oocyte-produced BMP15 and fibroblast growth factor 8B (FGF8B) synergistically regulate the expression of genes involved in the glycolytic pathway such as platelet phosphofructokinase (*Pfkp*) and lactate dehydrogenase A (*Ldha*) [27]. Furthermore, oocytes from *Bmp15* null mice or *Bmp15*/*Gdf9* double mutant mice show defective glycolytic activity [27].

Cholesterol biosynthesis is also a cooperative activity between oocytes and somatic cells [28]. The mRNA transcript levels of a number of genes involved in cholesterol biosynthesis pathway such as mevalonate kinase (*Mvk*) and phosphomevalonate kinase (*Pmvk*) are higher in cumulus cells than oocytes [28]. In vitro, oocyte paracrine factors (i.e., BMP15 and GDF9) can increase the biosynthesis of cholesterol in mouse cumulus cells [28]. Moreover, cholesterol biosynthesis is reduced in *Bmp15* null and *Bmp15*/*Gdf9* double mutant cumulus cells [28]. Taken together, these studies suggest that mouse oocytes are deficient in cholesterol biosynthesis and highlight the role of cumulus cells in supplementing cholesterol to

oocytes during development under the direct influence of the oocyte.

Besides glycolysis and cholesterol biosynthesis, the oocyte is also deficient in taking up certain amino acids, such as L-alanine and L-histidine. Because fully grown oocytes can stimulate the mRNA expression of solute carrier family 38, member 3 (*Slc38a3*) which encodes a sodium-coupled neutral amino acid transporter and the uptake of L-alanine in granulosa cells from both preantral and antral follicles [155], it is possible that the deficiency in amino acid uptake by oocytes is attributed to the lack of expression of the SLC38A3 and/or other transporters [155]. Therefore, cumulus cells play important roles in the uptake of certain amino acids and the subsequent delivery to oocytes via gap junctions, which couple the oocytes and the surrounding somatic cells [156]. Gap junctions can allow the passage of low molecular weight substances, such as metabolites and amino acids, from somatic cells to oocytes. However, the oocyte factors that regulate the expression of *Slc38a3* in cumulus cells remain to be elucidated.

Thecal Cell Development

A theca surrounds follicles that contain at least two layers of granulosa cells. Thecal layers include the theca interna, the place where androgens are synthesized, and theca externa, which is composed of smooth muscle-like cells and collagen and may play a role in follicle rupture during the ovulatory process [157, 158]. The role of oocytes in ovarian theca development comes from the evidence that *Gdf9* null mice do not form a thecal layer around the follicles, although a flattened layer of fibroblastic cells surrounding the follicles can be observed in *Gdf9* null mice [75]. Accordingly, *Lhcgr* and *kit* were undetectable in the theca. However, cells positive for CYP17 (a thecal marker) are dispersed in the interstitium and presumably belong to a thecal cell precursor population [31, 75]. Thus, GDF9 seems to play a role in the recruitment of thecal cell precursors during early follicular development. Unexpectedly, theca recruitment and formation of morphological thecal layer can occur in the absence of GDF9 as observed in the *Gdf9* and inhibin-α (*Inha*) double mutant mice [159]. Despite these observations, theca differentiation is impaired with no detectable thecal cell markers [159]. Therefore, a role of oocytes in the regulation of thecal cell function is plausible in the context of oocyte-somatic cell interaction in favor of thecal cell differentiation. GDF9 also stimulates the production of androstenedione in both the primary and transformed thecal cells in vitro [160], although conflicting results were reported using bovine thecal cells [161]. In agreement with the role of GDF9 in regulating thecal cell function, intraoocyte delivery of *Gdf9* antisense in the rat inhibits the growth of preantral follicles in culture, accompanied by reduced production of androgen and expression of *Cyp17a1* [162]. Treatment of rats with GDF9 promotes the development of follicles to small preantral stage and upregulates CYP17 expression [64]. These results are consistent with the hypothesis that GDF9 regulates thecal cell differentiation in vivo.

Others

Oocytes within follicles are under meiotic prophase arrest prior to ovulation. During follicular development, granulosa cells are indispensable for the oocyte to acquire its developmental competence and maintain meiotic arrest, the disruption of which leads to precocious maturation of the oocytes [163]. The mechanisms controlling meiosis initiation and meiotic arrest and resumption have been continuous interests of scientists [12, 164]. It is well established that two critical regulators maintaining the meiotic arrest are cyclic adenosine 3′, 5′-monophosphate (cAMP) and cyclic guanosine 5′-monophosphate (cGMP) [165–167]. The LH surge activates phosphodiesterase 3A (PDE3A) that causes the hydrolysis of oocyte cAMP, leading to meiotic resumption [168]. Although it has been known that fully grown oocytes undergo spontaneous (i.e., gonadotropin-independent) maturation after removal from follicles [169], the "inhibitory substance/signal" from the follicles that maintains oocyte meiotic arrest remains elusive until the discovery of the natriuretic peptide precursor type C

(NPPC)-NPR2 (NPPC receptor) pathway which prevents oocyte meiotic resumption [163].

Nppc is mainly present in mural granulosa cells, while its receptor *Npr2*, a guanylyl cyclase, is predominantly localized to cumulus cells adjacent to the oocytes [163]. Granulosa cell-derived NPPC promotes cumulus cells to produce cGMP, which is then transferred to oocytes through gap junctions to suppress PDE3A-mediated cAMP hydrolysis, thus maintaining the oocyte meiotic arrest [163]. Estradiol may play a role in promoting oocyte meiotic arrest through the regulation of NPR2 [170]. Consistent with the role of LH in initiating oocyte meiotic resumption, preovulatory LH/hCG reduces granulosa cell NPPC production [171]. Interestingly, oocyte-produced factors including GDF9 and BMP15 enhance *Npr2* levels in cumulus cells [163], suggesting that the oocyte may regulate its own meiotic status via paracrine signaling within the oocyte-somatic cell regulatory loop.

Oocytes Regulate Follicular Cell Function: Why

Because of the intimate relationship between oocytes and their surrounding somatic cells in the unique follicular units, physical and functional interactions between the two cellular compartments are of key importance for the development of ovarian follicles and oocytes [11, 19–21, 29]. By regulating granulosa cell functions, the oocyte orchestrates the rate of follicular development and creates an optimal microenvironment that is critical and beneficial to its development [11, 19, 29], because healthy cumulus cells can provide sufficient nutrients and regulatory signals. More specifically, the oocyte regulates follicular cell development and function for the following main reasons:

1. It has been proposed that gonadotropins induce granulosa cell differentiation to mural granulosa cell lineage as a default setting; however, oocytes are capable of abrogating this default pathway to drive a cumulus cell phenotype that is more favorable to oocyte development [11], as inappropriate differentiation of cumulus cells in vitro manifested by expression of LHCGR adversely impacts oocyte developmental competence [172]. Undoubtedly, oocyte development will benefit from a cumulus environment with minimal apoptosis and luteinization.
2. Regulation of thecal cell development will help to form an intact follicle that is the functional unit of the ovary and the "nest" of a growing oocyte.
3. Nutritional support provides driving force for follicular cell growth and development, without which the cells will lose the energy source to complete the developmental programs. Thus, outsourcing catabolic metabolisms may benefit the oocyte in two ways: first, it can prepare the oocyte for embryo development by anabolic metabolism, and second, it reduces the oxidative stress of the oocyte to maintain oocyte quality [173, 174].
4. Cumulus expansion is such an important event that it is not only beneficial to oocyte development and maturation but also a key step toward fulfilling the oocyte's final destiny: ovulation and fertilization.
5. It is amazing that oocytes regulate cumulus cell *Npr2* gene expression to set up the microenvironment conductive to the maintenance of oocyte meiotic arrest, the disruption of which will lead to precocious meiotic resumption of the oocytes, a disaster to fertilization and development [163].

Conclusion and Perspective

Follicular development is an enigmatic process, during which oocytes acquire their competence for fertilization and development. Studies from the last decades generated robust evidence that the oocyte actively regulates the companion somatic cell functions during the entire process of follicular development. By regulating follicular cell functions, such as proliferation, differentiation, apoptosis, steroidogenesis, cumulus cell expansion, and metabolism, the oocyte determines the pace of the follicular development and creates a favorable microenvironment for its own development. The oocyte can influence somatic cell function or gene expression via paracrine factors such as GDF9, BMP15, and FGF8B, and disruption of this regulatory circuit results in

Table 6.1 Recombinant BMP15 and GDF9 ligands reported in the literature

Name	Species	Wt/mutant	Epitope tag	Purification	Reference
BMP15	Human	Wt	C-terminal tag/mature domain	Purified	[65]
BMP15	Human	Wt	N-terminal tag/mature domain	Purified	[135]
BMP15	Human	Wt	No tag	Purified	[182]
BMP15	Ovine	Wt	No tag	Unpurified	[70]
BMP15	Mouse	Wt	No tag	Unpurified	[184]
GDF9	Human	Wt	No tag	Purified	[185]
GDF9	Human	Wt	N-terminal tag/proregion	Purified	[183]
GDF9	Human	Gly391Arg	N-terminal tag/proregion	Purified	[183]
GDF9	Ovine	Wt	No tag	Unpurified	[70]
GDF9	Rat	Wt	No tag	Unpurified	[22]
GDF9	Mouse	Wt	No tag	Unpurified	[63]
GDF9	Mouse	Wt	No tag	Unpurified	[184]
GDF9	Mouse	Wt	No tag	Unpurified	[111]
GDF9	Mouse	Wt	N-terminal Flag tag	Purified	[40]

catastrophic consequences on the oocyte competence and/or follicular development. Thus, the gene expression profiles of granulosa cells are reasonable predictors of oocyte developmental competence [18]. In humans, embryo quality is potentially correlated with granulosa cell gene expression such as *HAS2*, *PTGS2*, and *GREM1* [175, 176]. Additionally, both cumulus and mural granulosa cells are easily obtained, and they are excellent sources of materials for noninvasive diagnosis/assessment of oocyte developmental competence. This is extremely important in the context of the increasing demand on establishing more objective criteria for embryo selection for in vitro fertilization (IVF). Therefore, exploiting the potential of using granulosa cell genes as markers for assessing oocyte competence deserves continuous and combined efforts of scientists from both research laboratories and assisted reproductive technology (ART) clinics.

Oocyte competence influences early embryonic development, pregnancy outcome, and fetal development, which is highly associated with reproductive success. In vitro maturation (IVM) of oocytes has been applied for infertility treatment and fertility preservation, which eliminates the gonadotropin stimulation procedure and the associated risks such as ovarian hyperstimulation syndrome [177, 178]. However, the low success rate of IVM substantially hinders its otherwise wide application. Two oocyte-secreted factors, BMP15 and GDF9, are key regulators of oocyte-somatic cell interaction, ovarian function, and female fertility in multiple species including mouse and human [27, 31, 32, 57, 58, 81, 83–86, 88, 89]. Inclusion of exogenous recombinant mouse GDF9 during IVM increases the percentage of hatching blastocysts and the number of viable fetuses [179]. Additionally, recombinant mouse GDF9 or ovine BMP15 increases the number of blastocysts that develop from bovine oocytes during IVM [180]. As further support, disruption of GDF9 signaling pathway during IVM reduces fetal survival [181]. These studies bring excitement to the ART clinics. It has been a long-standing challenge to obtain purified, bioactive, and stable recombinant BMP15 and GDF9 [145]. Recently, a number of new recombinant proteins have become available which include bioactive human BMP15 with an N-terminal epitope tag [135] or without tag [182] and bioactive human GDF9 with a point mutation [183]. The recombinant BMP15 and GDF9 proteins that have been produced and used by a number of laboratories are listed in Table 6.1. Future studies should elucidate the contribution of individual or combinations of these factors to the development of oocyte competence using multiple culture systems.

Due to the dynamic intercellular communications between oocytes and their companion granulosa cells and the active role of oocytes in the regulation of follicular cell function, a better understanding of the oocyte-somatic cell interaction and the signaling pathways is important to

unravel the myth of the oocyte developmental competence. Particularly, translational studies in this research field may prove invaluable and guide the design of novel therapies for female reproductive disorders and infertility which are associated with defects in follicular development and oocyte competence.

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Changes of Large-Scale Chromatin Configuration During Mammalian Oocyte Differentiation

7

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Abstract

Mammalian oocyte development is characterized by impressive dynamic changes in chromatin structure and function within the germinal vesicle (GV). During meiotic arrest at diplotene stage, and particularly during the oocyte growth phase leading to the formation of the fully-grown and differentiated oocyte, the chromatin enclosed within the GV is subjected to several levels of regulation controlling both its structure and function. Morphologically, the chromosomes lose their individuality and form a loose chromatin mass, which in turn undergoes profound and dynamic rearrangements within the GV before the meiotic resumption. These large-scale chromatin configuration changes have been studied in several mammals and progressive condensation of the chromatin has been related to the achievement of meiotic and developmental potential. In this chapter we will give an overview of the scientific literature on this topic, highlighting how changes in chromatin configurations are related to both functional and structural modifications occurring in the oocyte nuclear and cytoplasmic compartments. Further, we will discuss the machinery regulating this complex process, including the fundamental role exerted by the follicular cells also throughout intracellular messenger dependent mechanism(s). Finally, we will discuss possible implications for the field of assisted reproductive technologies.

Keywords

Chromatin configuration • Karyosphere • Transcriptional activity - Meiosis
Developmental competence • Oocyte growth

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Introduction

In recent years, the process of remodeling of chromatin configuration within the nucleus of the oocyte, occurring toward the end of its differentiation phase before meiotic resumption, has received much attention and has been studied in several mammals. The aims of this chapter are to give an overview of the scientific literature on this topic and to highlight, to the fullest extent possible, the common features among the species studied so far. Thus, as classically described in textbooks, we first delineate the *temporal* context in which these changes occur in terms of meiosis and folliculogenesis. Then, we focus on observations made on several mammals and on how changes in chromatin configurations are related to the oocyte competence and to both functional and structural modifications occurring in its nuclear and cytoplasmic compartments. In further sections, we consider studies intended to explain the mechanism(s) regulating this complex process that is still far from being completely elucidated. Finally, we discuss possible implications for the field of assisted reproductive technologies.

Large-Scale Chromatin Configuration Changes: When Do They Happen?

In the process of meiosis, the most complex and temporally extended phase is the meiotic prophase I when homologous chromosomes pair, exchange genetic material by recombination, and prepare for meiotic division. Classically, meiotic prophase I is divided into five stages related to changes in chromosome morphology. During *leptotene*, individual chromosomes, each consisting of two sister chromatids, are visible as thin strands, and meiotic recombination is initiated by the formation of DNA double-strand breaks (DSBs). During *zygotene*, homologous chromosomes pair and align along their lengths; the proteinaceous synaptonemal complex (SC) forms and stabilizes the alignment between paired chromosomes, which are now referred to as bivalents, or tetrads. In *pachytene*, SC formation is complete, and meiotic recombination among DNA molecules of non-sister chromatids is accomplished through repair of DSBs. The following stage, *diplotene*, is usually described as the stage where SC disassembles and chiasmata become visible. Each chiasma corresponds to a position where crossover occurred and where non-sister chromatids remain associated, while the other regions of homologous non-sister chromatids are largely separated from each other. Finally, at *diakinesis*, bivalents continue to condense and reorganize into more compact and contracted structures in preparation for meiotic division.

Mammalian oocytes enter meiotic prophase I during fetal life, after a period of DNA replication. In the fetus, following leptotene, zygotene, and pachytene stages, when chromosome condensation and recombination occur, the meiotic cell cycle arrests at the diplotene stage until after puberty. This protracted *diplotene arrest*, also known as the *dictyate stage*, may last for several weeks months or years depending on the species. When the oocytes enter the dictyate stage, their chromosomes become dispersed, less distinct, and appear to form a faint network [1, 2], while a single layer of flattened granulosa cells surrounds the oocytes, thus forming the primordial follicles. These follicles are quiescent and constitute a pool where they will be recruited from for growth and cyclically selected for ovulation during reproductive life. Concomitant to follicle development, the growth phase of the oocytes occurs, when their diameters increase considerably due to massive synthesis of cytoplasmic components. This is a crucial phase for the subsequent early embryonic development. At this stage, chromosomes decondense and chromatin becomes transcriptionally active. In the oocyte nucleus, intense RNA synthesis is detectable until it stops toward the end of the growth phase. Timing of completion of the growth phase and of transcriptional inactivation in relation to follicular development is species-dependent. Thus, for example, the growth of mouse oocytes is already completed in the early antral follicles [3], while bovine oocytes are still in their growth phase [4]. Once the growth phase is concluded, the sole fully grown oocytes selected for ovulation resume meiosis in response to luteinizing hormone (LH), while subordinate follicles, enclosing oocytes that have not been selected for ovulation,

undergo degeneration through atresia. Although in several mammals – including mice, humans [5], and large domestic species [6, 7] – a certain follicular growth activity can be detected during prepubertal life, meiotic resumption occurs only after puberty is reached. In laboratory practice, meiotically competent oocytes are capable of spontaneous (LH-independent) meiotic resumption once they are removed from their follicle and placed in a suitable culture environment. Upon meiotic resumption, oocytes enter diakinesis; bivalents condense and individualize in preparation for the first meiotic divisions. The nuclear envelope breaks down, and meiosis continues to the metaphase of meiosis II, occurring when the oocytes are released from follicles. This process is referred to as oocyte maturation.

The nucleus of oocytes arrested at the diplotene I stage shows an intact nuclear envelope and is named *germinal vesicle* (*GV*), while the breakdown of this nuclear envelope is known as GV breakdown (GVBD). Authors use the expression "*GV stage*" to indicate when the oocyte is collected from the ovarian follicle, or even the period when the GV is clearly detectable within the oocyte before GVBD occurs. Note that the "GV stage" is not to be confused with the phase of meiotic arrest, or dictyate stage, since meiotic resumption is initiated well ahead of the nuclear envelope disappearance at GVBD. Therefore, even though GVBD is perhaps the first clear manifestation of meiotic resumption, these two events are not to be considered equivalent.

During meiotic arrest and particularly during the oocyte growth phase leading to the formation of the fully grown and differentiated oocyte, the chromatin enclosed within the GV is subjected to several levels of regulation controlling both its structure and function. These events include mechanisms acting both locally, on specific loci, and on a large scale to remodel wide portions of the oocyte genome. *Morphologically, the chromosomes lose their individuality as well as their characteristic appearance and form a loose chromatin mass* [2, 8], *which in turn undergoes profound and dynamic rearrangements within the GV before the meiotic resumption***.** In the next sections, we refer to these rearrangements as to "*large-scale chromatin configuration changes*."

(General bibliographic references for this part: [9–13]).

Large-Scale Chromatin Configuration(s) in Mammalian Oocyte

Different techniques for sample preparation and DNA staining have been used to assess chromatin configuration within the GV of mammalian oocytes. Early studies in rat [2], mouse [14], human [8], pig [15–17], cattle [18, 19], and horse [20] described chromatin configuration in growing, fully grown, and in vivo and/or in vitro maturing oocytes by using different methods that include DNA staining with orcein after acetic alcohol fixation, light and electron microscopy techniques, DNA staining with Giemsa after hypotonic treatment and acetic alcohol fixation, and other histological methods for preparation and staining of ovarian sections.

However, the characterization of mammalian oocyte's chromatin configurations to date is based on fluorescence microscopy, in which DNA is stained with fluorescent dyes such as DAPI (4′,6-diamidino-2-phenylindole), Hoechst, propidium iodide, SYBR Green, SYTOX Green, CMA_3 (chromomycin A_3-distamycin A-4, 6-diamidino-2-phenylindole), or with other commercially available fluorescent dyes. Usually oocytes are freed of cumulus cells and fixed in formaldehyde-containing solutions, stained, and finally mounted on a slide for epifluorescence or laser-scanning confocal microscopy analysis. The three-dimensional organization of the nuclear components is typically preserved by mounting the samples between a coverslip and a glass slide supported by small columns of a mixture of Vaseline and paraffin and gently compressing them between slide and coverslip. Alternatively, living (not fixed) oocytes can be observed under an inverted fluorescence microscope, while maintained in suspension in drops of medium containing Hoechst dyes, which are cell permeable and can bind to DNA both in live and in fixed cells [21–23]. Fluorescence microscopy is frequently used in combination with differential interference contrast microscopy and/or phase contrast microscopy in order to verify the presence of an intact

nuclear envelope and/or the nucleolus. Finally, chromatin configuration can be assessed in ovarian cryosections after DNA fluorescent staining.

Using these techniques, Matson and Albertini in 1990 [22] described changes in the organization of the mouse oocyte's chromatin with respect to progressive stages of follicular development. In their study, chromatin organization was classified into discrete patterns in which the chromatin mass is initially found dispersed throughout the nucleoplasm and appears mainly decondensed, with the exception of some aggregates of condensed chromatin. Thereafter, chromatin condensation increases, and few chromatin foci start to associate with the nucleolar periphery until a complete rim of heterochromatin is formed in close apposition with the nucleolus, which is not active at this stage and is referred to as "nucleolar-like body," or nucleolar remnant. Further studies confirmed these observations. Consequently, the presence or absence of a complete rim of condensed chromatin around the nucleolus is considered to be the morphological discerning element for the classification of mouse GV oocytes. The configuration in which the perinucleolar chromatin rim is absent is typically named "non-surrounded nucleolus" (NSN), while the configuration where the perinucleolar chromatin rim is present is termed "surrounded nucleolus" (SN) [24–26]. Intermediate configurations have been described as "partly NSN" and "partly SN" [27]. Notably, NSN-type configuration is typical of oocytes collected from primordial to growing preantral follicles. Nonetheless, NSN oocytes are also found in antral follicles that indeed enclose both NSN and SN oocytes, with the percentage of SN oocytes increasing with the increase in follicle diameter. The origin of the oocyte should always be carefully considered when studying mouse oocytes with NSN chromatin configuration, since oocytes from preantral follicles and oocytes from antral follicles differ significantly. Some authors refer to such oocytes as to "fully grown" NSN oocytes, indicating that they originated from antral follicles [11].

Starting from studies in the mouse model, GV chromatin configurations have been assessed in several mammals by means of fluorescence microscopy analysis. Thus, changes in large-scale chromatin organization during oocyte growth and differentiation as it occurs within the ovarian follicle have been reported in monkey [28], pig [29], human [30, 31], and horse [32, 34] and reviewed in [33], cattle [21, 35–37], goat [38], sheep [39], rabbit [40], buffalo [41], dog [42–44], ferret [45], and cat [46] (see Table 7.1).

The formation of the perinucleolar rim of condensed chromatin has been reported to be a typical feature in some of the mentioned above species, including human, pig, and monkey. However, notable exceptions have also been described. For example, in cattle and horse, chromatin condenses into a single compact clump instead of forming a clearly detectable rim around the nucleolus [21, 33, 34]. Further electron microscopy studies in cow, though, have revealed that chromatin in these oocytes is mainly located near the inactive nucleolar structure, or nucleolar remnant, that in turn appears to be partially or completely encapsulated by heterochromatin [47].

It is worth mentioning here that to describe GV chromatin configuration, many different criteria of classification together with a great variety of names and acronyms (Table 7.1) have been proposed, differences that, beyond some species specificities, can also depend on the research group. This might add complexity into the process and generate confusion in data interpretation. Therefore, whenever possible, labs are encouraged toward more uniformity on the issue.

Nevertheless, what is clear from scientific literature is that configuration of chromatin enclosed within the GV of the prophase I-arrested mammalian oocyte condenses and progressively rearranges passing through intermediate configurations. In fact, chromatin occurs in a less condensed state, in which it is dispersed throughout the nucleoplasm, and then remodels into a more condensed state where its mass is concentrated in a small area of the nucleoplasm, often in close association with the inactive nucleolus (Fig. 7.1).

This process of chromatin condensation and spatial reorganization typically occurs during growth and differentiation of the oocytes within the ovarian follicle, reaching high level of condensation before meiotic resumption. Numerous studies on different mammals report and demonstrate that increase in chromatin condensation can be related

Table 7.1 Chromatin configurations in diplotene I arrested oocytes of different mammalian species

Species	Chromatin Configuration	Reference
Mouse	Stage I GV; Stage II GV; Stage III GV; Stage IV GV	Mattson and Albertini [22], Wickramasinghe et al. [25]
	NSN; SN	Debey et al. [24], Zuccotti et al. [26]
	NSN; partly NSN; partly SN; SN	Bouniol-Baly et al. [27]
Monkey	GV1; GV2; GV3	Schramm et al. [28]
Human	Group 1; Group 2; Group 3	Miyara et al. [31]
	Pattern A; pattern B; pattern C; pattern D (occurring mainly after culture)	Combelles et al. [30]
Horse	Fibrillar; intermediate; loose condensed; tight condensed; fluorescent nucleus (=degenerated)	Hinrichs et al. [32, 33]
	Fibrillar; intermediate; condensed	Franciosi et al. [34]
Cattle	GV0; GV1; GV2; GV3	Lodde et al. [21]
	Category 1; Category 2; Category 3	Fuhrer et al. [36]
	GV I; GV II; GV III; GV IV; GV V	Chohan and Hunter [35]
	NSN; N; C; SN; F (occurring only after culture)	Liu et al. [37]
Goat	GV1; GV2n; GV2c; GV3n; GV3c; GV4	Sui et al. [38]
Sheep	NSN; SN; SNE	Russo et al. [39]
Pig	FC; SC; GVI	Bui et al. [29]
Rabbit	NSN; NL; LC; TC; SC	Wang et al. [40]
Buffalo	GV I; GV II; GV III; GV IV; GV V	Yousaf and Chohan [41]
Dog	GV I; GV II; GV III; GV IV; GV V	Lee et al. [42], Jin et al. [43]
	Type I; Type II; Type III	Reynaud et al. [44]
Cat	Filamentous; reticular	Comizzoli et al. [46]
Ferret	FC; ICC; CC	Sun et al. [45]

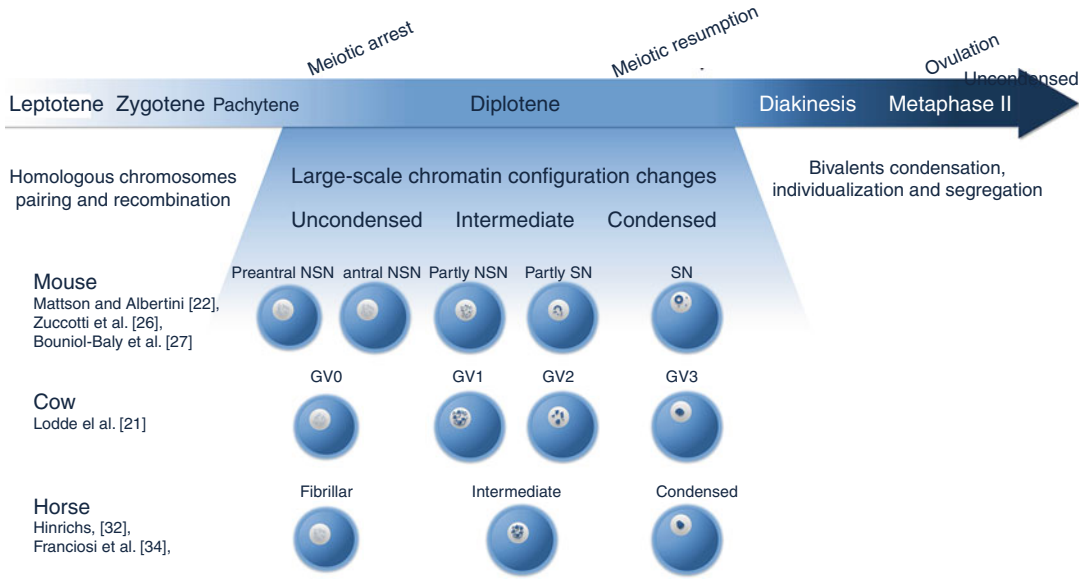

Fig. 7.1 Schematic representation of large-scale chromatin configuration changes in mammalian oocytes

to progressive increase in oocyte size and to formation and development of the antral follicle [21, 22, 24, 26, 28, 29, 31, 35–37, 39–41, 46, 48, 49].

Both the exact role of the perinucleolar rim of heterochromatin and the mechanisms of its formation are not known and remain thought-provoking

questions to answer. In mouse and human, this structure goes under the name of the "karyosphere" [8, 11, 50] that is regarded as "*the result of all chromosomes of the gametocyte joining in a limited nuclear volume with final formation of a single complex chromatin structure*" [51]. Considering this definition, it is clear that the single compact clump of chromatin found within the oocyte GV at the end of the process of chromatin condensation in bovine, in horse, and in other mammals resembles the karyosphere. Strikingly, a similar structure, named karyosome, exists in Drosophila as well as in many other phylogenetically distant organisms studied so far [51]. It has been hypothesized, but not experimentally proved yet, that this structure may fasten the chromosomes into a precise and restricted volume within the large GV [51].

The exact role of the karyosphere also requires further and careful studies. Undoubtedly, if we knew more about its composition, we would better understand its function. For example, studies in mouse oocytes have revealed that centromeres are an important component of the karyosphere [50, 52, 53]. This is crucial in that centromeres are the specific regions in each chromosome where the kinetochores that provide interaction sites with the spindle microtubules are assembled. In mouse SN oocytes, the transient disruption of the karyosphere, obtained by a pharmacological treatment using an inhibitor of histone deacetylases, trichostatin A (TSA), results in errors during the subsequent meiotic division. This is a clear indication of the importance of the karyosphere formation for the function of the centromeres [50]. Whether this mechanism is conserved among mammals, especially in those where the perinucleolar rim is not evident, remains to be established.

Significance of Large-Scale Chromatin Configuration Changes on Oocyte Competence

An important developmental step during mammalian oocyte growth and differentiation is represented by the transition from less condensed to more condensed states of chromatin configuration. In fact, oocytes showing high levels of chromatin condensation have been related to an increased embryonic developmental potential when compared with oocytes whose chromatin is less condensed, a relation that much experimental evidence has confirmed.

For example, in different species it occurs that oocytes have a higher capability to mature in vitro when isolated from large antral follicles that enclose a big proportion of oocytes showing advanced stages of chromatin condensation, in comparison with oocytes derived from smaller follicles where uncondensed chromatin is mainly noticed [17, 25, 28, 36, 38, 40, 41, 49]. Similar correlative relations are found when comparing chromatin configurations and meiotic competence of oocytes isolated from adult versus prepubertal or fetal ovaries [25, 35]. Moreover, in human and mouse, meiotically incompetent oocytes have been shown to retain GV with uncondensed chromatin after in vitro maturation [25, 48].

Further and direct evidence that oocytes with condensed chromatin are more capable of in vitro early embryonic development was firstly provided by murine studies, where living oocytes from antral follicles were divided into NSN and SN oocytes after staining with Hoechst 33342 and then subjected to standard procedures for in vitro embryo production [23, 54]. These studies demonstrated that after in vitro maturation and fertilization, NSN oocytes were incapable of any development beyond the two-cell stage, whereas SN oocytes progressed into the blastocyst stage. In cattle, a similar experimental approach gave further insights into this process [21]. For animals in this species, living bovine oocytes were considered and classified into four stages based on their chromatin configuration under fluorescence microscopy analysis and cultured in vitro with a system that enables embryonic development of denuded oocytes [55]. Specifically, the GV0 stage is characterized by a diffuse filamentous pattern of chromatin in the whole nuclear area; the GV1 and GV2 configurations represent early and intermediate stages, respectively, of chromatin remodeling, a process starting with the appearance of few foci of condensation in GV1 oocytes and proceeding with the formation of distinct clumps of condensed chromatin in GV2 oocytes; the GV3 is the stage where the maximum level of condensation is

reached with chromatin organized into a single clump. Importantly, when matured in vitro, oocytes with a GV0 configuration showed a very limited capacity to resume and complete meiosis I, while virtually all the GV1, GV2, and GV3 oocytes were able to reach MII stage, despite their GV configuration. On the contrary, after in vitro fertilization and embryo culture, only a limited percentage of GV1 oocytes reached the blastocyst stage, while GV2 and GV3 oocytes showed a higher embryonic developmental potential [21].

These results further support the notion that competencies are acquired concomitantly with changes in large-scale chromatin structure at sequential stages of oogenesis [11, 56] and, more precisely, they indicate that meiotic competence acquisition is timely related with the appearance of early signs of chromatin condensation, while embryonic developmental competence is acquired before the highest level of chromatin condensation is reached [21].

Whereas acquisition of meiotic competence at the beginning of the process of chromatin condensation seems to be a common feature in mouse and horse [23, 24, 57], the embryonic developmental potential in oocytes with intermediate chromatin configurations has not yet been assessed in species other than the cow. Moreover, it remains to be established whether transition to the highest level of chromatin condensation is strictly required for the successful completion of meiosis and early embryonic development.

Large-Scale Chromatin Configuration Changes and Transcriptional Activity

In all mammals studied so far, changes in chromatin configuration are temporally correlated with the onset of transcriptional silencing in the oocyte nucleus [27, 31, 38, 40, 46, 47, 58–60]. In the mouse, NSN oocytes are transcriptionally active and synthesize all classes of RNA, whereas the acquisition of the SN configuration correlates with global repression of transcription in vivo as well as in cultured cumulus-oocyte complexes [27, 31, 58–61]. More precisely, transcriptional activity seems to cease completely as soon as condensed chromatin begins to associate around the nucleolar-like body [27]. Likewise, in bovine oocytes, the GV0 configuration, with uncondensed chromatin, is always associated with high levels of transcription, which in turn is globally silenced in GV3 oocytes, where condensed chromatin is organized in a single clump. Importantly, a major drop in transcription is temporally linked to initial chromatin condensation that occurs during the GV0 to GV1 transition [47, 62].

These observations may indicate that chromatin architecture regulates transcriptional activity directly. The reciprocal connection of the two events is, to some extent, demonstrated by the recent finding that the NSN to SN transition is significantly impaired and transcriptional repression is incomplete in mouse oocytes in which the developmental pluripotency-associated protein 3 (Dppa3, also known as PGC7 or Stella) is knocked down [63]. However, studies in mouse have illustrated that transition into the SN configuration is not strictly required for transcriptional repression. In fact, both large-scale chromatin configuration changes and global transcriptional silencing can be experimentally dissociated in in vivo as well as in vitro models. For example, in oocytes from nucleoplasmin 2 null females, transcriptional silencing occurs even though chromatin does not organize into the SN configuration, while transcription remains silenced when the SN configuration is disrupted and reverted to an NSN-like configuration after treatment with TSA [50]. Moreover, transcriptional silencing fails to occur in oocytes from histone 3 lysine 4 methyltransferase (MLL2) conditional knockout females, even though chromatin reorganizes into the SN configuration [64]. Therefore, large-scale chromatin condensation does not seem to be the direct and primary cause of transcriptional silencing, and, conversely, the establishment of the SN configuration does not depend primarily on the acquisition of global transcriptional repression. Hence, these two events might be controlled, at least partially, by independent mechanisms. Accordingly, recent findings in the murine model indicate that RPB1, the largest subunit of RNA polymerase II, is bound to DNA in NSN transcriptionally active mouse oocytes, while it is found DNA-dissociated in SN transcriptionally inactive oocytes, which indicates that global

repression of transcription may be due to RNA polymerase II dissociation from the DNA of the oocyte [61]. These results also suggest that chromatin condensation state is the cause of RNA polymerase II dissociation from DNA. However, experimental disruption of SN configuration with TSA does not cause reassociation of RPB1 with DNA in loosely structured chromatin resembling NSN configuration. Additionally, when the SN configuration is experimentally induced by use of RPB1 inhibitor (5,6-dichloro-1-β-D-ribofuranosylbenzimidazole, DRB), RPB1 remains bound to DNA [61]. All together these data suggest that RNA polymerase II dissociation from DNA occurs independently of the chromatin condensation state [61], which might explain why transcription could persist in SN oocytes under certain circumstances [64]. On the other hand, the fact that transition from NSN to SN chromatin configuration occurs after treatment with the RNA polymerase II inhibitor DRB suggests that chromatin condensation may be a consequence of RNA polymerase II-dependent transcriptional silencing [63]. Interestingly, it has been proposed that increase in chromatin condensation may reinforce transcriptional repression once it is initiated [50]. A hypothesis to be confirmed, similarly, primary mechanisms triggering transcriptional repression in mammalian oocytes deserve intensive investigation.

Consistent with the state of global transcriptional repression that characterizes oocytes with highly condensed chromatin, their nucleoli are typically found as inactive structures, referred to as "nucleolar-like bodies" (NLB) or "nucleolar remnants." Ultrastructurally, these nucleoli appear compact and composed of dense material, as those described in mouse and human oocytes [8, 24, 27, 31], or as electron-dense fibrillar spheres with a remnant fibrillar center on the surface, as those described in bovine and pig oocytes [47, 65, 66]. On the contrary, nucleoli of bovine oocytes with decondensed chromatin (GV0 stage) show the typical morphology of nucleoli that are actively engaged in rRNA synthesis, with eccentric fibrillar centers and electron-dense granules aggregated in the form of electron-dense clusters. Nucleoli of NSN antral oocytes in mouse and human are in a more advanced stage of nucleolar inactivation than those described in bovine GV0 oocytes, and therefore they are still referred to as NLB, but possess vacuoles [8, 24, 27, 31] and, in mouse, accumulation of fibrillar/granular material at the periphery corresponding to rRNA transcription sites [27].

Clearly, such diversities reflect dissimilar metabolic properties of oocytes showing diverse chromatin configurations, which in turn could be the basis for different capacities of in vitro development, as the synthesis and storage of maternal transcripts before transcriptional repression enables the oocyte to complete meiosis and to initiate embryogenesis. In this view, oocytes with uncondensed chromatin are still in the process of accumulating transcripts and other molecules indispensable for further development.

Recently, by using a microarray analysis approach, the metabolic states of two classes of oocytes – metaphase II (MII) stage oocytes obtained after in vitro maturation of NSN and SN antral oocytes – were definitively confirmed to differ. Importantly, Oct-4 expression was shown to be dysregulated in MII oocytes obtained from NSN oocytes, when compared with MII from SN, which also leads to an altered expression of other important genes involved in several biochemical pathways [67–69].

Large-Scale Chromatin Configuration Changes and Cytoplasmic Maturation

Coordination between chromatin configuration changes and rearrangement of cytoplasmic components before meiotic resumption was first examined and described in mouse [22, 25]. Microtubules form an extensive interphase-like network in the cytoplasm of the oocytes of preantral follicles with highly uncondensed chromatin and start to localize to perinuclear areas of the ooplasm in concomitance with the initial increase in chromatin condensation. A step followed by a progressive reduction in the density of the microtubule network that is eventually replaced when prominent

microtubule-organizing centers (MTOC) appear in oocytes with SN configuration [22]. This correlation, together with the finding that more than 80 MTOCs form in the cytoplasm of the oocyte before GVBD, was definitively demonstrated by using high-resolution confocal imaging in living mouse oocytes [70]. This outstanding work also revealed that the vast majority of MTOCs form de novo from the cytoplasmic microtubule network and confirmed that this process is important for spindle assembly during later stages of meiotic progression [70].

Additional confirmation that chromatin configuration changes are temporally correlated with cytoplasmic events comes from studies in cow [47], in which the progressive increase in chromatin condensation and its large-scale reorganization have been related to key structural modifications and redistribution of the cytoplasmic organelles that are in turn necessary for the oocyte to achieve meiotic and developmental competences [47, 71, 72]. Typical features denoting completion of the oocyte growth phase, such as undulation of the nuclear envelope and reduction in size of the Golgi complex in the oocyte cortex, were found to be associated with high levels of chromatin condensation in oocytes with GV2 and GV3 configurations, thus resembling the process of "oocyte capacitation" [72] that typically characterizes the bovine oocyte differentiation within the dominant follicle, during preovulatory development before LH surge [72, 73]. It is interesting to mention here that structural changes associated with oocyte degeneration in subordinate follicles, usually referred to as "pseudo-maturation" [73], also show similarities to the process seen during capacitation in dominant follicles [73, 74]. Therefore, it is not surprising that some features of early atresia, such as degeneration of cortical granules and reduction in the intercellular coupling between the oocyte and cumulus cells (see below), are typical of GV3 oocytes, since oocyte developmental competence appears to be improved by low levels of atresia [75–79], reviewed in [74, 80, 81]. Thus, oocytes with the highest level of chromatin condensation could represent that proportion of gametes that had reached a high developmental capability during follicular growth and that were undergoing early events of atresia at the time of collection, as hypothesized in other species [21].

Mechanisms Involved in the Control of Large-Scale Chromatin Configuration Changes

The molecular mechanisms regulating changes in large-scale chromatin configuration in the mammalian oocyte still remain largely unknown. Nonetheless, it is clear that multiple factors can contribute to this process. In the following paragraph(s) we discuss some of the latest studies and their first success in approaching this complex mechanism, though aware that this review cannot possibly be comprehensive.

As discussed above in section "Large-scale chromatin configuration changes and transcriptional activity", interferences with the expression and function of several molecules can affect the process of chromatin reorganization into the SN configuration. Thus, nucleoplasmin 2 [50, 82] and DPPA3 depletions [63] all prevent the establishment of the SN configuration in mouse oocytes. Whether these molecules participate in the control of large-scale chromatin configuration remodeling through independent mechanisms or rather they are interconnected remains to be established. Moreover, the extent to which the function of these molecules is conserved among mammals is of utmost importance.

Histone tail modifications (acetylation, methylation, phosphorylation, ubiquitination, etc.) as well as changes in the global level of DNA methylation, known to influence chromatin structure and function, have been proposed as possible modulators of large-scale chromatin configuration in mammalian oocytes (for examples, see: [11, 34, 39, 83–87]). However, to date, there has been more research focused on identifying modifications rather than providing extensive insight into their function. To verify the specific role of each modification, research should stress on the function of the enzyme(s)

responsible for such modification, as already done for H3K4 methylation and MLL2 [64]. Among histone-modifying enzymes, histone deacetylases (HDACs) seem to participate in the maintenance of the SN configuration in mouse oocytes [50]; in fact, in oocytes exhibiting the SN configuration, pharmacological inhibition of HDACs with TSA induces chromatin decondensation and formation of NSN-like configuration. However, as TSA treatment does not change the levels of H3 and H4 acetylation, the mechanism by which HDACs alter SN configuration remains undetected.

Interestingly, the function of the histone H2A kinase (NHK-1) has been proved necessary for the formation of the karyosome, a structure that resembles the mammalian karyosphere (see section Large-Scale Chromatin Configuration(s) in Mammalian Oocyte") in Drosophila oocytes [86, 88], as chromatin of oocytes from nhk-1 mutant females fails to organize into a karyosome and is rather located at the nuclear periphery in a beaded aspect. Moreover, H2AT119 phosphorylation and H3K14 and H4K5 acetylation, while present in wild-type karyosomes, are altered in the chromatin of oocytes bearing nhk-1 mutation, which indicates that these modifications are likely to be involved in karyosome formation [88]. Whether these functions are conserved in mammalian oocytes is still an intriguing question.

Additional experiments in nhk-1 mutant Drosophila oocytes have revealed that disassembly of the synaptonemal complex and condensin loading are impaired in the absence of NHK-1 function, thus suggesting that these two processes may also be implicated in the formation of karyosome [86, 88]. Noticeably, studies in mouse and pig oocytes can confirm a critical role for condensin I and II in many aspects of chromosome segregation during meiosis such as individualization and resolution [89, 90]. Moreover, they can indicate that while some of the specific subunits of condensin I localize in the cytoplasm before GVBD, subunits of condensin II are present in the nucleus of the oocyte. Therefore, if condensin function in mammalian karyosphere formation is conserved, it is probably due to condensin I rather than to condensin II.

Role of Cumulus Granulosa Cells and Gap Junction-Mediated Communications in the Control of Large-Scale Chromatin Configuration Changes and Transcriptional Silencing

As discussed in the previous sections, to date, the mechanisms involved in the control of large-scale chromatin configuration changes and transcriptional silencing are poorly understood. Moreover, how these two events are coordinated during oocyte growth and differentiation remains largely unknown. Most likely, strategies set in place for the control and coordination of these events are part of a complex physiological process that ultimately confers the oocyte with meiotic and developmental competence.

Studies in mouse and bovine indicate that ovarian granulosa cells and their coupling with the oocytes through gap junctions are implicated in such a process. In the mouse, when gap junction-mediated communication (GJC) between the oocyte and cumulus cells is prevented, due to targeted deletion of the connexin 37 gene (Gja4), transcriptional silencing, normally occurring in wild-type mice during oocyte growth, is impaired and chromatin remains in an uncondensed state instead of acquiring the typical SN configuration [91]. Similarly, when mouse oocytes from preantral follicles are cultured in vitro, the presence of associated granulosa cells is necessary for the coordination of large-scale chromatin configuration with the onset of transcriptional silencing, at chronological stages equivalent to those of in vivo grown oocytes. On the contrary, if the oocytes are cultured in the absence of granulosa cells, as denuded oocytes, transcriptional silencing fails to occur and chromatin does not organize into the SN configuration, but remains uncondensed with transcription sites that appear to be uniformly distributed instead of being more pronounced in certain areas of the chromatin, as judged by Br-UTP incorporation [59].

The central role of oocyte-cumulus cells GJC in the modulation of chromatin configuration and transcription has been confirmed in bovine oocyte-cumulus cells complexes. At the time of

collection from antral follicles, the pattern of uncondensed chromatin in bovine GV0 oocytes associates with a fully open state of GJC between the oocyte and the surrounding cumulus cells, whereas the percentage of oocytes with functionally open communications significantly decreases with the increase of chromatin condensation, from GV1 to GV3 oocytes [21]. Furthermore, when cumulus-oocyte complexes from early antral follicles, in which chromatin is mostly decondensed (GV0), are cultivated in vitro with low doses of FSH, the maintenance of a patent bidirectional coupling allows chromatin to gradually organize into the GV1 configuration, thus acquiring the ability to mature and be fertilized in vitro [62]. On the contrary, in the absence of hormonal treatment, coupling is not sustained, and chromatin remains mainly in an uncondensed state. Strikingly, treatment with higher doses of FSH does not support functional coupling either, and it seems likely to force the oocyte to resume meiosis, without an orderly remodeling of chromatin configuration. Thus, the apparent increased ability to resume meiosis is not accompanied by an equal increase of fertilization competence [62].

Additional experiments in the bovine model, where low doses of FSH sustain GJC functionality in cumulus-oocyte complexes from early antral follicles, give further insight into the mechanisms by which GJC may modulate changes in large-scale chromatin configuration and transcription. In this system, treatment with the uncoupler 1-heptanol induces sudden condensation of chromatin and decrease in transcription. However, this effect is nullified by addition of cilostamide to culture medium [62]. This indicates that the functional status of GJC may affect both transcriptional activity and remodeling of large-scale chromatin configuration, potentially through cAMP-dependent mechanism(s), because cilostamide acts as a specific inhibitor of the oocyte-specific PDE3, an enzyme-degrading cAMP [92–94]. Besides the well-characterized mechanisms of action by which cAMP is known to regulate meiotic resumption (Reviewed in [95, 96], also see chapter by L. Mehlmann (Chap. 12) in this book), the present results may suggest that cAMP could be also involved in the control of the activity of factors that modulate transcription and large-scale chromatin remodeling during the final phase of oocyte growth and before the resumption of meiosis.

Interestingly, while in mouse the absence of a patent bidirectional communication caused the majority of oocytes to remain transcriptionally active with uncondensed chromatin [59, 91], in the bovine GJC disruption by means of 1-heptanol caused premature chromatin condensation and transcriptional interruption. However, it should be noted that these experimental models differ substantially in some aspects; thus, it remains to be fully investigated whether this discrepancy might be due to a different physiological status of the animal model or to the growth phase of the follicle from which an oocyte is isolated. Notably, it cannot be excluded that simply the timing when the functional coupling between oocytes and cumulus cells is interrupted could determine the effect on chromatin structural and functional changes [62].

Implications of Large-Scale Chromatin Configurations in Basic Science and in Assisted Reproductive Technologies

No doubt that the analysis of the functional and structural modifications that accompany large-scale chromatin configuration changes in the oocyte will have wide-ranging implications for understanding: the role of nuclear organization in meiosis, the events of nuclear reprogramming, and the spatiotemporal regulation of gene expression during oocyte and embryo development. Moreover, experimental manipulation of large-scale chromatin configuration in vivo and in vitro will provide a tool to determine the key cellular pathways and oocyte-derived factors involved in genome-wide chromatin modifications. In this context, micromanipulation techniques, such as GV transfer [46, 97, 98] that allows the transplantation of an isolated GV from one oocyte to another, would deepen the knowledge of fundamental processes in oocyte biology, for example, the coordination between nuclear and

cytoplasmic events and the relative contribution of the nuclear and cytoplasmic compartments to the competence of the oocyte. Using this technique, for instance, the nuclei of SN and NSN mouse antral oocytes were reciprocally exchanged, and the meiotic and developmental competences of the reconstructed oocytes were assessed. Results of these experiments indicate that cytoplasmic factors are responsible for the oocyte's meiotic competence, while nuclear factors are responsible for the embryonic developmental competence [97].

Assessment of large-scale chromatin configurations has also large implication in assisted reproductive technologies (ART) in humans and domestic species. It has been shown that different patterns of chromatin configuration are indicative of different metabolic properties; thus, they could represent a morphological marker to select a population of oocytes with different culture requirements. Several studies support the idea that treatments aimed to improve the developmental capability of immature oocytes can have a different effect with "prematuration culture" depending on the metabolic status of the oocyte at the moment of its removal from the follicular environment [99–101]. They suggest that prematuration culture may be of greater benefit to growing oocytes, allowing them to accelerate through their growth phase and achieve developmental competence [102]. This is confirmed also by morphological studies in bovine, which demonstrated that pharmacological pretreatment can negatively affect oocyte belonging to medium antral follicles when compared with those isolated from smaller follicles [103]. Accordingly, findings in the mouse indicate that it is crucial to consider the transcriptional activity of the oocyte when attempting to maintain the female gamete in meiotic arrest in vitro for protracted periods; in fact, experimentally extending the interval between transcriptional inactivation and resumption of meiosis may be deleterious to subsequent embryonic development [59]. In view of these considerations, success in attempting to improve the oocyte developmental capability in vitro may be dependent on the actual growth stage of the oocyte, when subjected to treatment. Surely, the possibility to predict large-scale chromatin configuration on the basis of morphological characteristics of the oocyte or of the cumulus-oocyte complex, as found in mouse and human [104, 105], would considerably improve the application of such cultural strategies.

It is of extreme importance noticing here that attempts to manipulate in vitro large-scale chromatin configuration must be performed cautiously. In fact, even though it is true that the chromatin configuration of an oocyte is indicative of its developmental capability *at the time of its collection from the follicle,* pharmacological treatments forcing chromatin abruptly into a high condensed state may not necessarily be beneficial to the oocyte competence although fundamental in basic science-type investigation [46]. Therefore, design of prematurative strategies must take into account that chromatin condensation and spatial reorganization should occur gradually and orderly, recapitulating the process that normally occurs in vivo. For example, maintenance of a proper functional coupling between oocyte and cumulus seems to be crucial in sustaining an orderly chromatin condensation in vitro [62]. Thus, if coupling is prematurely interrupted – that is, when oocytes have not yet acquired full competence as it is when they are still committed to accumulating transcripts and other molecules – unexpected chromatin condensation can be triggered, thus preventing proper and gradual differentiation of large-scale chromatin configuration and function. In view of all given considerations, knowledge of the molecular mechanism(s) leading the oocyte to remodel its chromatin configuration under physiological conditions will be of great help for ART.

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The Quest for Oogenesis (Folliculogenesis) In Vitro

8

Sergio Romero, Sandra Sanfilippo, and Johan Smitz

Abstract

Because of the improvements in efficacy of cancer treatments, the rates of cancer survivors are constantly increasing; however some of these more aggressive anti-cancer therapies endanger fertility.

Although the use of classical Assisted reproduction techniques, like *in vitro* fertilisation (IVF) and embryo freezing/transfer (Frozen embryo transfer, FRET), are an option for certain adult patients, these techniques depend on the amount of oocytes obtained after controlled ovarian stimulation. This treatment is not suitable for children or adolescent patients, and potentially unsafe when using it in breast cancer patients. Therefore, adolescent and adult female cancer patients are being offered the possibility to cryopreserve pieces of their ovarian tissue with the ultimate goal of restoring their fertility when they are disease free.

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Ovarian tissue grafting has proven successful, however the latent risk for reintroducing malignant cells, enforces the search for alternatives. *In vitro* culture of cryopreserved tissue, and of ovarian follicles isolated from this tissue, are challenging alternatives. Techniques for culturing ovarian tissue and follicles are under development and may benefit from advances in molecular techniques, innovative culture devices and artificial extracellular matrices.

This review focuses on state-of-the-art culturing techniques for ovarian follicles and reports on the most recent advances in the field in animal models and in human.

Keywords

Folliculogenesis • Oogenesis • Follicle • Oocyte • Follicle culture Oocyte competence

The constant progress in oncological diagnosis and treatments has greatly improved the long-term survival of adolescent and adult female cancer patients. However, the use of alkylating agents or ionizing radiation induces one of the most unbearable side effects: the premature ovarian failure (POF) [1]. Fertility preservation and restoration is a fastly evolving field in assisted reproductive technologies (ART): it aims to offer patients the possibility to bank gametes or gonadal tissue to restore their fertility after curing from cancer or other benign fertility threatening diseases.

Ovarian tissue cryopreservation is currently the only possible alternative to preserve the fertility of patients who cannot delay their treatment or who are not sexually mature to undergo ovarian stimulation for oocyte retrieval and/or embryo cryopreservation after fertilization. Once the treatment is completed and the patient is disease-free, the reimplantation of frozen/thawed ovarian cortical tissue offers the possibility to restore endocrine and reproductive function. To date, 18 live births have been reported worldwide after orthotopic transplantation of frozen/thawed tissue.

With transplantation, there is always a risk that in cases of aggressive metastatic cancers, malignant cells residing in the transplanted ovarian tissue pieces could retransmit the disease to cured cancer survivors [2]. In vitro folliculogenesis emerges as a safe technique, which would allow the growth of follicles and ultimately the production of fertilizable oocytes.

The fact that the gametes at the very immature stage (primordial follicles) are better suited to stand the cryopreservation procedure makes currently available ART techniques, like oocyte in vitro maturation (IVM) or IVF, insufficient as a stand-alone prophylactic procedure.

Growing primordial follicles in vitro, after cryostorage, imposes the need to develop in vitro systems, which at first would allow the reinitiation of growth and further sustain follicular development and the obtainment of healthy mature oocytes.

Over the last two decades, several culture systems have been developed with the ultimate aim to produce oocytes in vitro from small follicles. This in vitro production requires a good understanding of the physiological processes that occur within the ovarian follicle and determine the right environment for the development of the oocyte.

Although some studies had already proven the feasibility of growing follicles or oocyte-granulosa cell complexes in vitro, which were previously cryopreserved as follicles or within the ovarian tissue [3–8], the optimization of currently available techniques for both cryopreservation and in vitro culture will allow the mass production of oocytes that could be fertilized and give rise to live births (for review see Smitz et al. [9]).

In Vitro Folliculogenesis

Recruitment of primordial follicles into the growing pool is a tightly controlled event, which is responsible for the growth of a selected cohort of follicles. During follicular growth, the formation of an antral cavity is induced by the differentiation of granulosa cells into two populations with distinguishable characteristics. Cumulus cells surrounding the oocyte are responsible of its nourishment and contribute to the acquisition of oocyte competence [10, 11]. Mural granulosa cells (together with theca cells) are responsible for the steroid biosynthesis during the growing phase [12–14]. After the ovulatory stimulus, cumulus cells expand and secrete the extracellular matrix (mucification), while cells of the follicular wall (granulosa and theca cells) develop into the secretory corpus luteum, changing from being estrogenic to progestogenic [15].

The oocyte development was previously thought to be determined by the somatic cells surrounding it; however, there is compiling evidence that the oocyte rather drives the development of the ovarian follicle and therefore its own development by actively regulating the environment within the follicle. It has been shown that the oocyte induces the functional changes observed in cumulus and mural granulosa cells [16].

Acquisition of Meiotic and Developmental Competence In Vitro

In mammalian species, it is known that the oocyte initiates the prophase of meiosis before birth and gets arrested at the dictyate stage as soon as the primordial follicles are formed; this happens either before (human, bovine, ovine) or early after birth (murines and hamster) [17–19]. The meiotic arrest is maintained during the entire follicular growth, and meiosis resumes when the fully grown follicles respond to the LH ovulatory stimulus. The capacity of an oocyte to resume meiosis (named nuclear competence) is achieved when its volume has reached at least 80 % of its maximal size (mouse, [20], human [21]).

The ability of an oocyte to be fertilized and develop further into an embryo (developmental competence) is also acquired during follicular growth and after the oocyte has reached nuclear competence. It involves the relocation of ribosomes and mitochondria, the reorganization of the endoplasmic reticulum, the storage of essential transcripts required during early embryo development, and the ability to modulate both the Ca^{2+} release upon fertilization (calcium oscillations) and the calcium-dependent secretion of cortical granules [22–28].

Replication of these events in in vitro models has been the focus of several research groups, and some progress has been made in growing ovarian follicles starting from follicles at different stages of development (mainly, from late preantral follicles), especially in small animals. However, because cryopreserved primordial follicles are the main source of oocytes for fertility restoration, it still remains a challenge to culture these very immature follicles and to make them grow entirely in vitro. Furthermore, the species differences make it difficult to directly apply the expertise raised in one species to another [29].

Folliculogenesis from Primordial Follicles

Until today, the specific mechanism(s) responsible for initiation of growth of primordial follicles is yet unknown. Several oocyte or somatic cells-derived factors have been proposed as important for follicle activation to occur. For instance, Kit ligand (a granulosa cell-derived factor) has been shown to induce growth initiation [30, 31], while anti-Müllerian hormone (AMH, produced by granulosa cells of growing follicles) has been shown to inhibit follicular recruitment [32].

Few studies have attempted the culture of isolated primordial follicles of different species but with limited success [33–37]. This is most likely due to the need of follicles to progress to an adequate stage of development with some layers of granulosa cells that will support oocyte growth in an isolated environment. The advantage of culturing such small follicles within the ovarian tissue is

that follicles are grown in a physiological support which allows the interaction among growing follicles and with the stromal cells [38]. Nevertheless, some reports suggested that oocytes can be grown under the support of putative theca stem cells, without the need for granulosa cells, and potentially give rise to fertilizable oocytes [39, 40].

Studies of Eppig and collaborators have shown that mice newborn ovaries can be cultured to obtain follicles growing from the primordial to the primary or secondary stage [41, 42]. These growing follicles were enzymatically isolated and further cultured until they reached maturity, achieving sizes slightly smaller than their in vivo counterparts. Later studies have also succeeded in activating primordial follicles by culturing ovaries (or cortical tissue pieces) in presence or absence of extracellular matrices (murine [43, 44], bovine [45–47], human [38, 48–51], and others [52]).

Depending on the methodology used, a problem that may occur during tissue culture is that the basement membrane of growing follicles is not preserved, and therefore, oocytes would grow without the normal compartmentalization with granulosa and stromal cells. This issue has been solved by supplementation of ascorbic acid to culture media, which protects the basement membrane (by inhibiting matrix metalloproteinases), to stimulate collagen biosynthesis and to prevent apoptosis [51, 53–55].

A generalized concern of the tissue culturists is that growing follicles tend to inhibit the growth of other follicles in the tissue (growing or non-growing). Therefore, many strategies make use of culture systems including two or more steps: first starting as culture of ovarian tissue until follicles reach a multilayered preantral stage and then a second or more culture steps where preantral follicles are isolated and grown until the oocyte reaches its fully grown size and acquires both nuclear and developmental competence.

The idea of introducing a multistep culture for ovarian follicles was first proposed for mouse ovaries by Eppig and O'Brien [41] and later improved by the same group [42]. Although the improved protocol showed a higher offspring number after embryo transfer, the success rate was still low (6 % of the transferred two-cell embryos developed to term) and confirmed the principle that competent oocytes can be obtained from culturing primordial oocytes. Similar achievements in large animal species have not been proven yet. Consequently, it was not until recently that the efficacy of the stepwise-controlled growth of follicles from the primordial stage has been demonstrated by Telfer et al., [51] who developed a two-step serum-free culture system for human follicles that allowed the production of antral follicles in 10 days of culture. The belief until today is that a similar in vivo development would take several months in human [56]. The same principle was later applied to bovine follicles, demonstrating the interspecies validity of this culture system [47]. A similar approach involving secondary follicle culture in alginate beads has also been attempted in mouse [57].

Primordial Follicle Activation

The precise mechanisms underlying activation of a selected cohort of primordial follicles are yet unknown. Nevertheless, some studies have shown that follicular activation can be achieved in vitro by culturing ovaries or cortical pieces (depending on the species) in medium containing or not serum as protein source [mouse [41, 42], baboon and bovine [45, 58, 59], goat [52], and human ([38, 48, 51 and unpublished data from the Follicle Biology Laboratory; Vrije Universiteit Brussel, Brussels.]) (Fig. 8.1).

The primordial to primary transition characterizes the entry of an awaking primordial follicle into the pool of growing follicles. Morphologically, primordial follicle activation is defined by a significant oocyte growth (from around 30 μm to more than 60 μm in mouse) accompanied by a transition from flat to cuboidal granulosa cells and acquisition of a zona pellucida [56]. Although full knowledge of the precise biological mechanisms underlying primordial follicle activation is still lacking, there is evidence that a multidirectional communication among oocytes and surrounding somatic cells, acting in an autocrine and paracrine manner, is crucial in this process [60, 61]. Knowledge on the function of the majority of growth factors and hormones acting on the primordial follicle activation has been obtained mainly from animal models [62–71]. For instance, Activin A, GDF9, and BMP15

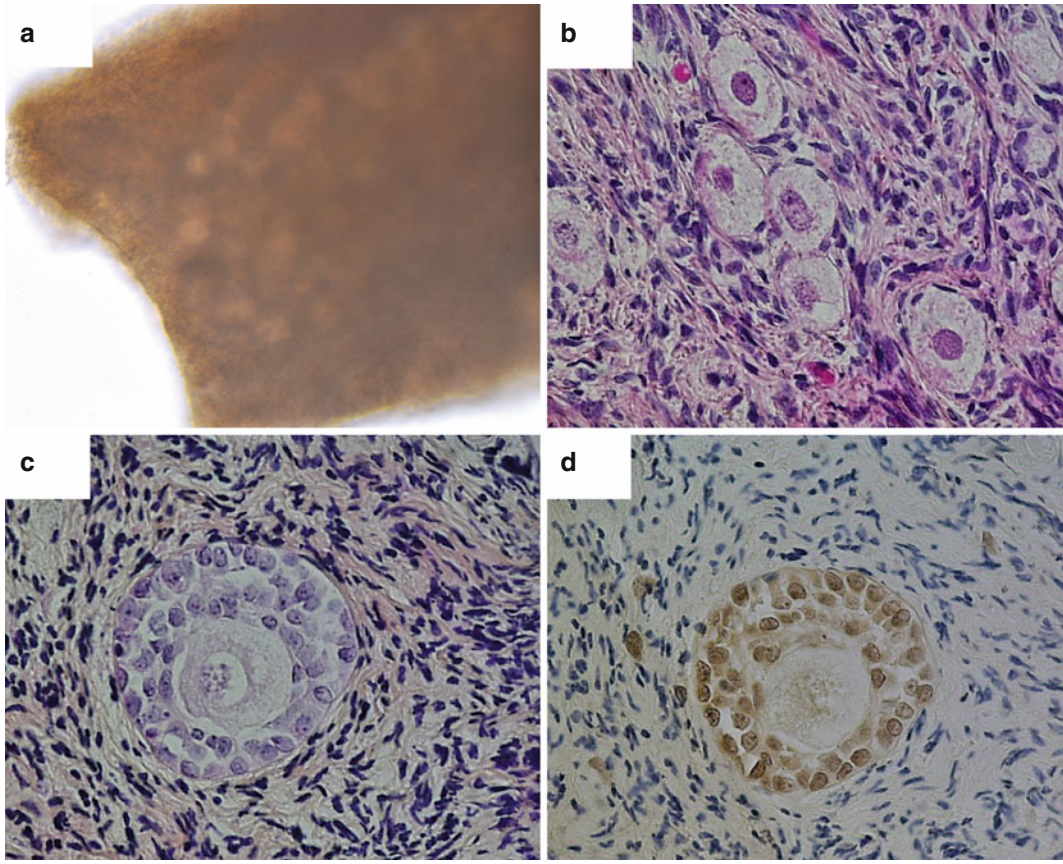

Fig. 8.1 Human ovarian tissue in culture. Tissue fragments were obtained from a patient (13 years old) that underwent oophorectomy for ovarian tissue cryopreservation before undertaking chemotherapy for cancer. Tissue fragments were cultured in medium containing 10 % fetal bovine serum as protein source and under a non-attaching condition. (**a**) Microscopical observation of ovarian tissue after 13 days of culture. Numerous translucent growing follicles can be observed within the tissue. (**b** and **c**) Hematoxylin-eosin staining of serially sectioned tissue before (**b**) or after (**c**) 13 days of culture. Tissue before culture contained mainly primordial follicles, while after culture time, some secondary follicles were observed. (**d**) Granulosa cells of the follicle depicted in (**c**) stained positively for PCNA, suggesting their proliferative status and the healthiness in the growing follicles (Data from the Follicle Biology Laboratory)

have been assessed for their potential physiological significance in regulating the earliest stages of follicular development in human [72, 73].

Control of Primordial Follicle Activation by the Phosphatidylinositol 3-Kinase (PI3K) Signaling Pathway in Oocytes

Recent genetic studies have reported that primordial follicle activation may require certain common pathways that are involved in cell proliferation and survival, such as the PI3K pathway, which can be activated in vitro by some growth factors [74, 75]. The PI3K pathway including Akt and FOXO3A (Forkhead box O3A) [76] establishes cascades of intracellular signaling that are fundamental for the regulation of cell proliferation, survival, migration, and metabolism. The phosphatase and tensin homolog deleted on chromosome ten (PTEN) antagonizes the activity of PI3K by converting PIP_3 to PIP_2 [77]. Thus, the balance between PI3K and PTEN activities determines PIP_3 levels, which affect the Akt pathway mainly involved in folliculogenesis [75, 78]. Particularly, PTEN indirectly inhibits the phosphorylation of Akt, which can no

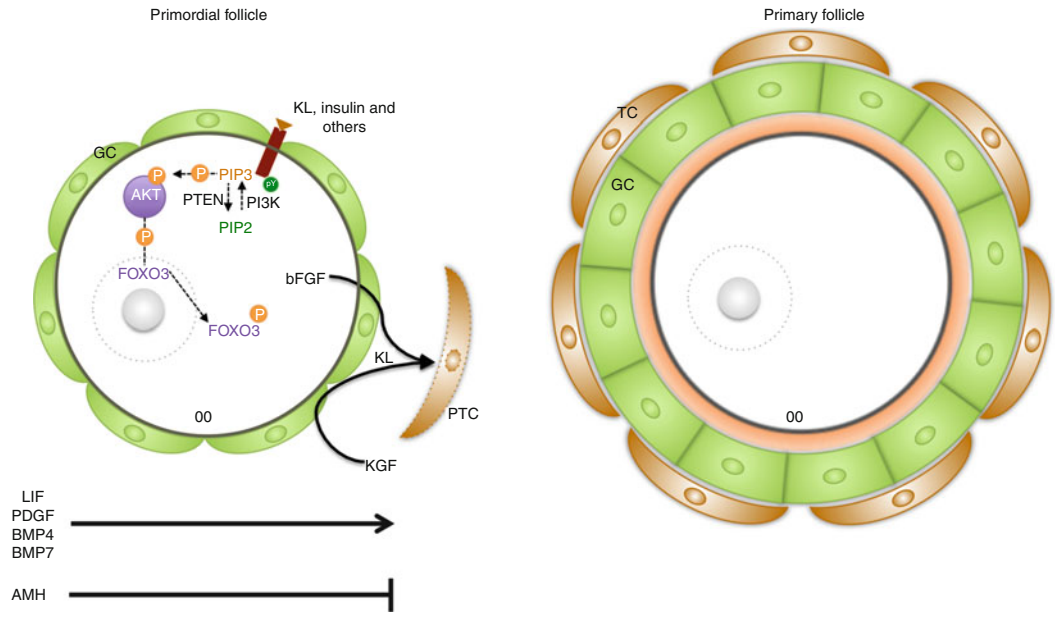

Fig. 8.2 Strategies to activate primordial follicles in vitro. Due to its nature (small size and volume), primordial follicles stand well the cryopreservation procedure; therefore, they become an important source of gametes for fertility restoration. The process of primordial follicle activation is not well understood; however, in vitro evidence has provided some information on the key players involved in the process. It has been proposed that somatic cells-derived factors may signal the primordial follicles to initiate the growth process. Factors like leukemia inhibitory factor (*LIF*), platelet-derived growth factor (*PDGF*), and bone morphogenetic proteins 4 and 7 (*BMP4* and *BMP7*) have been shown to promote the transition between primordial (*left in figure*) and primary follicles (*right in figure*). Among others, Kit ligand (*KL*), a peptide produced by granulosa cells and suggested to stimulate both oocyte growth and theca cell function, has been shown to be stimulated by factors secreted by the oocyte itself (i.e., basic fibroblast growth factor, *bFGF*) or by somatic stromal cells (i.e., keratinocyte growth factor, *KGF*). Recently, it has been reported that activation of the AKT pathway in the oocyte would phosphorylate and translocate FOXO3 (*forkhead box O3A*) from the nucleus to the cytoplasm, relieving its action as repressor of follicular activation and stimulator of apoptosis (detailed explanation in text). Moreover, KL and insulin had been suggested as potential triggers of the AKT pathway. In contrast, AMH (anti-Müllerian hormone) produced by granulosa cells of growing follicles has been reported to inhibit primordial follicular activation. *GC* granulosa cells, *OO* oocyte, *PTC* precursor theca cells, *TC* theca cell, *PTEN* phosphatase and tensin homolog deleted on chromosome ten, *PI3K* phosphatidylinositol 3-kinase; *PIP2* phosphatidylinositol 4,5-bisphosphate, *PIP3* phosphatidylinositol (3,4,5)-triphosphate

longer phosphorylate FOXO3A, prohibiting its nuclear export and the inhibition of transcriptional activities leading to apoptosis and cell cycle arrest [79] (Fig. 8.2).

Using an in vitro culture system, treatment of mouse and rat cultured oocytes with Kit ligand (KL, also known as stem cell factor) was shown to activate the PI3K network, leading to the phosphorylation of Akt and FOXO3A [76]. Moreover, initiation of follicular growth was dramatically increased by the addition of recombinant KL to the culture system of rat and mouse ovaries, relative to the unstimulated controls [43, 80].

Insulin has been found to influence mouse follicle development through the insulin receptor/PI3K/Akt signaling pathway [81]. Furthermore, the in vitro effect of insulin in the primordial to primary transition appeared stage-specific. In a 3-dpp mouse ovarian culture, continuous insulin exposure has been reported to repress the phosphorylation of Akt and the subsequent follicle activation, whereas it was promoted during culturing of the 16.5-dpc mouse ovaries [81]. Previous studies have shown that supplementing the medium with insulin and insulin-like growth factors (IGF) I and II may stimulate follicle activation when present during culturing of human ovaries. However, the precise mechanism remains unknown in human model [82, 83].

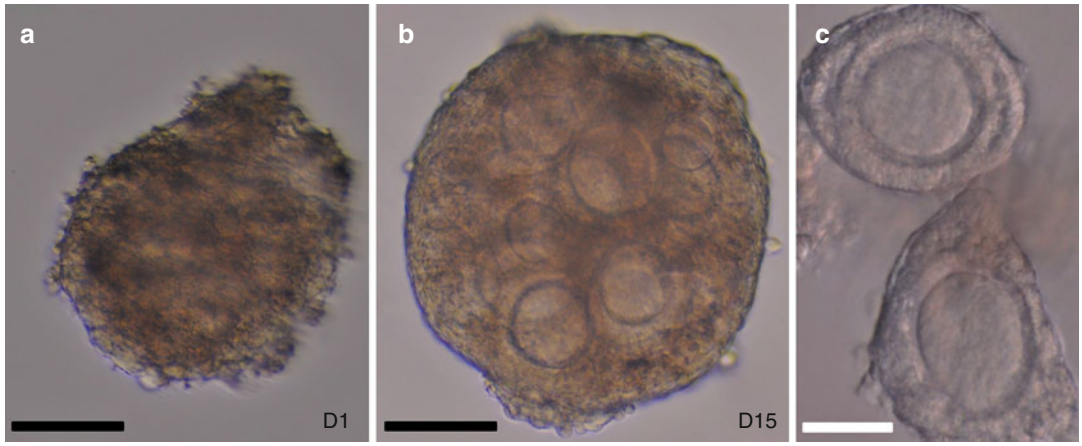

Fig. 8.3 In vitro production of secondary follicles from primordial follicles in mouse. Mouse ovarian primordial follicles can be activated and grown in vitro up to the secondary preantral stage. Preliminary data shows that these secondary follicles can be isolated and further cultured, reach the antral stage, and ovulate in vitro. Examples of the culturing steps are depicted in (**a**–**c**). (**a**) Piece of ovarian tissue treated for 24 h with a PI3K activator and a PTEN inhibitor. (**b**) Piece of ovarian tissue cultured for 15 days containing growing follicles in the primary and secondary stage. (**c**) Early secondary follicles isolated from ovarian tissue pieces after 18 days of culture. Scale bars: *black* = 10.0 μm, *white* = 5.0 μm

Manipulating key signaling pathway such as PI3K/Akt might be an original way to maximize in vitro yields of growing follicles from an ovarian tissue. In this regard, Li et al. (2010) [84] showed that after a short-term incubation of mice and human ovaries containing primordial follicles with the PTEN inhibitor and the activator of the PI3K, a larger amount of developing follicles was observed in comparison with an untreated ovary. Moreover, the addition of Activin A during the culture of ovarian tissue pieces pretreated with the PTEN inhibitor and the activator of the PI3K has been found to increase follicular growth in comparison with untreated controls. Treatment with Activin A alone led to improved follicle survival and larger harvest of secondary follicles (unpublished results of Follicle Biology Laboratory, Vrije Universiteit Brussel, Brussels) (Fig. 8.3).

Regulation of Primordial Follicle Activation by Growth Factors, Hormones, and Cytokines

The addition of FSH has shown positive effects on the development of the early stages of human and goat follicles [83, 85]; however, contradictory effects have been shown on the development of primordial follicles in pieces of bovine cortex [86] supported by the fact that FSH receptor message is absent in primordial follicles [87].

Keratinocyte growth factors (KGF) [68], leukemia inhibitory factor (LIF) [63], basic fibroblast growth factors (bFGF) [62, 67], and platelet-derived growth factor (PDGF) [69] were also shown to enhance the primordial to primary follicle transition in neonatal rat organ cultures.

Regulation of Primordial Follicle Activation by Some Members of the TGF-β(Beta) Family

AMH (also called Müllerian inhibitory substance) belongs to the TGF-β(beta) superfamily, which also includes activins, bone morphogenetic proteins (BMPs), and growth and differentiation factors (GDFs) [88]. It was demonstrated that AMH in culture inhibits the early development of human [49] and mouse [89] follicles in culture. In contrast, a study involving culture of human ovarian biopsies in the presence of recombinant AMH challenged the inhibitory role of AMH in the activation of primordial follicles [90].

The in vitro effects of GDF-9 in the primordial to primary transition are controversial. Although the in vitro culture of neonatal rat ovaries with GDF-9 did not affect primordial follicle development [64], GDF-9 has been shown to facilitate the activation of primordial follicles in cultured human ovarian tissues [91]. Similarly, GDF-9 has been reported to stimulate follicular activation in cultured goat and hamster ovaries [70, 92].

BMP-4 [66] and BMP-7 [93] were also shown to act as positive regulators of follicular activation by increasing the primordial to primary follicle transition in neonatal rat organ cultures.

Activin A and Primordial Follicle Activation

Effects of Activin A on primordial follicle activation are unclear: the few in vitro experiments have reported contradictory data. While it has been shown to regulate the formation of the initial pool of follicles after occurrence of the germline cysts breakdown in mouse ovaries [94], an inhibition of spontaneous follicular activation has been described in human [72]. Another study has shown that Activin A promotes the growth of transitional (primordial-primary) bovine follicles [95].

The Preantral to Antral Phase

Most of the models for in vitro growth of preantral follicles have been developed in mouse. The advantage against other models is that its follicular cycle is relatively short, which makes it feasible to be entirely reproduced in vitro. Therefore, most of the information provided here has been reported in mice, and when available, information on large species is pointed out.

Because oocyte competence in cultured follicles is still suboptimal when compared to in vivo grown oocytes [29, 42, 96–99], in vitro conditions need to be optimized, so that oocytes with good developmental competence can be obtained.

For instance, the presence of both insulin and FSH has been described to influence the regulation of the follicular environment, with physiological levels stimulating follicular survival and growth, while high levels were inducing inappropriate differentiation of granulosa cells and more importantly affecting oocyte competence [100–104]. The improvement of culture conditions should allow for the maintenance of a normal cumulus cell phenotype, which probably reflects the quality of the oocyte [101].

The culture of secondary follicles is relatively well standardized. Over the years, many models for culture of isolated secondary follicles have been developed and further optimized; however, culture of isolated primary follicles has proven more difficult to achieve [33, 38, 48, 105, 106]. An overview of the main achievements in the quest for the most adequate system for growing secondary follicles is provided hereafter.

Attachment Permissive Versus Non-attaching Culture Conditions

Protocols for culture of secondary follicles involve different procedures for follicle isolation, culture supports, and culture media; nevertheless they could be divided into two main categories: the attaching permissive systems (also referred to as bidimensional systems) and the non-attaching systems (also referred to as tridimensional). The main difference between these two ways of culturing is the spatial distribution of the follicular cells including the localization of the oocyte.

Methods for follicular isolation involve enzymatic or mechanical isolation, the latter being more advantageous when maintenance of basement membrane and theca cell layer matters.

In the attaching permissive systems, follicles are cultured in microdroplets or small volume of media, with/without oil overlay. The culture dishes (or plates) are usually treated for ensuring the adherence of cells to the plastics. Once in culture, granulosa cells quickly breach the follicular basement membrane, and both granulosa and theca cells adhere to the bottom of the culture dishes. The follicle remodeling results into the development of the oocyte in an open system where nutrients, hormones, and gases diffuse freely via the culture medium [24, 107, 108].

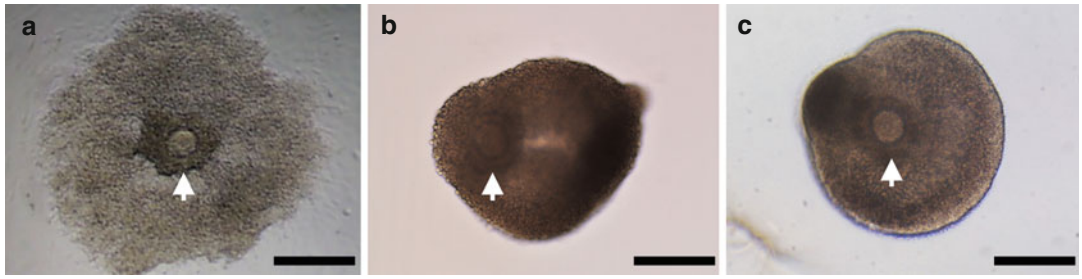

Fig. 8.4 Examples of follicle culture models. All follicles were isolated from 13-day-old mice (as type 3b follicles; Pedersen classification) and cultured for 9 days. (**a**) Mouse follicle in an attachment permissive system. (**b** and **c**) Mouse follicles cultured in a non-attaching condition, in ultra-low attachment plates (**b**), or embedded in an alginate bead (**c**). *Arrowheads* indicate position of the cumulus-oocyte complex. Scale bar, = 20.0 μm

The non-attaching systems prohibit the adherence of the follicles to the plastic. This can be obtained (1) by growing the follicles on cellulose or polycarbonate membranes [109–112]; (2) by frequently transferring the follicles from a culture well to another [113–115]; (3) by culturing follicles embedded in a matrix such as matrigel, collagen [116–118], or alginate [5, 119–125]; (4) by combining the use of non-adherent surfaces, like hydrogel-coated plates, and transferring the follicles daily to a new culture well [126, 127]; or (5) by using culture plates coated for ultra-low attachment with an hydrophilic hydrogel [128]. The embedding of follicles in alginate matrix is lately used the most and has proven to be useful for culturing follicles from different species including mouse, bovine, primate, and human. Examples of cultured mouse follicles under attaching and non-attaching conditions are shown on Fig. 8.4.

Evaluation parameters to monitor progress during culture include follicular survival, follicle and oocyte growth, morphology (sometimes accompanied by histological analysis), steroidogenesis (measurement of androgens and estrogens in spent medium), response to the ovulatory stimulus (mucification and expansion of cumulus cells and the capacity of the oocyte to reinitiate meiosis), fertilization, and embryo development [5, 107, 109, 110, 119–125, 129–131]. More recently, molecular biology analysis has also been used as a way to better characterize in vitro grown follicles and define in vitro conditions which will favor oocyte and embryo quality [103, 104, 124]. Having all these techniques at hand, they could all be used for a broad range of applications; however some models are more suitable for studying certain aspects of folliculogenesis than others. Obviously, follicle rupture and ovulation are better studied in models where the basement membrane is maintained during culture [111].

In general, it has been shown in mouse that preantral follicles cultured with these techniques grow and reach the preovulatory stage and can give rise to fertilizable oocytes that can develop further to form blastocysts [111, 114, 132, 133]. In mouse, live offspring has been reported [41, 42]. Moreover, few comparative studies assessing the benefits of one technique over the others had been reported [134].

Follicle Maturation and Length of Culture

Studies on oocyte competence in mice revealed that an optimal length of culture would guarantee a better chance to obtain developing embryos. For instance, an important parameter to be considered when determining the appropriate length of culture is the follicular diameter at the start of culture. Thus, studies have shown that inadequately prolonged cultures affect follicular development causing follicles to degenerate [135] and that oocytes of follicles cultured for an optimal period of time have better chances to produce blastocysts [133].

An event that precedes the acquisition of developmental competence is the reorganization of chromatin around the oocyte nucleolus [from the nonsurrounded (NSN) to the surrounded (SN)

stage] [136–138]. This reorganization is reproduced by cultured follicles; however, the time between the establishment of the surrounded nucleolus (SN) chromatin configuration and fertilization should be kept to the minimal, so that oocyte quality is not compromised [139].

The concept of an innate growth time for follicles is challenged by recent work including Activin in a two-step culture system of short duration [47, 51]. However, competence of cultured oocytes under those conditions remains as yet unproven.

Gonadotropins

Several factors have been claimed to be beneficial for follicular growth; among them, hormones like FSH had been shown to help not only as survival factor (preventing atresia) but also stimulating follicular growth when follicles reach the antral stage and estrogen production [140–142]. The dose of FSH administered to growing follicles varies from system to system; however, 10–100 mIU/ml is most commonly used. Some studies have also reported that addition of small doses of LH induces the differentiation of the follicle and determines the transition between preantral and antral stage [143] as well as changes in the expression pattern of the cumulus-oocyte complex [104].

The interplay of factors such as the dose and timing of FSH and the follicular configuration (imposed by attaching or non-attaching conditions) has an influence on the transcript levels of marker genes in the oocyte and cumulus cells, which reflects the oocyte developmental capacity [128]. Consequently, in vitro conditions that closely reproduce the physiological follicular environment will definitely favor the production of good quality oocytes.

Protein Source and Other Factors

Many culture systems make use of fetal bovine serum as preferred protein source; however, specific protein composition of the serum (hormones and growth factors) is mostly unknown and varies from batch to batch. Therefore, a more defined culture medium (certainly for clinical applications) requires a serum-free composition in which proteins and growth factors are added in known quantities. Some studies attempted to lower serum concentration in culture or its total or partial replacement by albumin, ovalbumin, synthetic serum supplements, or fetuin. Especially, fetuin was included to prevent zona hardening during culture under serum-free conditions [24, 120, 144].

Among several growth factors, insulin-like growth factor 1 (IGF-1) and Activin A have been reported as beneficial for follicular survival, growth, and differentiation to the antral stage in mouse and other species [131, 145, 146].

Studies with knockout mice for the androgen receptor revealed that androgens are also important for follicular growth [147, 148]. Depending on the stage of follicles development, a critical amount of androgens in medium contributes to the appropriate development of follicles. For instance, androgens have been reported as factors promoting the growth of small preantral follicles in mouse, bovine, and primates [110, 149–153]. Other studies have proven that excess of androgens during the antral phase causes follicular atresia and is detrimental for oocyte quality [154–157].

Periovulatory Period

Periovulatory events have also been well replicated in culture, and recent achievements have made clear that LH surge triggers different pathways within the follicle, preparing the oocyte to be released and fertilized. Shortly after the LH signal, membrane-bound EGF-like factors are produced in both mural granulosa and cumulus cells [158–162] and are further released into the follicular fluid so that they reach out the cumulus cells to induce mucification and expansion and to trigger meiotic resumption in the oocyte.

The use of follicle culture systems has provided information on the importance of the oocyte in preparing the cumulus cells for its ovulatory role [10, 16, 163]. Lately, the in vitro

culture of follicles revealed that ovulatory signaling via LH and/or EGF might play a distinct role to the one reported in in vivo grown follicles [164] and therefore points out to the fact that when optimizing culture conditions some responses might differ from the expected ones. Nevertheless, follicular rupture and the ovulation process have previously been successfully reproduced in vitro in a mouse model [111].

At the End of Folliculogenesis: Oocyte In Vitro Maturation (IVM)

Patients undergoing ovarian hyperstimulation for ART are given high doses of FSH followed by a high dose of hCG to trigger oocyte maturation. Although the majority of the recovered oocytes are at the metaphase II stage and ready to be fertilized, there are some that did not mature and remain in the germinal vesicle (GV) stage. IVM intends to provide culture time that allows for the oocyte to resume meiosis and reach the metaphase II stage. In spite of the fact that many of those initially immature oocytes can become mature in vitro, the developmental competence is not optimal; the embryos produced are often aneuploid [165] and therefore give rise to half or less pregnancies than routine in vitro fertilization (IVF) procedures [166, 167].

It is well accepted that the key strategy for improving the outcome of IVM is to provide an environment that will allow acquisition of developmental competence encompassing nuclear competence. An approach that has been implemented into IVM is the prolongation of the meiotic arrest of the oocyte, while it acquires its developmental competence, and the provision of a more physiological succession of positive maturation stimuli, in mouse [168, 169], bovine [170, 171], and human [172–175].

Fertility preservation may benefit from IVM in cases where ovarian hyperstimulation of oncological patients is unsafe and where the retrieval of immature oocytes from small antral follicles becomes the preferred option. Therefore, further research is required to improve the IVM outcome and to make it a more reliable technique in ART.

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Part III

The (Epi)Genetic Making of the Oocyte

Regulation of Oogenesis by Oocyte-Specific Gene Networks

9

Swamy K. Tripurani and Stephanie A. Pangas

Abstract

Although, development of traditional and conditional transgenic knockout mice models has been critical in revealing how oocyte-expressed transcripts regulate oocyte growth and embryo development, many aspects concerning reproduction remain to be determined. Recent studies have demonstrated that oocyte-specific transcriptional regulators play essential roles in regulating various stages of oogenesis, folliculogenesis and early embryonic development. In this book chapter, we categorized and reviewed transcriptional regulators based upon their known function in germ cells: (1) those that are specifically expressed in the female germline and appear to function only in oocytes (Figla and Nobox); (2) those that are expressed in both male and female germline and affect both male and female fertility (Sohlh1 and Sohlh2); and (3) those that are highly expressed in germ cells, but show additional expression in other somatic tissues (Lhx8, Pou5f1 and Yy1). These investigations will provide insight into the regulation of mammalian fertility.

Keywords

Transcription factor • Germ cell • Reproduction • Fertility

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Introduction

Oogenesis is defined as the formation, development, and maturation of an oocyte. In mammals, it is a process that is initiated during fetal development but completed many years later at the time of fertilization. The precursors to oocytes are the primordial germ cells (PGCs), which are specified early in embryogenesis outside the embryo proper. Subsequently, PGCs migrate and colonize the urogenital ridge to establish the undifferentiated gonad. Oogenesis consists of several sub-processes including oocytogenesis (transformation of oogonia into primordial oocyte) and ootidogenesis (development of primordial oocyte into primary oocyte). Folliculogenesis, or development of the ovarian follicle, accompanies and supports oogenetic development (Fig. 9.1). Folliculogenesis begins when the oocyte is surrounded by somatic cells to form primordial follicles, which are generally believed to represent the stock of oocytes available to the female during her reproductive years. Development of the oocyte within the ovarian follicle requires synchronous growth and differentiation of the oocyte and its companion somatic cells [1–3], and oocytes produce many factors that regulate granulosa cell development. In addition, oocytes must also synthesize and store maternal proteins that are important not only for successful folliculogenesis and oocyte maturation but also for early embryonic development [4–6].

The advent of advanced genomic methodologies and transgenic mouse genetic technologies has led to the identification of genes involved in transcriptional regulation within the oocyte [7–11]. Many of these germ cell- or oocyte-specific transcription factors have proved to be critical modulators in maintaining coordinated expression of oocyte-specific genes necessary for oocyte and follicle growth, as well as chromatin organization and reprogramming during early embryonic development [12–16]. In this chapter, we discuss these transcription factors based on their known function in germ cells (Table 9.1): (1) those that are preferentially expressed in the female germline [factor in the germline alpha (*Figla*) and newborn ovary homeobox gene (*Nobox*)] and appear to function only in oocytes; (2) those that are preferentially expressed in both

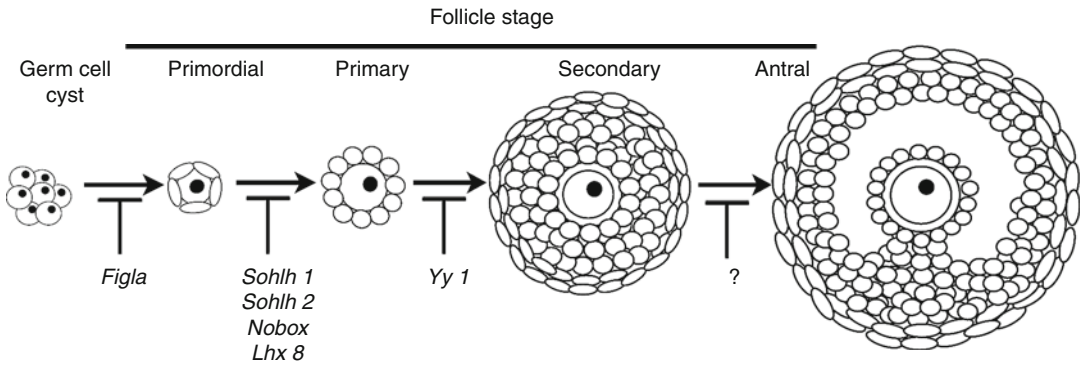

Fig. 9.1 The role of oocyte-expressed transcription factors during mammalian folliculogenesis. Oocytes initially develop in the ovary as clusters termed germ cell cysts (*GCC*). GCCs break down to form primordial follicles, which constitute the quiescent pool of follicles during the reproductive lifespan. Primordial follicles activate to become primary follicles. Primary follicles develop into secondary follicles with continued growth of the oocyte and the somatic granulosa and thecal cells. Stimulation of follicles by the pituitary gonadotropins results in the development of a follicular antrum (antral follicles), which defines a gonadotropin-dependent stage of growth prior to ovulation. Mice lacking oocyte transcription factors (*Figla*, *Nobox*, *Sohlh1*, *Sohlh2*, *Lhx8*, and *Yy1*) display arrest at different stages of the folliculogenesis. While many of the transcription factors such as *Nobox*, *Figla*, and *Lhx8* are also expressed at later stages of follicle development, their function at these times is currently unknown due to the postnatal loss of oocytes in knockout mouse models

Table 9.1 Oocyte-expressed transcriptional regulators required for oogenesis

Gene	KO phenotype	Germ cell loss	Mutations in POI	References
Functions exclusively in oocytes				
Figla	Infertile	Oocyte loss by PN day 2	Present	Soyal et al. [17]
Nobox	Infertile	Oocyte loss by PN day 14	Present	Rajkovic et al. [18]
Functions in male and female germ cells				
Sohlh1	Infertile	Oocyte loss by PN day 7	ND	Pangas et al. [47]
Sohlh2	Infertile	Oocyte loss by PN day 7	ND	Choi et al. [19]
Functions in multiple cell types				
Lhx8	Infertile	Oocyte loss by PN day 7	Absent	Choi et al. [20]
Pou5f1	Infertile	Early PGC depletion	ND	Kehler et al. [21]
Yy1	Infertile	Arrested follicle development	ND	Griffith et al. [22]

Abbreviations: *PN* postnatal day, *PGC* primordial germ cell, *ND* not determined

the male and female germlines, but not other tissues [spermatogenesis and oogenesis helix-loop-helix 1 and 2 (*Sohlh1* and *Sohlh2*)], and affect both male and female fertility; and (3) those that are highly expressed in germ cells, but show additional expression in adult tissues [LIM homeobox 8 (*Lhx8*), POU domain class 5 transcription factor 1 (*Pou5f1*), and Yin Yang 1 (*Yy1*)] and have known physiologic functions in other tissues. Knowledge of these differences in expression may be a key to understanding how these genes are individually regulated, as well as how they intersect to form the oocyte transcriptional network.

Transcription Factors Whose Function Is Limited to Oocytes: *Figla* and *Nobox*

Very few transcription factors function exclusively in oocytes. In mice, *Figla* and *Nobox* have the highest expression in the ovary, and mice null for *Figla* or *Nobox* have reproductive defects only in females. As might be predicted, these oocyte-restricted transcription factors regulate a distinct set of genes whose expression is largely limited to oocytes. Thus, *Figla* and *Nobox* are necessary for establishing an oocyte-specific gene expression pattern [18, 23]. In addition, recent data have also demonstrated that they may have additional functions to suppress the alternative testis-specific pattern [24]. As critical regulators of oocyte gene expression, not surprisingly, mutations in these genes are being found in patients with ovarian dysfunction, including primary ovarian insufficiency (POI).

Factor in the Germline Alpha (*Figla*)

Figla encodes a germ cell-specific basic helix-loop-helix (bHLH) transcription factor and was first discovered in a screen used to identify transcription factors that bind to the promoters of genes comprising the extracellular matrix that surrounds growing oocytes, the zona pellucida (ZP) [23]. *Figla* transcripts can be detected in the mice embryonic gonad as early as E15.5, shortly after the onset of sexual dimorphism of the gonads, but *Figla* expression undergoes a dramatic increase at the end of embryonic development to peak by postnatal day 2 – a time when oocytes have become enclosed in primordial follicles [17]. A similar timing of *FIGLA* expression during primordial follicle formation is found in humans, with *FIGLA* transcripts increasing between 17 and 19 weeks of gestation [25]. Functional homologs of *Figla* have been identified in fish [26, 27] and cow [28]. In bovine, *FIGLA* is expressed predominantly in ovaries and detectable when primordial follicles start to form, as early as 90 days of gestation [28]. Bovine *FIGLA* mRNA and protein are abundant in germinal vesicle and metaphase II-stage oocytes, as well as

in embryos from pronuclear to 8-cell stage, but barely detectable in embryos collected at morula and blastocyst stages, suggesting that *FIGLA* might be a maternal effect gene and may play a role during bovine early embryonic development [28].

Mice lacking *Figla* appear normal at birth and have normal mating behavior as adults [17]. In addition, aspects of PGC physiology, such as PGC specification, migration, and proliferation, are not affected in female *Figla*$^{-/-}$ mice, nor is embryonic gonad development [17]. However, *Figla*$^{-/-}$ females are sterile and lose all germ cells by postnatal day 7 [17]. In addition, primordial follicles do not develop [17]. As expected, the FIGLA target genes, *Zp1*, *Zp2*, and *Zp3*, are not expressed in the *Figla*$^{-/-}$ ovaries [17].

Additional genes beyond the zona pellucida genes are also regulated by FIGLA. By comparing gene expression patterns in normal and *Figla* null gonads, *Figla* was further identified as a key regulatory factor that coordinates expression of several known oocyte-specific genes, especially the *Nlrp* (*NLR* family of pyrin domain-containing) genes, which are known to be essential for early embryogenesis. In addition, FIGLA regulates a set of functionally unannotated genes specifically expressed in ovary [29]. Interestingly, proteomic analysis between normal and *Figla*$^{-/-}$ ovaries found 18 testis-specific proteins significantly upregulated in *Figla*$^{-/-}$ ovaries compared to normal ovaries [24]. By using a human sperm-specific promoter (*TSPY1*, testis-specific protein Y-encoded) to drive ectopic expression of *Figla* in spermatogonia and early primary spermatocytes, FIGLA was shown to downregulate a subset of testis-associated genes essential for spermatogenesis and subsequently causes age-related sterility associated with testicular dysmorphology, impaired meiosis, and germ cell apoptosis [24]. This suggests that FIGLA, in addition to acting as a positive regulator of oocyte-specific genes, acts as a repressor of testis-specific genes, thereby promoting the female sexual differentiation pathway during gonadogenesis.

Two plausible mutations in *FIGLA* have been found in patients with premature ovarian failure (a heterogeneous genetic disorder characterized by premature depletion of ovarian follicle before the age of 40) [30]. Molecular analyses of these mutations show that these variants adversely affect FIGLA's interaction with TCF3 helix-loop-helix domain and further substantiate the importance of *FIGLA* in the pathophysiology of ovarian failure. Such findings suggest that in addition to its role in the formation of primordial follicles, *Figla* may be critical in regulating primordial follicle activation, but further research is needed to verify this proposed functional role.

Newborn Ovary Homeobox Gene (*Nobox*)

Newborn ovary homeobox gene (*Nobox*) was identified by *in silico* subtraction of expressed sequence tags (ESTs) derived from the newborn ovaries [31]. The mouse *Nobox* expression pattern is very similar to that seen for *Figla*. Its transcripts are detected as early as E15.5 in the mouse embryo [18]. *Nobox* mRNA and protein are preferentially expressed in oocytes of germ cell cysts and primordial and later-stage follicles throughout folliculogenesis [18, 31]. Functional homologs of *Nobox* have also been identified in human [32], pigs [33], and cows [34]. Human *NOBOX* expression is restricted to oocytes within the ovary, but *NOBOX* has detectable levels in the testis and pancreas within adult human tissues [32]. Bovine *NOBOX* mRNA is restricted to ovarian samples with very minor expression in adult testis and can be detected in fetal ovaries harvested as early as 105 days of gestation period when primary follicles start to form [34]. Similar to mice, bovine NOBOX protein predominantly localizes to the nuclei of oocyte throughout folliculogenesis [34]. Furthermore, *NOBOX* mRNA and protein are expressed in a stage-specific manner during bovine oocyte maturation and early embryonic development and of maternal origin [34].

Similar to the *Figla* knockout mouse model, deletion of the *Nobox* coding region (including the homeodomain) in female mice results in infertility and disrupts early postnatal stages of folliculogenesis [18]. Although *Nobox*$^{-/-}$ mice have normal embryonic ovarian development (i.e., germ cell proliferation and initial formation of germ cell cysts), the majority of oocyte and follicle growth is inhibited beyond primordial follicle stage (Fig. 9.1), and oocytes are lost

rapidly 2 weeks after birth [18]. Some primordial follicles form, but most oocytes are retained in germ cell cysts prior to their loss [35].

A very clear pattern of gene expression changes quickly emerged during the analysis of *Nobox* null ovaries. There were no changes in genes implicated in initiation of primordial follicle formation (*Kitl* and *Kit*), meiosis (*Mlh1* and *Msh5*), or apoptosis (*Bcl2*, *Bcl212*, *Casp2*, and *Bax*) [18]. Moreover, normal expression was found for some secreted growth factors such as *Fgf2*, *Bmp4*, and *Wnt4*, as well as transcription factors, *Gcnf* and *Foxo3a* [18]. However, transcripts specifically expressed in oocytes are dramatically downregulated in *Nobox* knockout mice ovaries. These include *Mos*, *Pouf51*, *Rfpl4*, *Fgf8*, *Zar1*, *Dnmt1o*, *Gdf9*, *Bmp15*, and *H1oo* [18, 36]. Some of these genes are direct targets of NOBOX regulation, such as *Gdf9* and *Pou5f1*, while others are likely indirectly regulated [18, 37].

Several high-affinity cis-acting sites (TAATTG, TAGTTG, and TAATTA) have been identified as NOBOX DNA binding elements (NBEs) [37]. Three putative NBEs are found in the mouse *Gdf9* and *Pou5f1* promoters [37]. GDF9 is an oocyte-specific member of the transforming growth factor beta (TGFB) superfamily and a key mediator of granulosa cell function and follicle growth. Deletion of *Gdf9* in mice causes infertility due to an arrest during follicle growth such that only one layer of granulosa cells is formed (i.e., follicles arrest at the primary stage) [38]. In addition, because of disruptions in feedback between the granulosa cells and the oocyte, kit ligand (*Kitl*) is overexpressed and binds to its receptor, *Kit*, on the oocyte, resulting in precocious growth of the oocyte [39].

While GDF9 is a critical oocyte-expressed protein and appears to be directly regulated by NOBOX, *Nobox* null ovaries lose follicles earlier than the stage at which *Gdf9* null ovaries arrest, likely because NOBOX regulates additional genes necessary for proper germ cell cyst (GCC) breakdown [35]. During GCC breakdown, oocytes, which were previously connected via intracellular bridges, are invaded by somatic cells to form primordial follicles. Oocytes in *Nobox* null ovaries improperly separate, leading to the development of syncytial follicles [35]. While alterations in a number of different signaling pathways cause defects in GCC breakdown (i.e., somatic deletion of activin, altered Notch signaling, and exposure to estrogens) (reviewed in [40]), the mechanism by which this happens is still unclear.

Mutations in NOBOX have been found in women with primary ovarian insufficiency (POI), with some estimates as high as 6.2 % of idiopathic cases, which would give *NOBOX* the highest prevalence for any currently described gene in nonsyndromic POI [41]. These mutations occur as both nonsense and missense mutations, with variable severity in phenotypes [41, 42]. Many of these mutations occur in the homeobox domain and have a dominant negative effect on the binding of NOBOX to DNA [42] or show haploinsufficiency [41] depending on the mutation. There also appear to be some differences in the association of these mutations with POI in different populations, with most of the mutations found in Caucasian or African patients [41, 43–45].

Transcription Factors Whose Function Is Limited to Male and Female Germ Cells (*SOHLH1* and *SOHLH2*)

NOBOX and FIGLA are transcription factors that are predominantly expressed in oocyte and, as such, control the expression of the limited number of genes whose expression is, by definition, restricted to oocytes. Even though *Nobox* and *Figla* have low levels of expression in the testis, male mice null for *Nobox* or *Figla* have normal fertility [17, 18]. In contrast, there are several transcription factors with expression restricted to male and female germ cells and which function in both cell types. These include the basic helix-loop-helix transcription factors, SOHLH1 and SOHLH2.

Spermatogenesis- and Oogenesis-Specific Basic Helix-Loop-Helix 1 and 2 (*Sohlh1* and *Sohlh2*)

Sohlh1 and *Sohlh2* were identified by the same silico subtraction strategy used to identify *Nobox* [46, 47]. *Sohlh1* and *Sohlh2* are preferentially expressed in the male and female gonads and

detected in embryonic ovaries as early as E13.5 [46, 47]. In the ovary, *Sohlh1* and *Sohlh2* mRNA and protein are expressed exclusively in oocytes in germ cell cysts and primordial and primary follicles (i.e., they are undetectable by the secondary follicle stage and beyond). This peculiar expression pattern is different from other oocyte-restricted transcription factors, *Figla* and *Nobox*, and suggests that *Sohlh1* or *Sohlh2* play unique roles in early folliculogenesis [46, 47]. Similar to *Nobox* and *Figla* knockout mice, female mice lacking *Sohlh1* and *Sohlh2* have normal germ cell migration and proliferation but display defects in early folliculogenesis. *Sohlh1* null ovaries lack primary follicles by postnatal day 3. In addition, by postnatal day 7, mutant ovaries are significantly smaller than the wild type and contain only a few remaining oocytes enveloped by the flat somatic cells (i.e., primordial follicles) [19, 47].

Both *Sohlh1* and *Sohlh2* null ovaries have defects in expression of many oocyte-specific genes such as *Figla* and *Nobox* and their respective downstream target genes *Gdf9*, *Pou5f1*, *Zp1*, and *Zp3* [19, 47]. These data suggest that *Sohlh1* and *Sohlh2* function earlier than these other oocyte-specific networks (i.e., that for *Nobox* and *Figla*). Analysis of *Sohlh1* deletion in mice also uncovered a role for an additional homeobox gene, *Lhx8*, in oocyte development [47]. Interestingly, expression of *Sohlh2* is partially downregulated in *Sohlh1*$^{-/-}$ ovaries, and *Sohlh1* is absent in *Sohlh2*$^{-/-}$ ovaries, suggesting that *Sohlh1* and *Sohlh2* might share similar regulation during oogenesis or potentially cross-regulate each other's transcription.

There are many questions that remain with respect to SOHLH1 and SOHLH2 function. In general, basic helix-loop-helix transcription factors act as dimers, and it is currently unknown as to whether SOHLH1 and SOHLH2 can form heterodimers or whether cooperation exists between these two pathways. *Sohlh1* mutations have been identified in nonobstructive azoospermia in males [48], but *Sohlh1* or *Sohlh2* mutations in female reproductive disorders have not been reported. Further identification and characterization of the regulation of *Sohlh1* and *Sohlh2* will help to delineate germ cell-specific pathways that contribute to the molecular basis of the gonadal differentiation.

Transcription Factors that Are Highly Expressed in the Germ Cells but also Function in Embryonic or Adult Tissues [LIM Homeobox 8 (*Lhx8*); POU Domain Class 5 Transcription Factor 1 (*Pou5f1*); Yin Yang 1 (*YY1*)]

Very few transcription factors show a germ cell- or oocyte-restricted expression pattern, and the majority of transcription factors that are expressed in the oocyte are also utilized in other tissues. Therefore, their deletion in mice often results in embryonic lethality, unless their loss of function can be compensated by expression of other genes. This can be circumvented by the use of conditional knockout technologies such as the loxP-cre recombinase system. In reproductive biology, this has been facilitated by the use of two oocyte-restricted promoters (*Gdf9* and *Zp3*) to express cre recombinase to generate targeted deletion in oocytes in a stage-specific manner [49].

Lim Homeobox Gene 8 (*Lhx8*)

LIM homeobox (*Lhx*) transcription factors are unique to the animal lineage and highly conserved from nematodes to mammals [50, 51]. They play critical patterning roles during embryonic development in invertebrates and vertebrates, with a conserved role in specifying neuronal identity [52]. *Lhx8* mRNA is preferentially, though not exclusively, expressed in the male and female gonads and detected as early as E13.5 in the embryonic gonads, similar to the other oocyte-specific transcription factors discussed above [20, 47]. However, deletion of *Lhx8* causes a cleft palate in mice with incomplete penetrance resulting in the inability of newborn pups with a cleft palate to suckle and, subsequently, their neonatal death [53]. *Lhx8* is also expressed in the brain, and surviving *Lhx8*$^{-/-}$ pups live to adulthood but lack cholinergic neurons [54].

Lhx8 transcripts localize to oocytes in germ cell cysts as well in oocytes during postnatal folliculogenesis [20, 47]. Female mice null for *Lhx8* are infertile [20, 47]. When analyzed from newborn mice, *Lhx8*$^{-/-}$ ovaries do not show any differences in the number of oocytes; however, the number of oocytes enclosed as primordial follicles is significantly less in *Lhx8*$^{-/-}$ ovaries compared to wild type [20]. Moreover, by postnatal day 7, *Lhx8*$^{-/-}$ ovaries contain very few oocytes compared to the wild type, and most follicles in the *Lhx8*$^{-/-}$ ovaries are degenerating. These data implicate *Lhx8* as a critical transcriptional regulator of oocyte survival and differentiation [20].

Several oocyte-specific genes downregulated in *Sohlh1* and *Sohlh2* null ovaries were also downregulated in *Lhx8*$^{-/-}$ ovaries and surprisingly, this included *Nobox* [20, 47]. These data suggest that *Nobox* functions downstream of *Lhx8*. This clarified a question remaining from initial studies of *Sohlh1*, which indicated *Lhx8* was a direct target of SOHLH1, but which did not determine the relationship between *Nobox* and *Lhx8* [47]. Comparing gene expression changes between the various knockout mouse models was important in determining the potential epistatic relationship between these transcription factors. Microarray datasets from *Lhx8*$^{-/-}$ and *Nobox*$^{-/-}$ newborn ovaries show that not all genes misregulated in *Lhx8*$^{-/-}$ ovaries are also misexpressed in the *Nobox*$^{-/-}$ newborn ovaries [20]. For instance, *Nlrp5* and *Zp3* expressions are significantly downregulated in *Lhx8*$^{-/-}$ ovaries, but not affected by *Nobox* deficiency. In addition, *Nobox* deletion shows differences not seen in the *Lhx8* knockout. Thus, these data indicate that some, but not all, of the defects in *Lhx8* are due to loss of *Nobox*. The relationship between these transcription factors does not appear to be linear, and the networks they control yet to be determined.

POU Domain-Containing Class 5, Transcription Factor 1 (*Pou5f1*)

Nobox deletion results in loss of *Pou5f1* (*Oct4*), a nuclear transcription factor that belongs to class V of the POU transcription factor family [55]. Most of our knowledge of *Pou5f1* function comes from studies that describe its key role in regulating transcriptional networks essential for stem cell renewal and differentiation [56]. *Pou5f1*-deficient embryos develop to the morula stage, but do not form inner cell mass cells and fail to give rise to embryonic stem (ES) cell colonies in vitro [57]. Furthermore, *Pou5f1* determines the cell fate decisions of embryonic stem cells, and repression of *Pouf51* causes a loss of proliferation and induces differentiation of trophectoderm lineage [58]. In contrast, overexpression of *Pouf51* in ES cells leads to primitive endoderm differentiation [59], while a minor (1.5-fold) elevation of *Pou5f1* expression in germ cells leads to development of gonadal tumors [60]. Therefore, minute changes in the expression level of *Pou5f1* can have significant impact on cell fate and growth properties of ES cells.

At E7.5, *Pou5f1* expression is strictly confined to PGCs, which are the precursor of the gametes [61]. Targeted deletion of *Pou5f1* in PGCs at E7.5 (using TNAP-cre transgenic mice) results in premature apoptosis in PGCs prior to their colonization of the developing gonadal ridges [21]. *Pou5f1* mRNA and protein are expressed in the nuclei of proliferating PGCs present in the undifferentiated genital ridges at E11.5 and in the developing ovaries at E12.5 and E13.5; however, its expression ceases when the oocytes enter into prophase of the first meiotic division (E14.5) and reappears after birth to remain expressed throughout folliculogenesis [61]. It has been speculated that NOBOX regulates the re-expression of *Pou5f1* [18]. However, the importance of *Pouf51* during postnatal folliculogenesis is currently unknown.

Yin Yang 1 (*Yy1*)

Yin Yang 1 (*Yy1*) is a zinc finger-containing transcription factor that functions as an activator or repressor of gene expression and is essential for mouse development [62–67]. *Yy1* is expressed in multiple tissues including the

ovary, and *Yy1* null embryos die around the time of implantation [67]. In the ovary, *Yy1* is expressed in the oocytes and granulosa cells throughout folliculogenesis [22]. YY1 has been proposed to function as a modifier of target chromatin because it has been shown to recruit histone deacetylases and acetyltransferases [68] and also interacts with core transcriptional regulator proteins, such as CREBBP (p300/CBP) and PRMT1 [69] and members of the polycomb repressive complex [70]. In addition, YY1 has been shown to negatively regulate TGFB signaling by interacting with the SMAD transcription factor, SMAD4 [71].

Conditional deletion of *Yy1* in oocytes from primary follicles using *Zp3*-cre transgenic mice results in infertility in female mice. These ovaries contain defects in folliculogenesis and oocyte maturation [22]. When *Yy1* is deleted, a suite of germ cell-expressed transcription factors changes in a pattern not seen in previous knockout mouse models for oocyte-expressed transcription factors. *Sohlh1* is significantly increased, and *Figla*, *Lhx8*, and *Pou5f1* are significantly decreased [22]. Even though *Figla* is decreased, the zona pellucida genes are unchanged. In addition, *Nobox* expression does not change in *Yy1* mutant oocytes though the NOBOX target genes *Gdf9* and *Pou5f1* are decreased. This may suggest that YY1, perhaps by its ability to alter chromatin structure, is also required for NOBOX to appropriately regulate these genes [22]. Further complicating the understanding, the phenotype of *Yy1* null oocytes is that the deletion, unlike those for *Sohlh1*, *Nobox*, *Lhx8*, and *Figla*, occurs conditionally at the primary stage during postnatal follicle development. By using the *Zp3*-cre mouse to drive expression of the recombinase, the deletion thus coincides with the normal timing of *Sohlh1* suppression in oocytes of WT follicles. It has been proposed that *Sohlh1* is a master regulatory gene that specifies an oocyte-specific gene expression pattern by establishing the initial expression of a set of transcription factors (i.e., *Lhx8* and *Nobox*) that are capable of sustaining their own transcription throughout the remainder of folliculogenesis [47] (Fig. 9.3). It is possible, though unproven, that perhaps YY1 is required by *Sohlh1* to establish this pattern of gene expression.

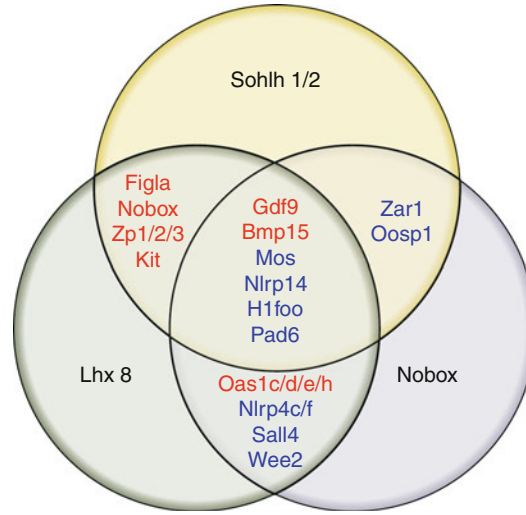

Fig. 9.2 Venn diagram of gene expression changes in *Sohlh1*, *Sohlh2*, *Lhx8*, and *Nobox* knockout mouse models. The genes represented in the intersections indicate that they were downregulated in two or all four mutants. Genes involved in folliculogenesis are highlighted *red* and in early embryogenesis, as *blue*

Conclusion

Over the past decade, the use of mouse genetic models has delineated the critical roles of multiple oocyte-expressed transcription factors in oogenesis, such as the *Sohlh1*, *Sohlh2*, *Nobox*, and *Figla*. These mouse knockouts display depletion of their entire oocyte pool at the very earliest stages of follicle formation and development. Because of their dramatic phenotype and their high degree of conservation between species, these genes and their downstream target genes are excellent candidates for analysis in primary ovarian insufficiency (Table 9.1) [72–74]. It is clear that *Sohlh1*, *Sohlh2*, *Lhx8*, *Figla*, and *Nobox* modulate the expression of genes essential for oogenesis (Fig. 9.2 and Table 9.2). However, it is not entirely understood whether, or how, these transcription factors cross-regulate each other and if they do so by interacting with input from other signaling pathways (Fig. 9.3). Recent advancements in conditional knockout

Table 9.2 Summary of germ cell-specific transcription factor gene expression changes in various mouse knockout (KO) models

	Germ cell transcription factor expression				
	Sohlh1	*Sohlh2*	*Lhx8*	*Nobox*	*Figla*
Mouse KO					
Sohlh1 $^{-/-}$	–	–	–	–	–
Sohlh2 $^{-/-}$	–	–	–	–	–
Lhx8 $^{-/-}$	+	+	–	–	–
Nobox $^{-/-}$	+	+	+	–	+
Figla $^{-/-}$	+	+	+	+	–

Abbreviations: (–) indicates a gene is downregulated, (+) indicates no change

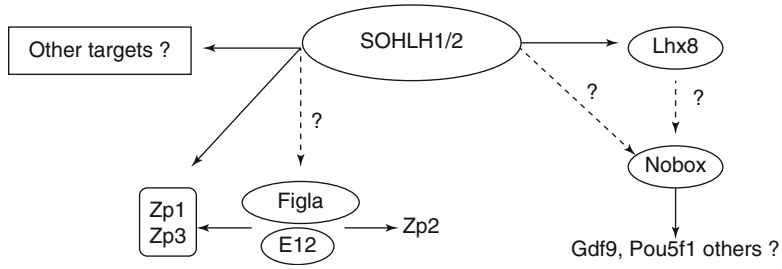

Fig. 9.3 A potential transcriptional regulatory network in early oogenesis. Based on the current literature, it is evident that *Sohlh1* and *Sohlh2* are at the beginning of a signaling cascade that regulates a number of oocyte-specific genes directly (*Zp1*, *Zp3*, and *Lhx8*) or indirectly (*Figla* and *Nobox*). *Lhx8* also regulates the expression of *Nobox* and *Figla*; however, the mechanism by which it does so is currently unknown, and the cross-regulation of *Nobox* with *Sohlh1* is unknown. Loss of *Nobox* in *Sohlh1*, *Sohlh2*, or *Lhx8* mutants results in the loss of *Gdf9* and *Pou5f1* via the NOBOX pathway. *Dotted lines* indicate unknown pathways. *Solid lines* indicate direct transcriptional regulation

technology and generation of stage-specific expression of cre recombinases will continue to provide new information about the involvement of these and other genes in regulating transcriptional networks in the oocyte. Future research also needs to focus on elucidating the molecular mechanisms of transcriptional regulation during oogenesis using a variety of biochemical, molecular, genetic, and high-throughput genomic approaches. Such studies will provide further insight into the transcriptional networks during oogenesis and may uncover new therapeutic opportunities for women who experience premature reproductive senescence and infertility.

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An Epigenomic Biography of the Mammalian Oocyte

10

Maurizio Zuccotti, Valeria Merico,
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Abstract

Successful fertilization and early development depend on the quality of the ovulated oocyte. Our knowledge lacks of links between morphological aspects described and the genomic and epigenomic features that work in the backstage. Bringing to light these links would permit the identification of molecular markers of the oocyte developmental competence. In this chapter, we will review our current understanding of the changes that occur to the oocyte epigenetic signature during folliculogenesis and in mature oocytes.

Keywords

Oocyte • Epigenomic • Chromatin organization • DNA methylation Histone acetylation • Histone methylation • ssRNA

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Introduction

Successful fertilization and early development depend on the quality of the ovulated oocyte. Even though this is endorsed by both the experimental and clinical practice, our still scarce knowledge of the biology of the mammalian oocyte makes it difficult to identify parameters that define the quality or "developmental competence" of an oocyte. The many attempts to establish markers of the oocyte's developmental competence have produced divergent results or have worked only in specific experimental contexts. To this respect, the oocyte morphology, the concentration of various factors in the follicular fluid, the role of the oocyte's mitochondria, the telomere length, and the transcription profile of cumulus cell-specific genes

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are some of the most studied aspects of the oocyte and of the ovarian follicle biology.

The analysis of the level of cumulus cell-specific transcripts has identified groups of genes that are directly (*PTGS2*, *HAS2*, *GREM1*, and *PTX3*) [1–3] or inversely (*GPX3*, *CXCR4*, *CCND2*, and *CTNND1*) [4, 5] correlated to human embryo preimplantation quality and pregnancy outcome. Recent studies analyzed the whole cumulus cell transcriptome in human [6] and bovine [7] cumulus cell-oocyte complexes bringing up a new set of putative marker transcripts.

The concentration in the follicular fluid of myoinositol (a serum trophic factor), inhibin B [8, 9], or AMH has been used for their predictive value of human preimplantation embryonic development, with the latter suggested as a better predictor of oocyte fertilizability [10] and pregnancy rate [11]. Some authors have proposed the presence of a high level of estradiol on the day of hCG administration as a candidate marker of low pregnancy rate [12, 13], but these data are conflicting with others that describe no correlations with the final pregnancy outcome [14, 15]. Similarly, a reduction at the time of oocyte collection in the level of progesterone receptor in human cumulus cells was associated with morphologically good oocytes [16].

Mitochondria have been the subject of a large number of studies, but how and whether they contribute to the determination of the oocyte developmental competence is still unclear. The whole preimplantation period is sustained by mitochondria produced during oogenesis, and only when the embryo begins implantation, their production is resumed. Therefore, an unbalanced number of these organelles, an incorrect distribution, or an altered function may have negative effects on the early stages of development (for a review, see [17]).

The number of mitochondria in mouse primordial germ cells (PGCs) is very small, being approximately 10–100/cell; then, by the mature oocyte they sum up to ~90,000 [18]. The total number of mitochondria seems to be critical to the developmental competence of an oocyte, since subnormal levels of these organelles correlate with premature maturation arrest of the oocyte and early death of the preimplantation embryo [19, 20]. A low mitochondrial complement may determine a bioenergetic/metabolic shortage with consequences on the oocyte's ability of meiotic resumption, fertilization, and to sustain the early phases of development (reviewed in [20–23]). Along with these studies, ATP values have also been associated with the oocyte's developmental competence; an ATP content of >2 pmol seems a threshold to distinguish between developmentally competent and incompetent human oocytes [24].

During folliculogenesis, mitochondria are located in different regions of the oocyte [25–28], and by the mature oocyte they will have an asymmetric polar distribution that will be maintained through segmentation resulting in blastomeres that will own a different number of mitochondria with a different spatial patterning.

A number of observations substantiate the involvement of these organelles not only as powerhouse, producing most of the ATP in the cell, but they may also regulate development by modulating Ca^{2+} signaling, reactive oxygen species (ROS), and intermediary metabolites and through their control of apoptosis. Oxidative stress and intracellular redox potential (IRP) have been shown to regulate the function of a number of transcription factors important in early development. For example, NF-KB (nuclear factor kappa-light-chain-enhancer of activated B cells) and GSK3β (glycogen synthase kinase-3β), expressed during preimplantation development, are activated by mitochondrial ROS production [29, 30]. S-glutathionylation of many transcription factors [31] occurs after the oxidation of the IRP; thus, the ability of mitochondria to modulate the IRP will possibly change the activity of these proteins. Altered mitochondrial Ca^{2+} cycling and ROS production are determinants for the oocyte to enter and accomplish the apoptotic program [32]. Oxidative stress induces mitochondrial dysfunction that triggers apoptosis in the mouse oocyte and zygote [33].

Specific oocyte morphological features are used to select female gametes of good quality. Oocytes are graded as good, when they possess a cytoplasm with fine granules, a single polar body, a narrow perivitelline space, and a ring-shaped zona pellucida [34]. On the contrary, they are

correlated with low fertilizability and developmental competence, when they own vacuoles and refractile bodies within the ooplasm. Although used in some laboratories that practice human assisted reproduction, these morphological markers have been the subject of many criticisms and are not widely accepted [34–40].

As for nuclear morphological features, a large literature demonstrated the possibility to select between developmentally competent and incompetent antral oocyte, depending on their chromatin configuration (see below). This selection, however, relies on the use of fluorochromes whose use, for obvious reasons, is not advisable in our species.

Up to date, our knowledge lacks of links between the morphological aspects described and the genomic and epigenomic features that work in the backstage. Bringing to light these links would permit the identification of molecular markers of the oocyte developmental competence. In this chapter, we will review our current understanding of the changes that occur to the oocyte epigenetic signature during folliculogenesis and in mature oocytes.

The Oocyte Epigenome

Chromatin Organization

While the oocyte grows within the follicle, its chromatin changes organization both locally (e.g., at specific promoter regions) and globally. These changes appear to be functional to the acquisition of a developmental competence. Since the first work by Mattson and Albertini [41] in the mouse, a large number of studies, based on the observation of the chromatin organization, have described the presence within the mammalian ovary of two main types of oocyte's germinal vesicles (GV): one with a ring of heterochromatin surrounding the nucleolus (surrounded nucleolus, SN, oocyte) and a threadlike nuclear chromatin, as evidenced after staining with the fluorochrome Hoechst 33342, and the latter, possessing a more diffused nuclear chromatin and lacking of a specific heterochromatic ring surrounding the nucleolus (nonsurrounding nucleolus, NSN, oocytes). Besides the mouse, SN and NSN oocytes have also been found in other species, like monkeys [42], rats [43], pigs [44], and humans [45]. Goats' [46] and horses' [47] oocytes represent an exception, as the SN type of chromatin configuration has not yet been described in their germinal vesicles.

The smallest primordial oocytes, with a size in diameter comprised between 10 and 20 μm, have an NSN type of chromatin organization that persists until the oocyte reaches a size of 40 μm. Then, at the time of follicle recruitment, about 5 % of oocytes assume an SN type of chromatin organization. The frequency of SN oocytes increases with oocyte growth, reaching about 50 % in the antral compartment of fully matured follicles. This equal frequency of the two classes is a characteristic of the ovaries of young females (4–6 weeks old); instead, during female aging, the frequency of SN oocytes greatly increases reaching about 90 % in >56-week-old mice [48].

What we see through the fluorescence microscope as a different chromatin organization has also a functional meaning. In fact, antral NSN oocytes are transcriptionally active, whereas in SN oocytes gene expression is downregulated. NSN is seen as an immature form that compacts the chromatin and acquires the SN type of chromatin configuration prior to meiosis resumption and ovulation. When mechanically isolated from the antral compartment, 48 h after pregnant mare serum gonadotropin (PMSG) treatment, SN oocytes complete preimplantation [49, 50] and postimplantation [51] development; on the contrary, NSN oocytes are developmentally incompetent and always arrest development at the 2-cell stage. An explanation for the developmental incompetence of NSN oocytes may reside in the role that the transcription factor *Oct4* plays in the acquisition, during folliculogenesis, of the oocyte developmental competence. Oct4 is an important factor in the maintenance of pluripotency. It is expressed in cells with a high differentiation potential such as the primordial germ cells, the type A spermatogonia, the blastomeres of the very early preimplantation embryo, and the cells of the inner cell mass and their derived embryonic stem

(ES) cells [52]. *Oct4* downregulation correlates with loss of pluripotency and cell differentiation [52–54]. Furthermore, a strong proof of its role as a key player in the induction of pluripotency is the demonstration that *Oct4* is one of four factors (together with *Sox2*, *Klf4*, and *c-Myc*) that, when induced in fibroblasts, will convert these terminally differentiated somatic cells into pluripotent cells [induced pluripotent stem (iPS) cells] [55].

The correct expression of maternal-effect genes such as *Stella* (*Dppa3*), *Npm2*, *Zar1*, *Smarca4* (*Brg1*), and *Oct4* is crucial for preimplantation development since mis-regulation of their expression ends in developmental block at the time of zygotic genome activation (ZGA) ([56–60], for a review see [61]).

Our studies have shown that the expression of Oct4 and Stella proteins is upregulated in oocytes with an SN type of chromatin organization, beginning with follicles at the time of their recruitment to start growth; on the contrary, Oct4 and Stella are both downregulated in NSN oocytes throughout folliculogenesis. This pattern of expression is maintained in MII oocytes derived from the maturation in vitro of SN or NSN fully matured antral oocytes [62, 63]. The downregulation of Oct4 in NSN oocytes leads to the upregulation of a number of Oct4-regulated genes involved in apoptosis and of the *Foxj2* gene that, interestingly, is located at the *Nanog* locus which includes also *Stella* and other genes involved in the regulation of cell pluripotency. The upregulation of Foxj2 has a deleterious effect as it provokes the arrest of preimplantation development at the 2-cell stage [64]. Our data on the expression of Oct4 in SN oocytes suggest that the molecular cascade that Oct4 activates during the oocyte's growth might govern the acquisition of the oocyte's developmental competence. Hypothetically, this regulatory pathway might represent the molecular ground that functions when, in nuclear transfer experiments, a somatic nucleus is inserted into an enucleated oocyte. Oct4 has been shown to be an important regulator of local chromatin organization [65, 66], the presence of maternally inherited Oct4 transcripts/proteins [60, 62, 63] in the enucleated oocyte may itself function to change the local epigenetic status of the transferred genome required to regulate the expression of genes necessary to initiate and continue development beyond the 2-cell stage. We have recently demonstrated the presence of an expanded Oct4 transcriptional network in oocytes and its persistence during early development and in ES cells [67].

DNA Methylation and Histone Acetylation and Methylation

These global changes observed in the chromatin configuration during oocyte growth coexist with a level of epigenetic chromatin modifications of the DNA (i.e., methylation of cytosines at CpG sites) and histones (methylation and acetylation) that are still poorly known.

DNA methylation is provided by DNA methyltransferases (DNMTs). DNMT3a and DNMT3b, whose activity is catalyzed by DNMT3L [68], are involved in the transfer of methyl groups to hemimethylated and unmethylated DNA in oocytes [69].

The overall DNA methylation profile, when analyzed with antibodies against 5-methyl cytidine, remains constant from the primordial stage to the time of follicle recruitment, when it increases progressively until the fully matured antral stage [70] (Fig. 10.1). This data was recently confirmed by identifying the presence of about 1,600 CpG islands in mouse oocytes and assessing their methylation status during oocyte growth with the bisulfite method: 0.5 % of the CpGs are highly methylated in oocytes from day 5 mice, 11.3 % in day 20 females, and 15.3 % in ovulated oocytes [71]. More specifically, these CpG islands are preferentially located within active transcriptional sites, and their methylation is not completely removed after fertilization. Instead, by the blastocyst stage, a variegated profile of methylation is observed. While so far it has been thought that the maintenance of CpG methylation after fertilization was a characteristic that pertained only to imprinted sequences, this study suggests that also non-imprinted regions may account for the lack of methylation erasure [71].

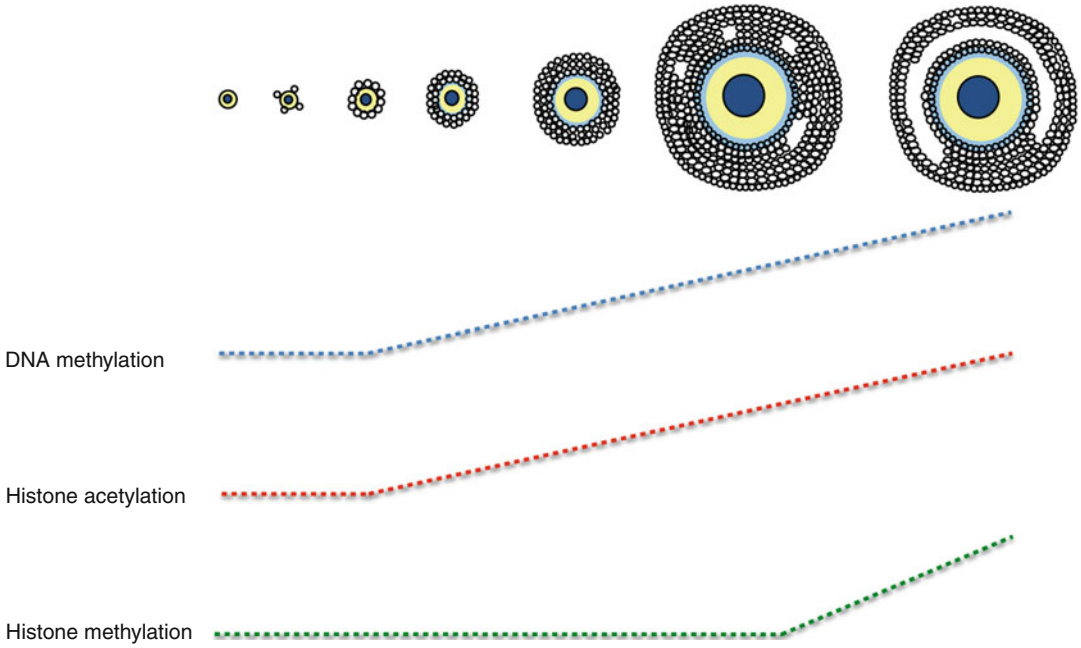

Fig. 10.1 Profile of DNA methylation, histone acetylation, and histone methylation in the mouse oocyte during folliculogenesis

At the single gene level, the group of imprinted genes that has been more extensively studied includes the majority of about 100 genes that have been identified as maternally imprinted, i.e., that are epigenetically modified at specific time points during oogenesis. Some of these genes are methylated during the transition from primordial to primary follicles (*Znfl27* and *Ndn*) and others in secondary (*Peg3*, *Igf2r* and *p57kip2*) and tertiary to early antral (*PegI*) or antral (*Impact*) follicles [72]. The study of the *Snrpn* gene has taught that methylation of the two alleles may not occur synchronously, suggesting that a memory of their parental origin, other than the methylation, is maintained and recognized. The maternal allele is methylated first in early growing preantral follicles, whereas the paternally inherited allele is methylated later at the antral stage [73]. Methylation of non-imprinted sequences occurs at the early stages of follicle growth [74], and it appears to involve different types of DNMTs [75]. Once established, the methylation parental imprint is maintained during preimplantation development and in the somatic line and is erased sometime between the genesis of the PGCs, their travel to the primordial gonads, and the beginning of the meiotic process in oocytes.

The level of histone acetylation (H3K9ac, H3K18ac, H4K5ac, and H4K12ac) remains low during the early stages of growth, but, as for DNA methylation, it increases abruptly following follicle recruitment, consistently with an increase of gene expression [70, 76, 77] (Fig. 10.1). SN antral oocytes have a higher level of histone acetylation than NSN oocytes [78], despite the former being transcriptionally inactive. The maintenance of a propitious transcriptional epigenetic condition, in the heterochromatin of transcriptionally silent SN oocytes, might be functional to the beginning of preimplantation development when transcripts from these heterochromatic regions are functional to preimplantation progression [79].

Instead, histone H3 is dimethylated (K4me2, K9me2) or trimethylated (K4me3 – controlled by the MLL2 methyltransferase [80] – and K9me3) much later during oocyte growth, when follicles reach the antral compartment and oocytes have a size in diameter of 70–80 μm (Fig. 10.1). Histone methylation is more stable and plays an important

role in the establishment and maintenance of genomic imprinting.

The Role of Small Silencing RNAs

During growth, the oocyte synthesizes a large number of transcripts that are partially used during folliculogenesis and in part stored for further use during preimplantation development. Soon after fertilization and by the time ZGA occurs [81], 90 % of the maternal transcripts are selectively inactivated or degraded because their presence may be unnecessary or even detrimental during preimplantation development. Inactivation occurs through processes of deadenylation [82], association with RNA-binding proteins [83, 84], and elimination through the action of a class of small silencing RNAs (ssRNAs). These are short length (20–30 nucleotides) RNAs that, following their transfer to the cytoplasm, are processed by an RNase III-like enzyme, Dicer, and then joined to proteins of the Argonaute family to constitute a ribonucleic complex that binds to the target mRNAs to degrade them or inhibit their translation [85]. ssRNAs are distinguished in three main classes: small interfering RNAs (siRNAs or endo-siRNAs), Piwi-interacting RNAs (piRNAs), and microRNAs (miRNAs). The few studies that have analyzed the presence of ssRNAs in oocytes have showed that their average amount does not vary during maturation, although single miRNAs may vary considerably [86]; instead, it shows a three-fold increase after ovulation in MII oocytes. These maternal ssRNAs represent the only contribution to the zygote, since the sperm does not carry a significant contribution [87]. Within the miRNAs, the let-7 family (miR-17-92 cluster) is the most abundant [88, 89]. With the beginning of segmentation, coincidentally with a global RNA degradation that occurs between 1-cell and 2-cell stage of development, the amount of miRNA decreases by 60 %, suggesting an active process of degradation [90]. The following stages of preimplantation development are characterized by an abundant and increasing production of miRNAs that, for some specific clusters (miR-290 and miR-295), reaches a 15- to 24-fold change. As for other cell types, Dicer plays in oocytes a central role in the production of miRNAs. Lack of Dicer leads to the production of developmentally incompetent oocytes that, after fertilization, do not proceed beyond the 1-cell stage [86]. These oocytes have an abnormal spindle organization, and the expression of some key genes such as *C-mos*, *H2Ax*, *H1foo*, and *SCP3* is altered.

The role that miRNAs play during early development has been further supported by recent studies that have demonstrated their crucial involvement in regulating ES cell pluripotency and differentiation [91]. The miRNA pathway is predominant in ES cells whose main function is the regulation of cell cycle progression during stem cell differentiation. Specific miRNAs, such as miR-290–296, play a role in the induction of mouse ES cells differentiation, whereas levels of miR-21 and miR-22 increase following the induction of differentiation. A recent study has shown that the mRNAs of the pluripotency genes *OCT4*, *SOX-2,* and *KLF4* are direct targets of miR-145 whose function represses pluripotency in human ES (hES) cells; furthermore, *miR-145* promoter is bound and repressed by OCT4 in a double-feedback loop that involves also the three pluripotency factors and switches hES cells between self-renewal and differentiation [92].

Conclusions

The role of the oocyte in supporting and regulating the life of the mammalian embryo during its early stages of development is renown. To put it in the words of E.B. Wilson, “Embryogenesis begins during oogenesis” (as quoted in [93]). It is therefore surprising how little we know of the molecular processes, the molecules, and the mechanisms that underlay the acquisition of the oocyte developmental competence. In this chapter, we have taken an oocyte-centered view, describing the first evidences that are emerging indicating the critical role played by the epigenome. Clearly, our future challenge will be to consider the changes that the oocyte epigenome undergoes during folliculogenesis within the context of the whole follicle structure, thus considering

the complex interactions between the female gamete and its companion follicle cells. To this regard, we foresee that an important contribution will be given by the use of a combination of ovarian follicle culture and micromanipulation techniques [94]. For example, the injection of demethylating substances such as 5′-azacytidine or of supravital fluorochromes, to track the modifications of particular cellular or nuclear structures during follicle growth, will allow the monitoring of the changes induced within each single oocyte/follicle. Furthermore, this approach would make possible the injection of a variety of molecules such as plasmids containing specific gene sequences (to induce the expression of specific genes), signaling proteins, siRNAs (to interfere with translation), and antibodies (to inactivate proteins). Altogether, these micromanipulation experiments will aid the dissection of the composite interactions existing between the oocyte and the surrounding follicle cells and help in the identification of the factors that regulate the acquisition of the oocyte developmental competence.

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Epigenetic Regulation of Oocyte Function and Developmental Potential

11

Wendy Dean

Abstract

Epigenetic regulation is complex, integrating transcriptional states of chromatin together with structural information influencing function and genome integrity. Some of these modifications are temporal lasting minutes or hours while others are heritable. The DNA content of all nucleated cells in an organism is nearly identical yet myriad cell types derive from the zygote, having followed the blueprint to generate all cell types of the adult. The orderly unfurling of this program of development with progressively restricted cellular plasticity, while maintaining cellular identity, requires mechanisms that uphold these heritable changes. Remarkably within these strict guidelines the germline and the early embryo reprogram their epigenetic information to restore pluripotency. While detailed information is not yet available for high-resolution analysis of chromatin profiles focussing on histone modifications, great advances have been made investigating DNA modifications during oogenesis and early development. These studies confirmed that differentially methylated regions, well beyond the cohort of imprinted genes, were affected with >1000 CpG islands acquiring their DNA methylation late in oogenesis. Moreover, in the mouse, as many as 15% of these methylated targets are maintained up to the blastocyct stage and are hence transgenerationally inherited. These reprogramming periods may be particularly sensitive to environmental disturbance and nutritional states making procedures such as those in the treatment of human infertility especially susceptible to epigenetic alterations that may be inherited and transmitted to future generations. This chapter describes some of the most important aspects and exciting new discoveries in epigenetic regulation in oocytes and embryos and highlights their implications in the treatment of human infertility by assisted reproductive technologies.

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Epigenetic regulation of cellular function is complex, involving a cast of versatile mechanisms that may seamlessly share roles in maintaining tissue-specific differentiation and cellular identity. This is no less the case for the mature female gamete. Charged with the immortality of the species, the oocyte has by necessity maintained a broad repertoire of mechanisms that instruct the underlying genetic code while treading the precarious pathway between meiotic and mitotic requirements. In order to support this versatility, the oocyte must be endowed with an extraordinary reserve of molecular resources. Yet, this too remains part of her 'feminine mystic' as it is still not clear how these reserves of proteins and activities are used and whether they are in fact used at all during the preimplantation period of development.

Epigenetic regulation was postulated by Conrad Waddington to explain the stepwise generation of the myriad of cell types found in the adult organism arising from the common origin of the fertilized oocyte. Waddington gave the analogy of cellular differentiation with a series of descending channels, which he called canalization, that directed cells from the totipotency restored in the zygote through graded stem cell gates to ensure developmental progression in the 'forward only direction'. In contemporary terms, epigenetic mechanisms regulate cellular specialization through developmentally and tissue-specific gene expression and provide a mechanism of maintaining that cellular identity once it has become established upon completion of differentiation [1].

What Constitutes Epigenetic Regulation- More than Just Transcriptional Regulation

In the postgenomic era, increasing attention has turned to the understanding of genomic regulation by epigenetic modifications that interpret the underlying genetic code often by specifying transcriptional states that define cellular identity. Perhaps the most interesting place in which to look at the influences of these epigenetic marks is in the reproductive axis. While many varied definitions have been supplied for the study of epigenetics, here our discussion will focus on the more canonical definition. Epigenetic modifications refer to those processes whereby a heritable change in the phenotype is achieved by changes to the instructional content of the genome without any change to the underlying DNA sequences and hence the genetic code [2]. As such, these changes in both DNA-based modifications and those of chromatin, especially the histones of nucleosomes, supply an overlying informational layer that allows for the myriad cell types of developing organisms and the adult. In short, epigenetics explains the complexity of multicellular organisms from a single genetic blueprint.

In mammals, DNA methylation has long been recognized as the major epigenetic modification in the genome [3]. DNA methylation has essential roles in genomic imprinting, a non-Mendelian parent-of-origin form of inheritance; in genomic integrity through silencing of retrotransposons; in structural integrity supporting centromeric function and in whole X chromosome inactivation. DNA methylation may also serve to regulate certain key lineage-specific genes and hence serves an essential role in the reinforcement of lineage specification [4].

Beyond this immediate covalent modification of the cytosine base, additional complexity and genomic plasticity can arise from dynamic changes at the level of the nucleosome mediated by the inclusion of histone variants [5, 6]. These encompass both replication-dependent and replication-independent forms of histone variants that alter the readability of the genome and hence influence transcriptional states. Multiple classes of RNA molecules including long non-coding RNAs and the small RNA families are also able to exert control over genome functions both in cis

and trans that affect chromatin organization [7]. Collectively, they describe a highly complex but integrated set of instructions that guide and maintain cellular processes essential for growth, development, and reproduction.

How to Read the Genome? The Histone Code and All That Jazz

The epigenetic code (1999) and quickly thereafter the histone code (2000–2001) were proposed to explain the means by which transcriptional states could be rationalized to account for the phenotype of differentiated cells. These hypotheses suggested that covalent modification of N-terminal histone tails by addition of acetylation, methylation, phosphorylation, ADP-ribosylation and ubiquitinylation would confer specific instructions to every gene, at any given moment, thus orchestrating transcriptional states specifying cellular identity [8–11]. Some residues were found to be modified differentially; for example, lysine residues can be acetylated, a modification long known to correspond to transcriptional activity, or it can be methylated, a modification usually associated with silencing and the heterochromatic state. Furthermore, methylation of specific residues can adopt mono-, di- or trimethylated states, which confers additional layers of instruction and discrimination between euchromatin and facultative heterochromatin [12]. Yet other specific signatures such as H4 K20 me3 are associated to the largely gene poor regions of the genome or constitutive heterochromatin with essential roles in maintaining centromeric function [13].

Another layer of complexity in chromatin organization comes in through the use of different histone variants, where the contribution of core histones H2A and H3 has gained most attention as they are integral to the functional nucleosome. At present, histone variants have been found for H2A and H3 but interestingly not for histone H4. In the oocyte, a restricted number of the 7 H2A variants are incorporated into newly formed chromatin presumably in keeping with specific function and totipotency and the impending activation of the zygotic genome at the two-cell stage of development [14].

A consensus for specific chromatin signatures with definitive structural features in somatic cells quickly emerged. The generation of an extensive battery of specific antibodies permitted biochemical and cellular analysis revealing the composition of chromatin in somatic cells [15]. However it became apparent that eggs, oocytes, and early embryos made use of these same components but created distinctive signatures that were associated with their unique totipotent and pluripotent status [16, 17].

Epigenetic Reprogramming: How Embryos Make Eggs and How Eggs Make Embryos

In the mouse, there are two well-documented developmental periods when the genome is profoundly remodeled [18]. One occurs on fertilization and is completed by the blastocyst stage; the other takes places in the germline shortly after specification of germ cells at day 6.25 post-coitum and culminates, after an indefinite hiatus, in the generation of the oocyte and, by a somewhat different path, in functional spermatozoa [19, 20].

Epigenetic Remodeling During the First Cell Cycle

On fertilization, the essential restoration of the diploid genome is initiated in the zygote [21]. This encounter triggers the requisite remodeling of both the haploid sperm nucleus, packaged in the compacted nucleoprotamine-rich configuration, and the oocyte that is widely arrayed in a somatic-like nucleohistone chromatin [22]. This initial asymmetry of organization is believed to require resolution in order to restore the zygote to a functional diploid cell. However, in so doing, a series of other significant epigenetic asymmetries must arise presumably to generate the only truly totipotent cell, all the while maintaining parental identity and chromatin isolation within the individual pronuclei prior to the first mitosis. For more than 20 years, it has been appreciated that fertilization triggers a transition from highly methylated gametes that lose DNA methylation

in a very deliberate manner by first an active and then a passive mechanism [23–25]. Now we have a much more detailed description of this process with molecular data that help unravel the targets and developmental implications. Despite our enhanced knowledge of the subject, this process still remains controversial and remarkably enigmatic.

Chromatin remodeling is initiated within an hour of penetration by the capacitated sperm and results in the remodeling of the sperm nucleus by the maternal environment [26, 27]. This process involves the removal of protamines, replacing them with nucleohistones in a replication-independent manner. As such, the nucleoprotamine is replaced with nucleohistones containing the specific H3 variant H3.3 [28]. At the same time, paternal-specific active demethylation of most DNA methylation in the CpG context is initiated [29, 30]. Prior to the initiation of the first S phase, the male pronucleus is largely demethylated except for the centromeric satellites that surround the nucleolar precursor bodies (NPBs). This essential residual DNA methylation is presumably maintained in part by chromatin marked by the H3K9me1 modification in conjunction with heterochromatin protein-1 beta (HP1β), which is found in the centromeric region [31]. The functional homologue HP1α is not found in the early mouse embryo. Other overt heterochromatin hallmarks such as H3 K9me2, H3 K9me3 and H4K20me3 are absent from the male pronucleus but abundant in the female at this stage [21, 31]. Although the chromatin of the male pronucleus is largely comprised of building blocks supplied by the maternal store, the chromatin of the male pronucleus remains highly distinctive suggesting an essential role for the maintenance of parental origin.

Polycomb-group (PcG) chromatin proteins, a further layer of transcriptional repressors, also becomes asymmetrically arrayed with enhanced accumulation of the Polycomb repressive complex 1 (PRC1) component Ring1b in heterochromatin of the male pronucleus [32]. Here, in the absence of DNA methylation, PRC1 mediates genomic silencing through a polycomb-specific response in the male but not the female pronucleus. Interestingly, PRC2 proteins Ezh2/Eed and Suz12 are found in both compartments along with the H3K27 me3 they supply although their pattern clearly differs between the male and female pronuclei [31, 32]. Here too, these instructions are sequentially complied in the absence of overt transcription. Whether they serve another role outside of transcriptional repression in this context remains an interesting question.

Chromatin instructions are important for nuclear architecture and higher-order chromatin organization. Given the highly differential nature of the chromatin of the male and female pronucleus, the *a priori* expectation would be that chromatin and nuclear organization should be profoundly different. In vitro fertilization (IVF) provides a means of studying temporal events in a detailed manner. IVF-generated pronuclear-staged oocytes revealed an unexpected synchrony of the array of centromeric satellite sequences during epigenetic reprogramming events in the first cell cycle. Despite their remarkable differences, the dynamic organizational changes were synchronous in male and female pronuclei revealing a striking pattern of intercalation of major and minor satellites arrayed around the NPBs [33]. This pattern is completely distinctive to the chromocentric coalescence of centromeric regions which become apparent as a hallmark of a differentiated somatic cell [34]. Therefore, at synkaryogamy, the male and female chromosomes align uniquely, maintaining parental origins and restoring the totipotent diploid nucleus of the zygote ephemerally at anaphase.

Thus, at chromosome congression, the genome is able to maintain genomic integrity and parental identity through the combined efforts of DNA methylation (in the female), maternal specific histone modifications, histone variants and, in the male, PcG- ensured heterochromatin function in the absence of DNA methylation.

Remodeling Chromatin in the Absence of Transcription

Relatively few opportunities allow for the ordinary study of epigenetic instructions in the absence of transcription. Details of heterochromatin landscapes are particularly relevant in the

first cell cycle as it takes place largely in the absence of transcription.

In the initiation of what may continue to be heritable patterns, epigenetic marks are dynamically changing but, importantly, not in order to support transcriptional requirements. Even those instructive elements associated to transcription at later stages are given to other tasks during the chromatin remodeling of pronuclei. The unique situation of these events occurring in a transcriptionally silent state implies that they occur by direct modification or active histone replacement in the nucleosome.

The oocyte is well equipped to handle the modulation of epigenetic marks, both adding and removing histone modifications as part of the layers of information. Shortly after fertilization, histone acetylation is asymmetric with an initially higher intensity of staining in the male pronucleus but quickly becomes equalized with the female pronucleus confirming the presence and function of histone deacetylases (HDACs) in the oocyte [35]. However, histone methylation is also in place, especially at lysine residues, confirming the presence of histone methyltransferases (HMTs often referred to as lysine methyltransferase KMTs or PMTs) [16, 31, 36].

While an activity capable of orchestrating the direct loss of the covalent methyl group of the nucleotide cytosine (5 meC) has yet to be found, activities that remove methyl groups from protein lysine and arginine residues in nucleohistones have been described. These activities work by a number of mechanisms ranging from direct removal of methyl groups from lysine residues via oxidation to the proteolytic removal of symmetrical and asymmetrical methyl groups of arginine residues by deamination [37]. We now appreciate that individual classes of histone demethylases are active on a narrow range of lysine residues and add an additional layer of regulation at this level of chromatin function [38]. Perhaps more importantly, early reports suggesting that histone modifications possessed the same heritable status of DNA methylation are no longer regarded as true and point to further potential for flexibility and susceptibility to environmental influences beyond DNA methylation. Interestingly, the oocyte does not possess two major histone methyltransferases associated to modification of H3K9, a hallmark of heterochromatin. Euchromatin methyltransferases (G9a/GLP) and the constitutive heterochromatin modifier SUV39h1 are not expressed in the oocyte, potentially reinforcing the impending pluripotent state during preimplantation development [32].

Other Histones Modifications and Variants

A unique chromatin configuration is further evident as the oocyte is missing some of the H2A class of variants later used in somatic tissues while possessing oocyte enriched variants of others. Absent from the oocyte is the variant H2A.Z and macroH2A [39] while H2AX is found in abundance, a situation dramatically different from somatic tissues. This is thought to reflect the unique totipotent stage occupied by the fertilized oocyte. Histone variants of H3 are also differentially accumulated leading to significant differences in chromatin distribution between male and female pronuclei. The replication-independent H3.3 variant allows for response-based remodeling and is first found in the male pronucleus and later in the female. However, incorporation of the replication-dependent variants H3.1 and H3.2 occurs simultaneously on entry into S phase. Other reports suggest something to the contrary. Flag-tagged H3 variants were shown to actively exclude H3.1 incorporation in favor of H3.3. DNA hypomethylation induces H3.3 incorporation into pericentric heterochromatin [40]. The assimilation of H3.3 into paternal chromatin consequently influences the distribution of H3K27me1 in pericentric heterochromatin and in turn permits expression of pericentric dsRNA leading to the reinforcement of heterochromatin in the male pronucleus, which is otherwise largely devoid of canonical heterochromatin marks [41]. Remodeling of maternal chromatin may also take place with the suggestion that epigenetic information encoded by H3.3 in growing oocytes is erased upon fertilization and then replaced [40]. This chromatin configuration may be essential to ensure pericentric stability and proper chromosome segregation following the first S phase.

While some of the histone modifications are differentially deposited and may reinforce parental identity over the entire period of preimplantation, a recent report identifies another heterochromatic mark, H3K64 me3, that is inherited maternally and contributes to pericentric heterochromatin only during the first cell cycle, before becoming rapidly degraded. Suggestions that in somatic cell types there is a connection between H3K9me3 and H3K64m3 and that K9me3 may be epistatic to H3K64me3 would not be the case for the oocyte nor the early embryo as H3 K9me is present in maternal chromatin and appears in paternal chromatin only from the two- cell stage onward despite the absence of H3 K64me3 [42].

Comprehensive surveys of the additional 200 plus modifications are slowly being compiled for the fertilized egg during the first cell cycle [43]. While biochemical studies to investigate the precise nature of the chromatin of these relatively rare samples and the sequences they influence are still largely in their infancy, the antibodies essential to revealing these structural principles abound. They have been powerful tools in learning about the earliest stages of newly fertilized eggs and reveal in part the signatures that are characteristic of pluripotency, an idea that has validity in translation of basic research to regenerative medicine and novel therapeutic approaches.

DNA Methylation: Marking the Genome – Fidelity and Heritability Through the Generations

Centrally important to the study of epigenetics is the idea of heritability. This justifies the importance given to the study of the establishment of chromatin signatures that first support pluripotency and then permit its step-wide decline during early development. In considering this unique window for studying establishment of epigenetic signatures, the relevance of the immediate history of the oocyte becomes essential.

A second developmentally relevant wave of epigenetic reprogramming is known to take place in the germline [18, 44]. While definitive experimental confirmation is still awaited for germline reprogramming, it is widely accepted that this period of resetting is ultimately required in order to first erase and then re-establish instructional marks associated to genomic imprinting [45–48].

Consistent with the requirement for lifelong heritability, this erasure involves the removal of the methyl group from 5-methylcytosine such that at its culmination at the time of meiotic arrest in prophase at day 13.5pc, female germ cells have effectively little or no DNA methylation remaining [49]. Yet, by the time oocyte maturation and growth is completed, the mature oocyte, in advance of fertilization, must be replete with fully restored DNA methylation at germline differentially methylated regions (DMRs) (Fig. 11.1). It is important to note that the vast majority of those imprinted genes reliant upon DNA methylation for their allelic instructions acquire this methylation in the female germline. A small number of enzymatic activities – in the context of a specific chromatin template – are known to be required to restore imprinted instructions to the growing oocyte in a temporally regulated manner [51–53].

As such, these activities should be regarded as essential for oocyte maturation supporting full-term development. Further, as they are heritable throughout the life of the organism, these processes are among some of the earliest and most assiduously maintained epigenetic marks found in mammals. Elegantly constructed genetic analyses using conditional deletion of Dnmt3a and its non-catalytic family member Dnmt3L have confirmed that germline imposition depends on these activities to establish maternal methylation imprints [45, 54, 55]. Operating as a tetrameric complex, these DNA methyltransferases effectively ‘embrace’ the targeted regions specified by chromatin marks H3K4 in conjunction with KDM1B (also known as AOF1 or LSD2), H3K4me2, H3K4me3 demethylase to restore DNA methylation to CpG dinucleotides in imprinting control regions (ICRs) and DMRs. The so-established DMRs designate oocyte-specific promoters. These promoters generate long coding transcripts that target DNA methylation in a transcription-dependent process to restore of ICRs [50, 56, 57]. Other imprinted genes seem to use additional specific activities as cofactors. It has been known for some time that ZFP57, a KRAB domain-containing protein, is essential for establishment in oogenesis of the genomic DMR of Snrpn and maintenance of a number

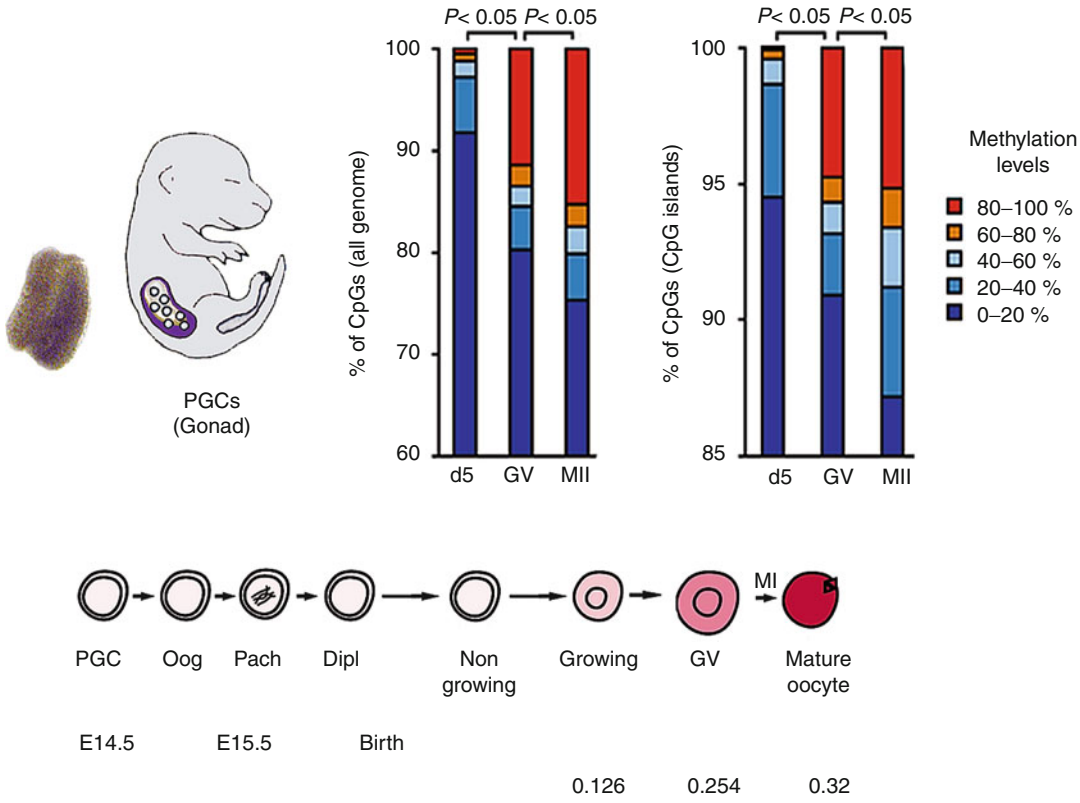


Fig. 11.1 Epigenetic reprogramming cycles establish DNA methylation between generations. The two defined reprogramming cycles during mammalian development are bounded by the period following meiotic arrest in the female germline. Oocyte growth and maturation is characterized by the restoring of DNA methylation in defined windows. Imprinted DMRs and imprinting centers are restored during this period. Two recent studies have revealed that DNA methylation is restored to non-imprinted loci with significant numbers of loci undergoing de novo methylation between the germinal vesicle stage and the mature MII oocyte. Estimates of the changing dynamic methylation are included for select stages and presented as a proportion of total CpG dyads '$P< 0.05$' signifies statistical significance between pairs (Portions of this figure are reprinted by permission from Macmillan Publishers Ltd: Nature Genetics [50], copyright 2011)

of other imprinted genes post-fertilization [58]. A breakthrough in understanding the relationship of ZFP57/KAP1 has led to the understanding that maintenance of histone signatures H3 K9me3 and DNA methylation at imprinted ICRs are intimately associated through KAP1. Thus, depletion of ZPF/KAP1 will result in hypomethylation at ICRs and identify another essential feature that must remain intact and functional during ART [52, 59].

Re-instruction of the oocyte methylome begins in haste during the postnatal period (Fig. 11.1). Thus, there is a switch from the maternal nutritional influence of the placenta to maternal nutrition mediated by lactation. The earliest stage for which there is robust information about this programming phase is postnatal day 1 when DNA methylation at imprinted loci can be detected [60]. DNA methylation can also be detected in IAPs, a specific group of retrotransposons, some of which have important roles in regulating placental development [61–63]. Until recently detailed information about DNA methylation programming during oocyte growth was largely restricted to CpG islands (CGIs) of imprinted loci and repeat families of the centromeric satellites, as well as other retroelements of medium- to high-copy number. Using the combined value of reduced representation by selective enrichment of genomic fragments containing CpG-centered restriction sites (MspI/HpaII) together with bisulphite mutagenesis and direct sequencing (RRBS), Smallwood and colleagues have supplied a richer and much deeper profile of the oocyte methylome [50].

Remarkably, RRBS identified >1,060 CGI with >75 % DNA methylation in mature oocytes. This unexpectedly high number of DMRs extends the genomic sites for this specific signature substantially as there are only around 90 imprinted genes. However, in contrast to imprinted genes whose DMRs are maintained during the preimplantation period, methylation of most of these other ~1,000 CGIs were not fully maintained up to the blastocyst stage. Many CGIs were found to have a mosaic pattern of DNA methylation lending credence to the provocative suggestion that on fertilization, reprogramming of the genome by active and passive mechanisms leads to the possibility of epigenetic heterogeneity at the time of lineage specification [64].

Thus, the oocyte methylome is a critical juncture for the brokerage of DNA methylation that may have impact throughout the preimplantation period and beyond.

Of special significance in the Smallwood et al. [50] results was the comparison of germinal vesicle (GV)-staged oocytes and the fully mature ovulated oocyte. Eighty-nine CGIs were methylated in MII oocytes that were not fully methylated at the GV stage. These results would highlight the importance of the final stages of oocyte maturation when nearly 10 % of methylatable GCIs are still actively acquiring DNA methylation marks by de novo processes. These findings should serve to extend substantially the experimental targets available to evaluate the efficacy of oocyte maturation in vitro as part of the many procedures associated to assisted reproduction technologies (ART) in the treatment of human infertility. Furthermore, these data reveal that the maintenance of DNA methylation at imprinted loci represents a minority of genomic targets that may be sensitive to manipulation around this critical time point.

Mechanisms for Epigenetic Reprogramming

There has been a renaissance for the importance and instructional significance of methylcytosine in DNA over the last few years. Genome-wide investigation by next generation sequencing in pluripotent embryonic stem cells (ESCs) and oocytes has identified the presence of significant non-canonical modification of DNA methylation. These have included asymmetric DNA methylation as well as the report of a new epigenetic modification, hydroxymethylcytosine, which is an oxidative derivative of 5mC and provides a substantial conceptual shift in the field of covalent DNA modifications after nearly 60 years.

Asymmetric DNA methylation has been widely accepted in the epigenome of plants, but robust demonstration of these signatures in mammals has not been consistently reported until now. Asymmetric DNA methylation may include hemimethylation of a CpG dinucleotide, the canonical context for most DNA methylation in mammals. However, the classes of DNA methylation recently reported included CpHpGp and CpHpHp, both common to the plant kingdom. Anecdotal reports of asymmetric DNA methylation have frequently cropped up when reporting bisulphite-modified DNA results in oocytes and ES cells [65]. Yet, the absence of rigorous investigation has made it impossible to distinguish it from unconverted sequences, and as such it has remained an unresolved issue. Indeed, analyses that relied on restriction enzyme digestion in target regions would have failed to detect such arrays of asymmetric DNA methylation.

High-resolution analysis of imprinted DMRs and ICRs in gametes and early embryos has now offered convincing evidence in oocytes, but not in sperm, of non-CpG methylation [66]. Interestingly, in a similar fashion to the non-imprinted DMRs reported in oocytes, asymmetric DNA methylation does not get maintained throughout the preimplantation and is substantially reduced by the blastocyst stage. While no specific function has been assigned as yet, in mouse oocytes there is a preferred context, CpHpH, and not the symmetrical trinucleotide CpHpG, that might lend itself to some underlying role in the genome [66].

Demethylation and Demethylase-Hydroxymethylation as a Mechanism for DNA Methylation Reprogramming

The idea of the process of active demethylation has courted adherents and skeptics in equal measure [67]. Most of the interest in active demethylation has been focused on the process of

paternal-specific active demethylation in the male pronucleus in the newly fertilized oocyte, best described in the mouse but evident among a wider group of mammals [21, 29, 30, 37]. Originally defined by loss of DNA methylation prior to replication, or outside of S phase, active demethylation should be strictly regarded as loss of DNA methylation due to removal or modification of the base or methyl group prior to, or during, S phase. As such, it has to occur as a consequence of some modifying process and not due to the exclusion of the maintenance methylase, Dnmt1, from the replication fork.

But what of the other mechanisms which have been proposed for this enigmatic process of demethylation? To date, a reasonably wide and diverse list of candidate activities have been proposed as active DNA demethylases [68]. Activities have included DNA methyl-binding proteins (Mdb2, Mbd4) and de novo DNA methylases themselves (Dnmt3b). These classes of proteins have specificity for cytosine in DNA (e.g., Mbd2) and are able to bind to and accommodate the methyl-leaving group by delivering it to an acceptor molecule (Dnmt3 and S-adenosylhomocysteine) as part of a direct mechanism. However, fertilized oocytes null for Mbd2 and Mbd4 readily undergo demethylation. Attention has also focussed on an indirect mechanism that relies upon deamination of the methylcytosine base generating thymidine [69]. This mechanism would require a downstream repair of the mismatched T-G base pair. A family of activities have the ability to deaminate methylcytosine and are found in the mammalian oocyte. Activation-induced cytosine deaminase (Aicda) and its family members, Apobec 1–3, are expressed in a wide number of tissues and serve various functions including the predominant role of AID in activated B cells where it brokers the variation essential to the repertoire of antibodies of the immune system by class switch recombination and somatic hypermutation. Experimentally it is not yet clear what role it may have as part of the DNA demethylation machinery in the fertilized mouse oocyte. A detailed review that considers these and other mechanisms has recently been published [70].

Of the growing list of possible candidates for a demethylase, among the most plausible is TET3, an oocyte-enriched activity that uses methylcytosine as a substrate and modifies it through its capacity as an oxidase into hydroxymethylcytosine [71–73].

Arguably the identification of hydroxymethylcytosine has been a ‘eureka’ moment in the field of epigenetics. At the time of its discovery, methylcytosine was originally called the fifth base of DNA. Hence, a significant modification of this base invites considerable interest and excitement in the realm of the epigenetic landscape. Indeed, very early on the potential for this modification was seen as a means of achieving active demethylation in a single step under physiological conditions [74]. Initial reports using immunofluorescence (IF) in fertilized oocytes described a concomitant increase in hydroxymethyl (hm) cytosine with a loss of methylcytosine predominantly in the male pronucleus [75, 76]. This seemingly symmetrical dynamic transition was thought to supply sufficient evidence for an active demethylation pathway.

Suggestions that TET3 may further modify the hydroxyl group through two further oxidative stages, formyl and carboxyl methylcytosine, have been made following similar results in ES cells [77, 78]. Thereby methylcytosine would be restored to cytosine- qed. In oocytes, experimental evidence using a genetic deletion of the TET3 activity results in loss of hydroxymethylation in the male pronucleus. Indeed, molecular analysis confirms the results observed by IF. Genetic loss of TET3 results in an increase in DNA methylation among single-copy genes as well as genomic repeat elements of the retrotransposon family. However, while methylation increases in the male pronucleus as suggested by IF, some specific demethylation still occurs revealing a more complex regulation of methylation reprogramming during early development [79]. Beyond the initial one-cell stage, recent analysis confirms that hydroxymethylation diminishes in parallel with DNA methylation in accordance with the expectations of a passive mechanism for demethylation [80]. These are early days for understanding how this process works and the targets it can modify. Much remains to be worked out about the role and function of this oocyte-specific hydroxymethylase and its significance for later development.

Transgenerational Epigenetic Inheritance: Fidelity, Erasure and Heritability in the Germline

The generation of gametes is the guarantee for any species that immortality is assured. In this regard, it may not be surprising that after reprogramming in the germline, a second genome-wide reprogramming is initiated triggered by the completion of meiosis at fertilization. This places the oocyte firmly between the generations, and its reprogramming provides a form of genomic refreshing after what may be a >30-year hiatus without the benefit of the resetting afforded by the replication of DNA.

Transgenerational epigenetic inheritance is concerned with those genomic targets ordinarily subject to germline erasure but which from time to time may elude this erasure event and consequently transmit epigenetic information into the next generation that may result in a heritable phenotype.

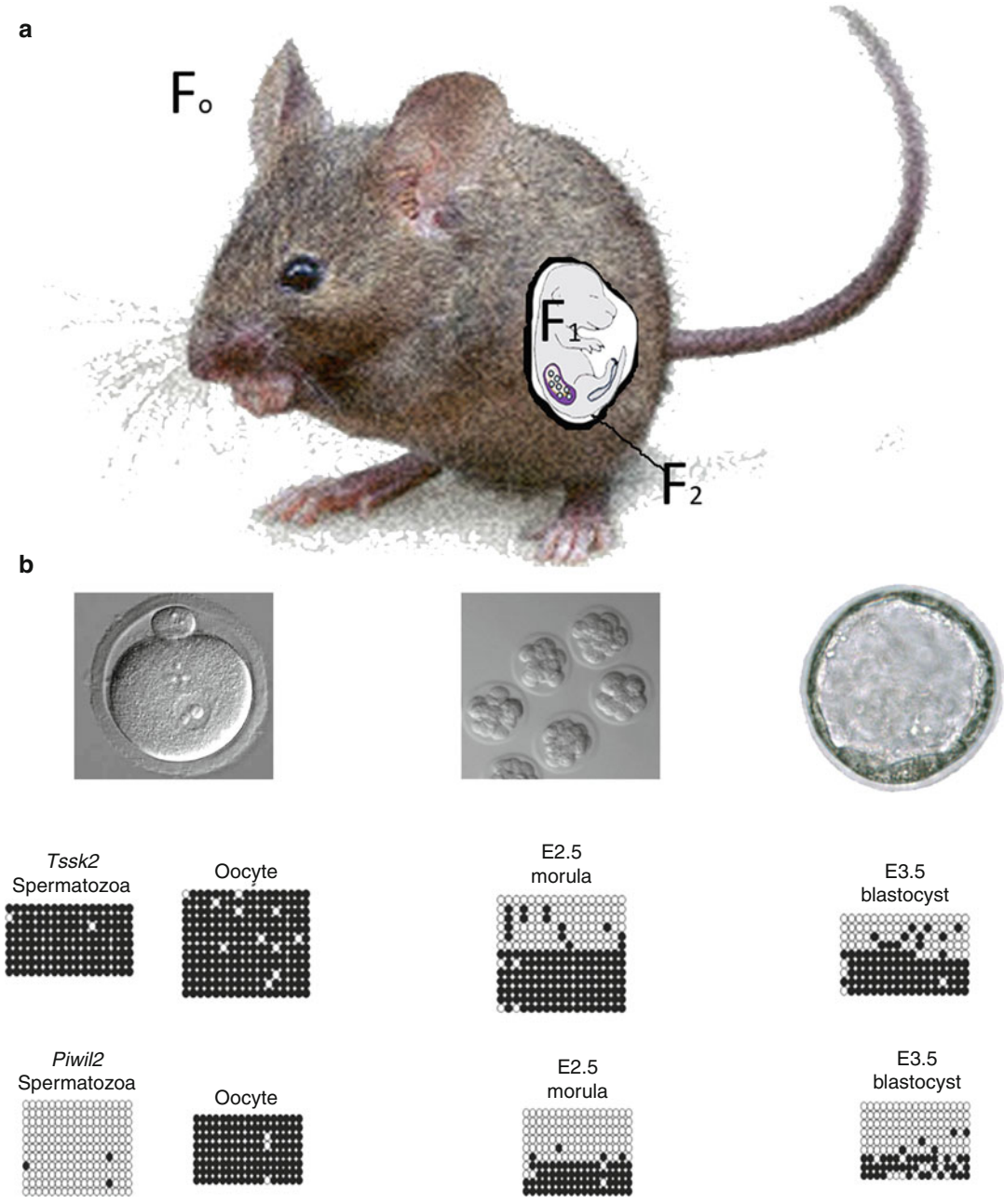
DNA methylation has been demonstrated to play an important role in this process. Among the most clearly studied genomic regions where this spurious inheritance can be demonstrated is the agouti locus in the mouse. In studying inbred genetic strains of mice, it became apparent that agouti locus controlled coat color variation was observable and was differentially heritable. This metastable behavior of the agouti-viable yellow locus is conferred by an intracisternal A particle (IAP) retrotransposon LTR linked to the gene which is able to affect agouti gene expression depending on the DNA methylation state of the cryptic promoter. Importantly, this effect is transmitted along the maternal line such that the phenotype of the mother affects the phenotype of the offspring. Moreover, this effect can be further influenced by the genetic background of the mouse as well as nutritional aspects of dietary supplements that comprise part of the biochemical pathway associated to methylation [81, 82]. Collectively these genomic targets act as biosensors for the sum of these effectors and produce what has been called ‘intangible variation’.

These ideas have sparked a renewed interest in Lamarckian inheritance [83]. This controversial form of inheritance suggested that adaptation over a lifetime to environment and as a consequence of behavioral influences could be transmitted heritably throughout generations. What remains unknown is the mechanism by which the effect registered by the somatic tissue is transduced to the germline where the heritable instruction would be laid down. Separating these issues can be difficult, and examples of how it might occur can be appreciated by considering a preg-

Fig. 11.2 Epigenetic changes may be acquired by environmental exposure influencing multiple generations. (**a**) Lamarckian inheritance has long been controversial in the wake of the Mendelian inheritance and a modern view of genetic and epigenetic mechanisms. However, the potential for the environment to influence multiple generations via inheritance through the female may be potentially more complicated. The example below is a pregnant female. Ingestion or inhalation of a genotoxic substance may simultaneously affect the pregnant mother (F0) her developing foetuses (F1) and the germline of these foetuses (F2). Substances that might be mutagenic to the mother may in turn influence epigenetic mechanisms essential to the erasure of the germline of the fetus that will only have impact on the F2 generation. Nutrition regulation, rarely seen as detrimental, may well have the potential to influence transgenerational epigenetic instructions by perturbing the genome-wide erasure of the maternal germline during its development. The combination of both gametic effects and post-zygotic effects may still have transgenerational impact but with varying penetrance and phenotypic severity. These possibilities suggest that a wider and more comprehensive consideration of family histories including socioeconomic consideration may prove useful in the treatment of human infertility. (**b**) Following fertilisation, both active and passive demethylation processes serve to modulate levels of DNA methylation for subsequent development. DNA methylation inherited by oocytes is largely lost by the blastocyst stage; however, >15 % of methylated sites are retained at the blastocyst. Two examples from Borgel et al., where DNA methylation is inherited from the oocyte, are retained at the blastocyst stage. The intermediate morulae stage is included to infer that this loss of DNA methylation occurs by a passive mechanism. Thus, a significant contribution of DNA methylation is inherited across generations highlighting the potential for the inheritance of transgenerational epimutations (Portions of this figure are reprinted by permission from Macmillan Publishers Ltd: Nature Genetics [84], 2010)

nant mouse at midgestation. The pregnant female, her offspring, and their germline, especially the female progeny, could all be influenced by an external environmental event where exposure or ingestion of food or a toxic substance may have enduring impact over generations (Fig. 11.2a).

Genome-wide studies are beginning to challenge the view that gametic epigenetic inheritance may be a relatively rare event in mammals. Recent evidence points to a much deeper and wider selection of both germline and somatic genes that resist the reprogramming events that were believed to reset the methylomes of oocytes and sperm and thus become inherited [50, 84]. These genes are distinctive from imprinted genes and may be methylated in one or both parental alleles but fail



to be maintained throughout development (Fig. 11.2b). Notwithstanding that these genes are subsequently demethylated, these results would indicate that a substantial portion of the epigenome may fail to be cleared during a significant developmental window ordinarily typified by dynamic DNA methylation reprogramming. The implications are profound. Should these genes retain their parental methylation, this opens the way for many additional genomic targets to be subject to variable phenotypic possibilities.

Epigenetic Regulation and Infertility in Humans: Causes and Cures

While it has been more than 30 years since the first baby was born by assisted reproductive technologies (ART) in the treatment of infertility, very little information is available to systematically assess the potential risks that may arise from conception ex vivo. Indeed, this original pioneering technology may now claim that a future generation of children has since been born from this original cohort of ART births. At present, it is too early to know whether these children will also have requirements for the same procedures that facilitated their birth.

In the developed world, greater than 1 in 60 live births is achieved as a consequence of one of the many procedures available for the treatment of human infertility. As such, it is imperative that we recognize and continue to identify genomic targets, as well as the underlying mechanisms that are able to alter the epigenome if subjected to extraordinary conditions, and therefore may result in perturbed phenotypes leading to disease pathologies.

The prospect of significant issues pertaining to epigenetic regulation as a function of ART may well have been overlooked early on owing to limited genomic information. Indeed, the frequency of multiple births and their consequent reduced birth weight contributed to the ideas we now know as developmental origins of adult health and disease. However, the defining conjunction of the misregulation of epigenetic marks and ART came to the attention of biologists and biomedical scientists in 2003 with a flurry of reports suggesting that children born following ART were found to have an increase frequency of Beckwith-Wiedemann syndrome (BWS). BWS is a fetal-overgrowth syndrome associated to the loss of imprinting of H19 and the KvDMR both allelic targets with inappropriate DNA methylation on the maternal allele. These reports originating from the UK and the USA were regarded as cause for concern about the procedures of in vitro fertilization (IVF) and intracytoplasmic sperm injection (ICSI). In the years immediately following these initial results, a steady stream of results continued to seemingly support the assertion that children born after ART procedures were at significantly increased risk of the likelihood of having an imprinting disorder [85–88]. However, these studies were universally retrospective in nature and reported only small increases in risk for disease.

More recent studies have addressed these limitations, and prospective analyses have been conducted together with comprehensive retrospective meta-analysis.

These studies suggest that rather than ART being a causal factor in these increased incidences of imprinting disorders, in fact the underlying infertility may be to blame for the aberrant epigenotype [89]. To date, reports have identified nine imprinted syndromes associated with ART births, but only a minority are statistically linked to these procedures [90]. Among those linked to ART are loci where maternal alleles are most severely affected [90]. Interestingly, 90 % of patients with BWS born following ART have associated imprinting disorders [91].

Recent studies in animal models have identified that superovulation may have a dosage-dependent effect on imprinted loci affecting both maternal and paternal alleles [92]. This type of outcome is consistent with reports that imprinting errors associated to both hypermethylation (e.g., BWS) and hypomethylation (Silver- Russell syndrome) have been reported from ART births resulting in fetal growth-associated syndromes. These effects cannot be regarded as restricted to the germline alone but may have downstream impact during the later post-fertilization period of reprogramming

during preimplantation thought to be critical for lineage determination. Growth restriction such as that observed in Silver-Russell syndrome may arise as a consequence of placental epimutations [93]. Hypomethylation of the paternal H19 gene has been reported to arise owing to the instability of imprinting maintenance during early development [94]. This instability may result from a mutated version of the de novo methylase Dnmt3b that has been reported to arise specifically under conditions of in vitro culture in the mouse [95]. These reports can now be better understood in light of mouse studies that reveal that even DNA methylation imprints, thought to be robustly heritable, are dynamic and undergo constant renewal during the preimplantation period of development [66]. Indeed, studies focusing on the effect of ovarian hyperstimulation in the mouse model concluded that establishment of imprinted methylation proceeded correctly but that the machinery required for the regulation of imprinting maintenance was found to have been altered. These results highlight that studies including the embryo, and not just the ovulated oocyte, are vitally important to properly study potential effects of procedures routine in the treatment of infertility [96].

It is likely that while attention has been focussed on changes in the epigenome associated predominantly to altered imprinting, other non-imprinted targets also sensitive to exposure from environmental modulations may also have been affected. These effects, while subtle in their initial impact, may lead to more serious health-related outcomes that only become apparent in later life [97]. That multiple imprinted loci may be misregulated routinely would be consistent with the metabolic alterations now thought to be a consequence of environmental exposure in vitro as part of the treatment of infertility [98].

In light of significant new results demonstrating the wide array of methylation targets that acquire their modification immediately prior to oocyte maturation, the assessment of many more subtle epigenetic changes affecting both short-term and long-term effects is warranted in the clinic; several lines of evidence point in this direction. In vitro maturation of immature oocytes (IVM) is employed clinically in the treatment of infertility where ovarian hyperstimulation syndrome is suspected [91, 99]. If in humans during the latter stages of oogenesis >1,000 targets must still acquire DNA methylation as is the case in the mouse [50, 84], this may pose additional complications in ensuring that the epigenome is maintained when using IVM. Indeed, studies in human and bovine where IVM was investigated reported increased incidences of imprinting errors associated to maternal methylation [100–102].

Beyond the procedures of direct consequence to the oocyte in the course of ART is an interesting report that identifies critical nutritional aspects for the mother during the late maturation period of the oocyte prior to retrieval. Studies have suggested that as little as an 8 % reduction in maternal protein consumption 3 days prior to ovulation may affect the final stages of meiotic maturation [103, 104].

This fits neatly with data from Smallwood et al., indicating a large number of genes are methylated between the GV and MII stages in the mouse [50]. Interruption of this process as a consequence of changes in maternal nutrition immediately prior to the harvest of oocytes in addition to the usual changes encountered with in vivo handling may collectively introduce deleterious alterations in the oocyte methylome that lead to the epimutations often identified at imprinted loci.

Collectively there is growing evidence that in humans, children born following ART procedures are phenotypically and biochemically different from those conceived naturally [98, 105]. Whether these changes involve deleterious effects remains to be seen. The extent to which these changes may be inherited over generations is also a key issue. Clearly much of the underlying causes arise from subtle and not so subtle changes in the epigenome, the instructional interpreter of the underlying genetic blueprint. Suggestions that DNA methylation changes in late-stage oocyte maturation involve a vast number of targets lend credence to the likelihood of these changes having a lifelong impact on reproductive ability and healthy aging. Still, much of what we know derives from studies of animal models. Recent genome-wide studies have revealed some cause

for concern with important implications for growth and development beyond the well-studied targets of genomic imprinting. What remains is for concerted and integrated studies that allow us to learn more about the specific differences as they apply to human reproductive biology in the context of the human genome as a prelude to specific direct improvements in all aspects of the treatment of human infertility. Internationally coordinated initiatives such as IHEC, the International Human Epigenome Consortium, will play an essential role in the path to such understanding in this area.

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Part IV

Oocyte Meiosis and Cytoplasmic Dynamics

Signaling for Meiotic Resumption in Granulosa Cells, Cumulus Cells, and Oocyte

12

Lisa M. Mehlmann

Abstract

Mammalian oocytes are arrested in prophase of meiosis I until a surge of luteinizing hormone from the pituitary signals them to resume meiosis and progress to metaphase II. Prophase arrest prior to the LH surge, and meiotic resumption following the LH surge, are regulated by an interplay between the oocyte and the surrounding follicle cells. This chapter reviews what is currently known about the maintenance of meiotic arrest and the mechanisms that bring about meiotic resumption in the rodent oocyte.

Keywords

Follicle • Oocyte • cAMP • cGMP • Luteinizing hormone • Oocyte maturation

Introduction

The preovulatory mammalian follicle is comprised of a meiotically competent oocyte, arrested in prophase I, surrounded by two different types of granulosa cells. The cells immediately surrounding the oocyte are cumulus cells, and the cells that line the outer wall of the follicle are mural granulosa cells (Fig. 12.1). The two types of granulosa cells are separated from each other by a large, fluid-filled antral cavity. The mural granulosa cells communicate with each other and with the cumulus cells through gap junctions composed of connexin 43 (Cx43), while the cumulus cells maintain contact with the oocyte via gap junctions composed of connexin 37 (Cx37). Gap junctional communication is essential for maintaining meiotic arrest.

The pituitary gonadotropin, follicle-stimulating hormone (FSH), causes the growth of follicles once they reach the preantral stage. The oocyte grows as well, reaching a diameter of ~75 μm in the mouse, but it remains arrested in prophase I until luteinizing hormone (LH) from the pituitary signals the oocyte to resume meiosis. Toward the end of follicular growth (~500 μm diameter in the mouse), FSH stimulates the expression of the LH receptor on the mural granulosa cells. The LH receptor is not expressed on the cumulus cells or on the oocyte [1, 2]. Therefore, the actions of LH to stimulate meiotic resumption are not a direct effect on the oocyte.

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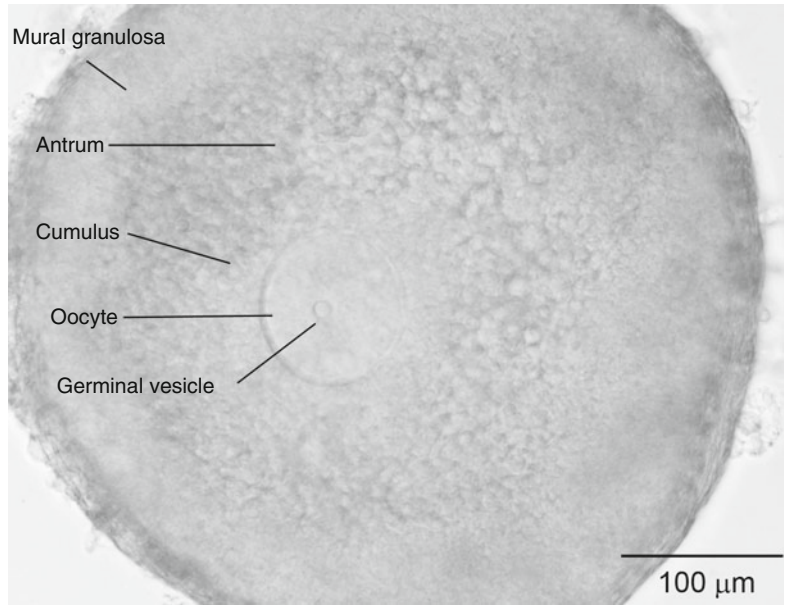

Fig. 12.1 Preovulatory follicle from a mouse ovary

In the oocyte, meiotic resumption is characterized by the disappearance of the large nucleus (GV), termed "germinal vesicle breakdown" (GVBD). The oocyte then completes the first meiotic division and continues to metaphase II, where it undergoes a second arrest that lasts until fertilization.

The mechanisms that regulate meiotic arrest and resumption have been examined for decades. Recently, convincing evidence has been obtained to explain how the oocyte and follicle cells communicate to regulate follicular growth, meiotic arrest, and meiotic resumption. Most of these studies have been performed in rodents; therefore, the discussion here will be limited mainly to meiotic arrest and resumption in rodent oocytes.

Prophase I Arrest: Regulation by the Oocyte

It has long been known that levels of cyclic adenosine monophosphate (cAMP) within the oocyte regulate both meiotic arrest and resumption. Release of the oocyte from the follicle causes the oocyte to mature spontaneously [3, 4] and is associated with a decrease in oocyte cAMP [5, 6]. This spontaneous maturation can be inhibited by incubating oocytes in the presence of phosphodiesterase inhibitors or analogs of cAMP [7, 8]. During meiotic arrest, cAMP levels within the oocyte are relatively high, whereas they fall in response to LH, prior to meiotic resumption [5, 9].

Cyclic AMP levels regulate meiotic arrest and resumption by affecting the balance between an inactive and an active protein complex made up of cyclin B and the kinase CDK1 [10, 11]. When cAMP is high in the oocyte, protein kinase A (PKA) is activated. Meiotic resumption proceeds in isolated oocytes in the presence of phosphodiesterase inhibitors when oocytes are injected with PKA inhibitors, and meiotic arrest is maintained by injecting the catalytic subunit of PKA in isolated oocytes in the absence of phosphodiesterase inhibitors [12]. The basal level of cAMP in the oocyte, ~650 nM [9], is high enough to stimulate both PKAI and PKAII [13, 14], both of which are expressed in the oocyte [15–17]. Protein kinase A phosphorylates and inactivates the phosphatase, CDC25B, and phosphorylates and activates the kinases WEE1B and MYT1 [11]. This causes a net phosphorylation on tyrosine and threonine residues in CDK1, resulting in its inactivation. Low cAMP levels that occur following the LH surge or in an oocyte released from the follicle cause a decrease in PKA activity and subsequently the dephosphorylation and activation of

CDC25B, such that there is a dephosphorylation of tyrosine and threonine and activation of the CDK1/cyclin B complex. This protein complex, termed MPF (for meiosis promoting factor, mitosis promoting factor, or metaphase promoting factor), is responsible for GVBD as well as other early events of meiotic resumption [11].

It was once thought that cAMP was produced in the cumulus cells and diffused into the oocyte through gap junctions to provide the oocyte with cAMP to maintain meiotic arrest [18]. Evidence to support this hypothesis came from studies in which gap junctional communication was disrupted. The gap junction inhibitor carbenoxolone effectively inhibits gap junctional communication and stimulates meiotic resumption [19, 20]. In addition, experiments in which gap junctional communication between the cumulus cells and the mural granulosa cells was disrupted within an intact follicle demonstrated that patent gap junctions must be maintained between the cumulus and mural granulosa cells to maintain meiotic arrest [21]. However, if follicle cells produce enough cAMP to "feed" the oocyte, it is unclear how they would do so, as PKA activity in the follicle is low prior to stimulation by LH [22]. The permeability of gap junctions comprised of Cx37 and Cx43 to cAMP is also unknown; it is thus possible that cAMP cannot pass through gap junctions.

Recently, the ability to microinject an oocyte while it is still in an intact follicle has provided strong evidence that cAMP is generated within the oocyte itself. The first evidence of this came when oocytes within isolated antral mouse follicles were microinjected with an antibody that inactivates the $G\alpha_s$ G-protein. This antibody stimulated meiotic resumption within follicle-enclosed oocytes as well as in isolated oocytes maintained in meiotic arrest with the phosphodiesterase inhibitor hypoxanthine [23]. Additionally, a dominant negative form of G_s stimulated meiotic resumption in follicle-enclosed oocytes [24]. An essential role for oocyte-derived adenylate cyclase in maintaining meiotic arrest is supported by the observation that oocytes from adenylate cyclase type 3 knockout mice undergo spontaneous meiotic resumption in vivo [25]. Interestingly, the ability of G_s inhibition to stimulate meiotic resumption is not limited to mouse oocytes, and inactivation of G_s has also been shown to trigger meiotic resumption in *Xenopus*, zebrafish, and human oocytes [24, 26, 27].

G_s activity requires stimulation by a G-protein-coupled receptor. Therefore, the next step in determining how cAMP might be produced by the oocyte was to identify this receptor. A search of an EST database from fully grown, prophase I-arrested oocytes yielded a list of 15 candidate receptors, of which one in particular was of interest due to its known ability to stimulate G_s activity [28]. This receptor, called GPR3, had been found to produce high levels of cAMP when overexpressed in mammalian cells, in the absence of exogenous agonist [29, 30]. *Gpr3* mRNA is expressed in the mouse oocyte and is ~10–20 times more abundant in the oocyte than in the surrounding follicle cells [28, 31]. Importantly, oocytes from *Gpr3* knockout mice undergo spontaneous meiotic resumption within intact preovulatory follicles in the absence of an LH surge [28, 32, 33]. Exogenous expression of GPR3 in follicle-enclosed knockout oocytes restores the ability of these oocytes to maintain meiotic arrest, and reducing *Gpr3* RNA within wild-type follicle-enclosed oocytes using RNA interference causes spontaneous meiotic resumption [10, 28]. In the rat, GPR3 is absent and a related receptor, GPR12, appears to be responsible for maintaining meiotic arrest [31], whereas human oocytes express GPR3 and not GPR12 [27]. In oocytes overexpressing a fluorescently tagged GPR3, GPR3 is localized in the plasma membrane of the oocyte and within early endosomes, and signaling at the plasma membrane appears to be necessary to produce enough cAMP to maintain meiotic arrest [34].

Prophase I Arrest: Regulation by Granulosa and Cumulus Cells

If cAMP is generated within the oocyte, what is the role of the follicle cells in maintaining meiotic arrest? The follicle cells are clearly important, as removal of the oocyte from the follicle causes the oocyte to mature spontaneously in culture [3, 4]. As mentioned earlier, patent gap junctions between

the mural granulosa and the cumulus cells are essential for maintaining meiotic arrest. These observations led to the hypothesis that follicle cells provide the oocyte with a meiosis inhibitory substance that diffuses through gap junctions to somehow inhibit meiotic resumption. This inhibitory substance is unlikely to be cAMP because oocyte-derived cAMP is sufficient to maintain meiotic arrest in oocytes from *Gpr3*$^{-/-}$ mice [28], and depleting GPR3 specifically within the follicle-enclosed oocyte prevents the oocyte from maintaining arrest [10, 33]. LH stimulating the follicle could then reverse the effect of this meiosis inhibiting substance, either by inhibiting its production or by closing gap junctions through which the inhibitory substance diffuses into the oocyte.

Early experiments investigating meiotic arrest found that in addition to cAMP, levels of another cyclic nucleotide, cyclic guanosine monophosphate (cGMP), decrease in the oocyte following removal from the follicle and in response to LH treatment [6]. In addition, isolated mouse oocytes microinjected with cGMP undergo spontaneous meiotic resumption at a slower rate than uninjected oocytes [6]. Because cGMP inhibits cAMP phosphodiesterase activity in oocyte lysates [35] and inhibitors of an enzyme required for the production of cGMP (inosine monophosphate dehydrogenase) cause GVBD in follicle-enclosed oocytes [36], cGMP thus became an attractive candidate for the meiosis inhibitory substance.

More recently, using FRET-based sensors to measure cGMP within follicle-enclosed oocytes, it has been shown that oocyte cGMP levels are ~900 nM prior to LH but fall to ~40 nM within an hour after LH treatment, before GVBD [9]. This basal level of cGMP is sufficient to inhibit the activity of phosphodiesterase 3A (PDE3A), the main phosphodiesterase involved in meiotic arrest [37, 38], whereas the drop to 40 nM should lead to a fivefold increase in PDE3A activity [9, 37, 39]. Additional evidence that cGMP could be the meiosis inhibitory substance includes the finding that injection of the catalytic domain of the cGMP-specific phosphodiesterase 9A causes GVBD in follicle-enclosed oocytes, but only if PDE3A is active [9]. Both LH and the catalytic domain of PDE9A cause a decrease in oocyte cAMP, and these oocytes undergo meiotic resumption.

Evidence that cGMP from the follicle cells diffuses to the oocyte through gap junctions was provided by the finding that experimental closure of gap junctions in intact follicles decreases cGMP in the oocyte [9] and stimulates meiotic resumption [9, 19, 40]. Moreover, recent measurements of follicular cGMP have demonstrated that prior to LH, cGMP levels are relatively high (2–3 μM), whereas they fall rapidly after the LH surge (to ~100 nM at 1 h) [9, 39, 41].

How cGMP is generated in the somatic follicular cells is beginning to be understood. Recent work has shown that a ligand–receptor interaction exists between the follicle cells that generate cGMP. Mural granulosa cells express mRNA encoding C-type natriuretic peptide (CNP, or NPPC); its cognate receptor, NPR2, is expressed predominantly in the cumulus cells, with lower expression in the mural granulosa cells [42, 43]. NPR2 is a membrane-bound guanylate cyclase [44], and CNP elevates cGMP levels in isolated cumulus–oocyte complexes [42, 43, 45]. Levels of *Nccp* mRNA and protein change throughout the rat estrus cycle, being highest at proestrus, when large preovulatory follicles are present, prior to the LH surge [46]. mRNA and protein are also higher in ovaries from eCG-primed mice, which contain many preovulatory follicles, compared to unstimulated ovaries [45, 47]. CNP inhibits spontaneous GVBD of oocytes within cumulus–oocyte complexes [42, 43, 47]. Importantly, oocytes from *Nppc* or *Npr2* mutant mice undergo spontaneous meiotic resumption in vivo [42], demonstrating the essential role of both ligand and receptor in the maintenance of meiotic arrest.

The interaction of the oocyte and somatic cells is critical for the proper expression of NPR2. For example, *Npr2* mRNA levels are significantly lower in cumulus cells from which oocytes are microsurgically removed [42]. *Npr2* mRNA levels are restored when such cumulus cells are co-cultured with fully grown isolated oocytes. In addition, paracrine factors are involved in the maintenance of *Npr2* expression. For example, *Npr2* mRNA levels are high in freshly isolated

cumulus–oocyte complexes obtained from hormonally stimulated mice but fall over time in culture; *Npr2* levels can be sustained in cumulus–oocyte complexes in the presence of estrogen or testosterone, which is produced during the period of follicular growth, when meiotic arrest must be maintained [43].

In summary, meiotic arrest depends on high levels of cAMP within the oocyte. Mammalian oocytes produce the cAMP through the constitutive activity of the G_s-linked receptor GPR3 (or GPR12). For their part, the follicle cells produce cGMP, which diffuses into the oocyte through gap junctions to inhibit PDE3A and thus prevent the hydrolysis of cAMP.

Meiotic Resumption: Pathways by Which LH Stimulates Oocyte Maturation

Luteinizing hormone (LH) from the pituitary is the stimulus for meiotic resumption. In response to LH, the oocyte undergoes the dissolution of the germinal vesicle ("germinal vesicle breakdown," or "GVBD") and progresses to metaphase II, where it undergoes a second arrest until fertilization. The first effect of LH on the follicle is to bind to and activate the LH receptor. LH receptors are located only on the mural granulosa cells, not on the cumulus cells or oocyte [1, 2]. It is well characterized that the LH receptor is coupled to G_s [48, 49]. LH binding to its receptor stimulates an increase in adenylate cyclase activity through G_s activation, leading to the production of cAMP. Thus, paradoxically, an increase in cAMP production in the follicle cells leads to a decrease in cAMP in the oocyte; the decrease in cAMP in the oocyte is due to an increase in cAMP phosphodiesterase activity [9, 39, 50]. Meiotic resumption can be stimulated by transiently elevating follicular cAMP levels using forskolin, which activates adenylate cyclase [31, 51]. The LH receptor also couples to $G_{q/11}$, which activates phospholipase C, resulting in Ca^{2+} release [48, 49, 52]. This is based on the observation that *Xenopus* oocytes or cultured mammalian cells overexpressing the LH receptor can mobilize Ca^{2+} when stimulated with LH. In addition, LH stimulates the formation of inositol 1,4,5-trisphosphate (IP_3) and Ca^{2+} release in dispersed granulosa cells from porcine [53] or rat [54] ovarian follicles in vitro. Meiotic resumption can be stimulated by increasing Ca^{2+} in the follicle [55, 56], and there is evidence that Ca^{2+} signaling is important for FSH-induced meiotic resumption in cumulus–oocyte complexes [57–59]. However, whether LH acting on intact follicles causes Ca^{2+} release and triggers meiotic resumption under physiological conditions has yet to be investigated.

LH Stimulates a Decrease in Gap Junction Permeability as well as a Fall in Follicular cGMP

LH stimulation of the mural granulosa cells could reverse the inhibitory effect on the oocyte from the follicle cells, could produce a signal that overrides the inhibitory substance, or could have effects on specific pathways in the oocyte that lead to cAMP production. In support of a positive stimulus that promotes meiotic resumption, isolated cumulus cells incubated in the presence of phosphodiesterase inhibitors or cAMP analogs undergo GVBD when treated with FSH or EGF [60]. In the oocyte, there is evidence that LH signaling does not act at the level of cAMP production in the oocyte to stimulate meiotic resumption. This is based on the observation that GPR3-dependent G_s activity does not change in response to LH [61]. In addition, LH-induced meiotic resumption in the oocyte is independent of a G_i G-protein, the activation of which would cause a decrease in cAMP, nor does it act through a Ca^{2+}-dependent pathway in the oocyte [62] that might inhibit adenylate cyclase activity. However, there is evidence that the AKT/PKB pathway could be involved in phosphorylating and activating PDE3A in the oocyte [63, 64]; how this pathway might be stimulated in response to LH is unknown. More recent studies have focused on the role that LH plays to reverse the inhibitory effect the follicle

cells surrounding the oocyte exert to promote meiotic arrest.

As discussed above, there is strong evidence to support the hypothesis that cGMP from the follicle cells diffuses through gap junctions to inhibit meiotic resumption until the LH surge. The effects of LH on the follicle are twofold. First, LH stimulation leads to a decrease in gap junction permeability between the follicular somatic cells [19, 20], which then leads to a decrease in oocyte cGMP [9]. The decrease in oocyte cGMP following gap junction closure permits the activation of PDE3A in the oocyte, which hydrolyzes cAMP, and meiotic resumption is able to proceed. Second, LH stimulation causes a decrease in total [cGMP] in the follicle, which also lowers oocyte cAMP and permits meiotic resumption to occur.

It has been known for years that disrupting gap junctions using nonspecific gap junction inhibitors stimulates meiotic resumption [19, 20, 40]. Connexin 43 (Cx43) is the main connexin comprising the gap junctions between follicular cells [65], and its permeability is regulated by phosphorylation [66, 67]. Treating intact rat or mouse follicles with LH stimulates Cx43 phosphorylation on several serines [20, 68–70], with maximal phosphorylation occurring at 30–60 min following LH treatment, a time that precedes GVBD [20]. Phosphorylation of Cx43 leads to a decrease in gap junction permeability between the follicle cells [20, 70], but not between the oocyte and surrounding cumulus cells, which are composed of Cx37 [20, 71]. Breakdown of communication between follicle cells contributes to GVBD.

LH stimulation also causes a decrease in the concentration of follicular cGMP [9, 39]. The basal concentration of cGMP in preovulatory follicles prior to the LH surge is ~2–3 μM, whereas this level falls to ~100 nM within 1 h following LH treatment [9, 41]. The decrease in cGMP content in the follicle could result from a decrease in guanylate cyclase activity or from an increase in cGMP PDE phosphodiesterase activity. As already discussed, CNP is the ligand expressed in the mural granulosa cells that stimulates cGMP production via its interaction with the transmembrane guanylate cyclase, NPR2, which is located on the cumulus and mural granulosa cells [42, 43]. Ovarian CNP protein levels fall in response to LH stimulation [47], with a time course that precedes GVBD [45]. Mechanisms by which CNP levels fall are currently unknown; one possibility is a decrease in *Nppc* expression [47]. In addition to a fall in CNP levels, NPR2 activity also decreases in response to LH [45].

Role of the MAP Kinase Pathway in Stimulating Meiotic Resumption

One of the early events associated with LH stimulation of the follicle is the activation of mitogen-associated protein kinase (MAPK, or ERK1/2). MAPK activity has been observed in isolated, intact rodent follicles within 15 min following stimulation of the LH receptor [69, 70, 72]. The MAPK kinase (MEK) inhibitor, U0126, prevents LH-induced meiotic resumption in intact follicles [73], although the dose required for half maximal to complete inhibition is in the range where the inhibitor has nonspecific effects on other protein kinases [74]. At this dose, U0126 also inhibits LH-stimulated expression of *Has2* and *Ptgs2*, two proteins required for cumulus cell expansion, in cumulus cells [73]. Oocytes from granulosa cell-specific *Erk1/2*$^{-/-}$ mice fail to resume meiosis when the mice are injected with hCG to stimulate the LH receptor, and these mice do not ovulate [75], supporting an important role for MAPK in more than one LH-stimulated pathway.

At the level of the oocyte, MAPK is also activated during maturation, but not until after GVBD [76], and inhibiting its action with U0126 does not inhibit spontaneous oocyte maturation [77]. MAPK activity in oocytes is ultimately stimulated by the oocyte-specific proto-oncogene product c-MOS. Oocytes from *mos*$^{-/-}$ mice are competent to mature, and in these oocytes, MAPK is not activated during maturation, yet they undergo GVBD in vitro [58, 76, 78]. Thus, oocyte-derived MAPK is not essential for the early stages of meiotic resumption.

MAPK Is Stimulated by the Activity of EGF-Like Ligands Through Activation of the EGF Receptor

The transmission of the LH signal requires the activity of the EGF receptor (EGFR) on follicle cells. EGF receptors are expressed on both the mural granulosa and cumulus cells [79, 80]. It has long been known that EGF stimulates meiotic resumption in both intact follicles [70, 81] and cumulus-enclosed oocytes cultured in the presence of hypoxanthine, which would normally hold them in meiotic arrest [60]. However, the relationship between LH and EGF in meiotic resumption was not fully appreciated until relatively recently.

There is now strong evidence that rather than EGF, LH stimulates the expression of EGF-like ligands in both the mural granulosa [72, 82, 83] and the cumulus cells [84]. Two of these EGF-like ligands, amphiregulin (AREG) and epiregulin (EREG), have been implicated in transmitting the LH signal [72, 82, 83, 85]. AREG and EREG are small (~5 kDa) peptides that are synthesized as precursor proteins that are cleaved by the transmembrane matrix metalloprotease ADAM17/TACE and shed in their mature forms [86]. *Areg* and *Ereg* mRNAs are rapidly induced by LH in rodent follicles in vivo [82, 83] and in vitro [72], prior to GVBD. *Areg* mRNA has also been shown to be induced by FSH and forskolin within 30 min in cultured cumulus–oocyte complexes [84]. AREG and EREG precursor proteins have been identified in mural granulosa and cumulus cells following stimulation with LH or FSH, respectively [82, 84]. Incubating follicles with AREG or EREG stimulates meiotic resumption with a time course faster than or similar to that of LH [41, 82], and incubating isolated cumulus-enclosed oocytes with AREG stimulates GVBD when cultured in the presence of hypoxanthine or dbcAMP, which would normally inhibit meiotic resumption [85].

EGFR phosphorylation occurs rapidly in vivo [82] and in vitro [72, 83] following LH receptor stimulation, and the specific EGFR kinase inhibitor AG1478 inhibits the effects of AREG or EREG on meiotic resumption [82]. AG1478 likewise inhibits LH-induced meiotic resumption [41, 83]. At the level of the cumulus cells, AG1478 inhibits FSH- as well as AREG-induced meiotic resumption [85]. Disrupting the EGF signaling network either by utilizing double mutant $Areg^{-/-}$ mice in combination with mice homozygous for a hypomorphic EGFR allele ($Areg^{-/-}$ $Egrf^{wa2/wa2}$) [87] or by deleting the EGFR specifically in the granulosa cells [79] impairs both meiotic resumption and ovulation.

The oocyte plays a pivotal role in EGFR signaling, as secreted factors from the oocyte regulate follicular EGFR expression. For example, cumulus cells from cumulus–oocyte complexes in which the oocyte was microsurgically removed have lower levels of *Egfr* mRNA and protein than cumulus cells within intact complexes [80]. Co-culture of cumulus cells with wild-type oocytes restores the expression of *Egfr* mRNA, whereas oocytes deficient in two oocyte-specific factors, growth differentiation factor 9 (GDF9) and bone morphogenetic protein 15 (BMP15), fail to restore the expression of the *Egfr*. The addition of GDF9 and BMP15 to oocyte-removed cumulus cells promotes *Egfr* mRNA expression [80]. GDF9 and BMP15 act through a SMAD2/3 signaling pathway in cumulus cells and, interestingly, the EGFR–MAPK pathway in cumulus cells enables GDF9–SMAD3 signaling [88]. Thus, cross talk between the oocyte and cumulus cells is key for successful EGFR signaling in response to EGF-like ligands.

LH Acts Through Redundant Pathways to Stimulate Meiotic Resumption

The activation of the EGFR and subsequent MAPK activation are important for both the decrease in follicular gap junction permeability and the decrease in cGMP concentration in the follicle. With respect to gap junction permeability, stimulating the EGFR with EREG and AREG closes gap junctions, albeit only partially [41]. EGFR activity is clearly necessary for gap junction closure, as the EGFR inhibitor AG1478 prevents gap junction closure in response to LH [41]. MAPK activation is also important for gap junction closure. MAPK activation causes Cx43 phosphorylation on several

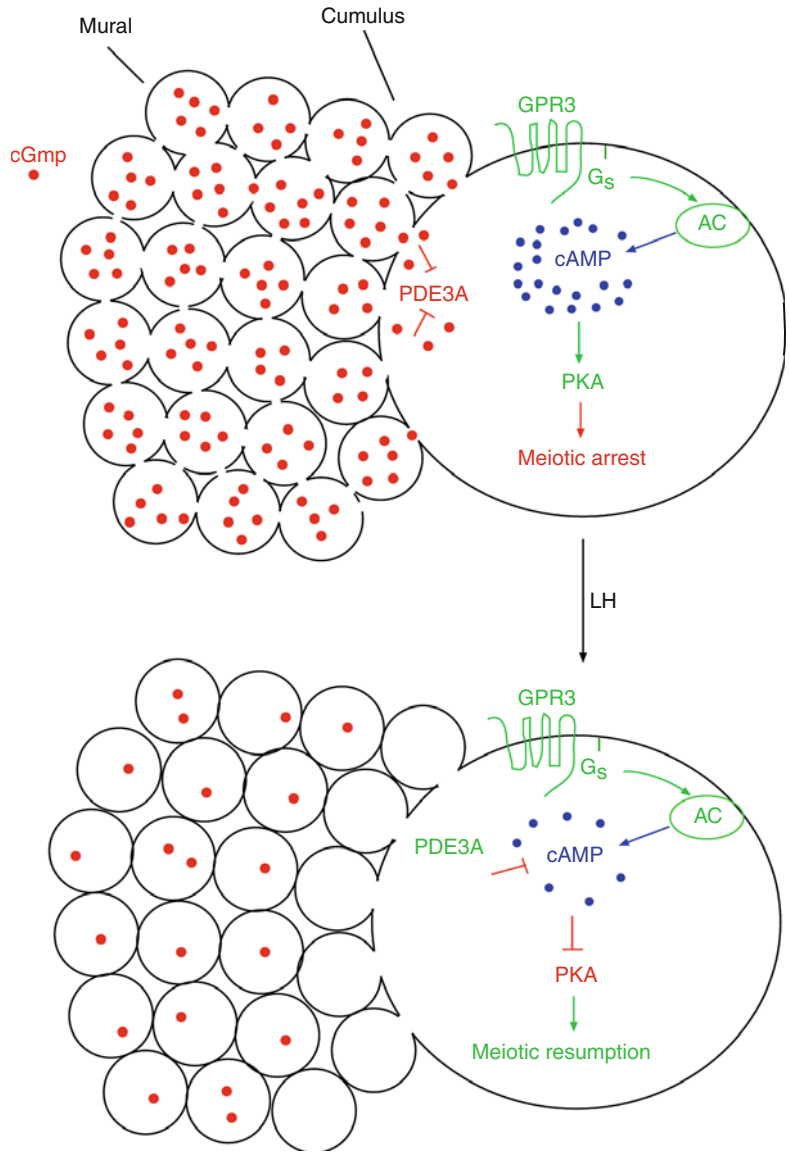

Fig. 12.2 Model for the maintenance of meiotic arrest and resumption. Before LH, levels of cGMP in the somatic cell compartment are high, and cGMP diffuses freely into the oocyte, where it inhibits PDE3A and maintains high levels of cAMP. Following LH stimulation, gap junctions between the follicle cells close and cGMP levels in the follicle fall, both of which lead to a decrease in cGMP in the oocyte; this allows the activation of PDE3A and the hydrolysis of cAMP

serines [20, 70], and inhibiting MAPK using the inhibitor UO126 abolishes Cx43 phosphorylation and prevents gap junction closure in response to LH stimulation [20]. Interestingly, preventing the closure of gap junctions with a dose of U0126 that is highly specific is ineffective in inhibiting LH-induced meiotic resumption [20, 73], illustrating that LH acts through separate, redundant pathways to initiate meiotic resumption.

EGFR activity is also necessary for at least part of the fall in cGMP that occurs in response to follicular stimulation by LH. The LH-induced decrease in follicular cGMP is reduced by a concentration of AG1478 that is sufficient to completely inhibit EGFR kinase activity [41]. In addition, follicular cGMP levels fall in response to LH in granulosa cell-specific *Egfr*$^{-/-}$ mice [79]. However, in both of these cases, the effects are only partial, further demonstrating that LH acts through multiple mechanisms to lower cGMP.

Conclusion

It has long been known that the maintenance of meiotic arrest as well as meiotic resumption is the result of a highly coordinated system between the follicle cells and the oocyte.

Recent work has shed light on how these follicular compartments function together to maintain meiotic arrest until the surge in LH that stimulates meiotic resumption (Fig. 12.2). Prior to LH elevation, prophase arrest depends on high levels of intra-oocyte cAMP. This cAMP is generated by the constitutively active G-protein-coupled receptor in the oocyte membrane, GPR3 (or GPR12), and its breakdown is prevented by cGMP, which inhibits PDE3A in the oocyte. Cyclic GMP is generated in the somatic cells by the ligand CNP that interacts with the membrane-bound guanylate cyclase, NPR2, which is located on the mural granulosa and cumulus cells. Cyclic GMP diffuses into the oocyte through gap junctions. Following LH receptor stimulation of the follicle, cGMP in the oocyte falls as the result of gap junction closure as well as a decrease in total follicular cGMP content. As a result of decreased cGMP in the oocyte, PDE3A becomes activated and phosphodiesterase activity increases, thereby hydrolyzing cAMP and permitting meiotic resumption. Future studies will no doubt unravel the questions that remain, including the mechanisms by which CNP levels are regulated in the follicle, as well as all of the intermediate steps through which LH stimulates meiotic resumption.

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Start and Stop Signals of Oocyte Meiotic Maturation

13

Keith T. Jones, Simon I.R. Lane, and Janet E. Holt

Abstract

Oocytes are made in the fetal ovary and are only ever fertilized some considerable time later in the adult. During this time, they have to undergo two meiotic divisions (meiosis I and II), which must be executed faithfully and on time so as to produce a mature egg, with a haploid chromosome content, that is ovulated into the fallopian tube ready to be fertilized. The two meiotic divisions are controlled by both internal and external (hormonal) triggers, principally executed by changes in the activity of the kinase CDK1 in the oocyte. Here, we focus on how the oocyte controls CDK1 activity at three important time points: (1) the arrest at prophase I in the ovary and the hormone-driven release from this arrest, (2) the progression through meiosis I, and finally (3) the rearrest at metaphase II and subsequent completion of meiosis triggered by a sperm calcium signal.

Keywords

Anaphase-promoting complex • Bivalents • CDK1 cyclin B1 • Fertilization • Meiosis • Meiotic maturation • Metaphase • Oocytes • Spindle assembly checkpoint

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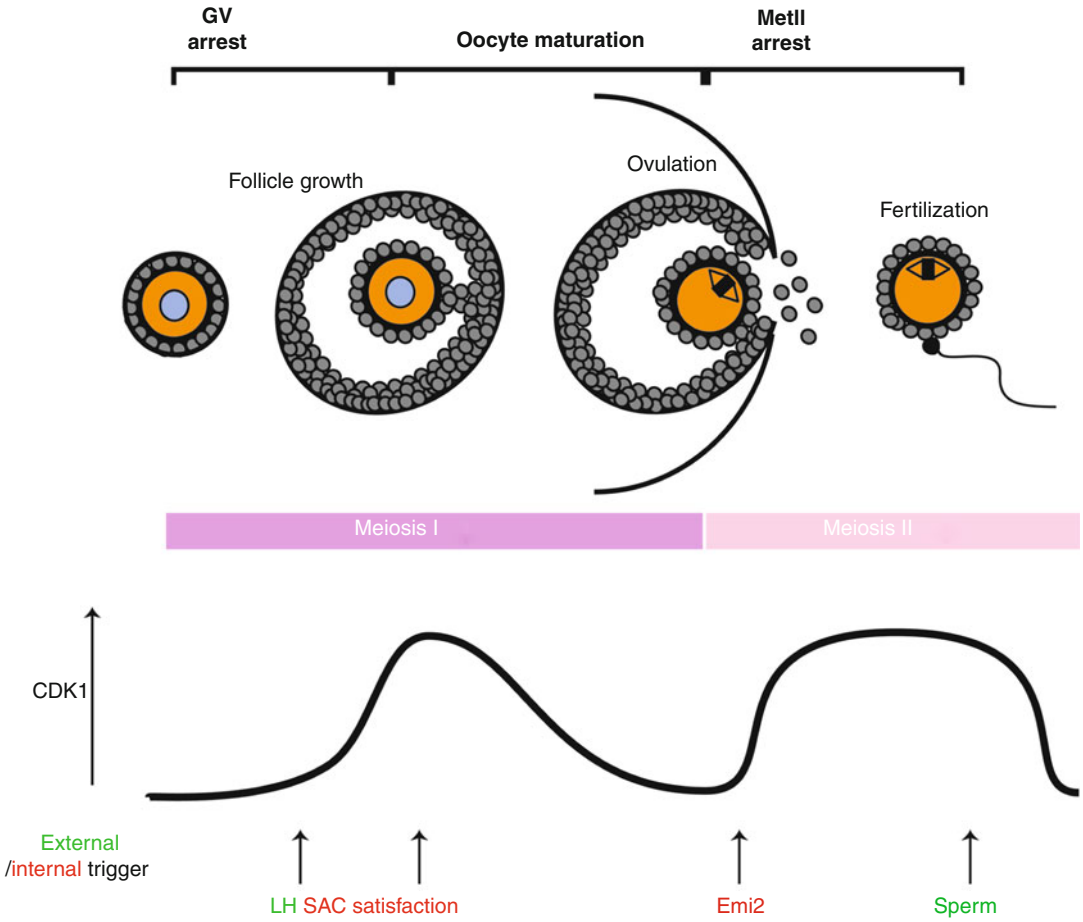

Fig. 13.1 The stops and starts of meiosis in mammalian oocytes. This chapter focuses on three distinct periods of meiosis: (1) GV arrest and hormonal release from this arrest; (2) oocyte maturation during meiosis I, which results in the separation of homologous chromosomes; and (3) rearrest at metaphase of meiosis II (metII arrest) and the sperm induced trigger for meiotic completion. All three periods are principally regulated by changes in the activity of the kinase CDK1, which is the focus of this chapter. The triggers driving these alterations in CDK1 activity are highlighted and detailed in the text

Introduction

The passage through meiosis in mammalian oocytes is not smooth but instead punctuated by a series of stops and starts. These remarkable events collectively highlight a very sophisticated life for this unique cell. The processes ensure that the mature oocyte (in later sections referred to as an egg) gets ovulated at the right time ready for fertilization and that this is ultimately done in a synchronized way that ensures the female remains endowed with oocytes for the entirety of her reproductive life. This has to be so given all oocytes ovulated in the adult are made in fetal life.

There are three distinct phases therefore that will be discussed here (Fig. 13.1). First, the period of prophase I/Germinal Vesicle (GV) arrest. An oocyte spends the vast majority of its life arrested here, and it is initiated in the fetal ovary following completion of meiotic recombination and terminated only in the hours preceding ovulation, when a surge in luteinizing hormone (LH) signals for the prophase I-arrested oocyte to undergo GV breakdown (GVB) and resume its first meiotic division. The second period, the resumption of meiosis I, is

the ensuing time during which homologous chromosomes (also known as bivalents) are segregated following the hormonal surge. In contrast to the first period, meiotic resumption lasts just a few hours but nonetheless is vitally important in making sure that the final preparations are made to generate a fertilization-competent oocyte. The third period begins in the final few hours before ovulation and is marked by the rearrest of the mature oocyte at metaphase of the second meiotic division. Unfortunately, despite all the above events, the ovulated oocyte is most likely to degenerate within the uterine tube, unless fertilized by a sperm. Fertilization breaks this metaphase arrest, causing the oocyte to complete its second meiotic division. Only at this point does the oocyte, having undergone two meiotic divisions, actually become haploid. The union with the fertilizing sperm generates a diploid embryo.

The journey of the oocyte through these three transitions is timed primarily by the activity of just one kinase – cyclin-dependent protein kinase 1/CDK1 (previously P34CDC2 or CDC2). For a more detailed examination of CDK1 biochemistry and function in the mitotic cell cycle, excellent reviews exist [1, 2]. During GV arrest, the primary goal of the oocyte is to keep CDK1 activity low, since an increase in its activity is the trigger for entry into meiosis I. During meiosis I, CDK1 activity rises, and once the oocyte is ready to segregate its homologous chromosomes, CDK1 activity falls. Entry into meiosis II requires a CDK1 activity resurgence, then the goal of sperm at fertilization is to cause CDK1 loss. In this chapter, our focus will be on how CDK1 activity is controlled with emphasis on the mouse oocyte, the mammalian model for which most is known, putting CDK1 control in the context of the unique nature of meiosis timing in females.

Maintenance of Germinal Vesicle Stage Arrest

The ability of the oocyte to maintain a lengthy prophase I arrest represents a regulatory phenomenon of the cell cycle unique to the female gamete. Oocyte prophase I arrest encompasses the developmental period from the nongrowing primordial oocyte onward, until the oocyte becomes a fully grown GV oocyte contained within an antral follicle. Despite this cell-cycle status quo, the oocyte remains intrinsically incapable of undergoing meiotic resumption until it reaches at least ~80 % of its full size [3]. In the mouse oocyte, CDK1 protein and that of its regulatory subunit, cyclin B1, accumulate during growth, and as such it has been suggested that small oocytes are meiotically incompetent due to limited CDK1/cyclin B1 content [4]. Since cyclin B1 levels always appear to be in excess of CDK1 in immature oocytes, it is likely CDK1 is the rate-limiting factor [4–6]. Additionally, the ability of CDK1 to associate with cyclin B1 also seems to be acquired with oocyte growth in the mouse [6]. Limited CDK1 and/or cyclin B1 protein levels certainly appear to underlie the meiotic incompetence of immature oocytes in some larger mammalian species where meiotic resumption requires protein translation even once the oocyte is fully grown [7–9].

Once the oocyte achieves meiotic competence, LH signals or removal of the oocyte from its maturation-inhibitory follicular environment can precipitate the CDK1-mediated events of meiotic resumption including chromosome condensation, nuclear membrane breakdown, and assembly of the meiotic spindle [10, 11]. Therefore, prior to an ovulatory LH surge, the oocyte must limit CDK1 activity to prevent precocious meiotic resumption. CDK1 regulation in the oocyte is achieved in a similar manner to the mitotic cell cycle yet involves important differences which reflect the need for the oocyte to coordinate meiotic resumption with the somatic cells of the ovarian follicle.

By definition, all cyclin-dependent kinases must bind a cyclin subunit in order to become active, with cyclin B1 being the predominant CDK1-binding partner during mitosis and meiosis [12]. However, cyclin B1 binding alone is not sufficient for activation, with phosphorylation required at threonine 161 of CDK1. In mitotic cells, the responsible kinase is a CDK-activating kinase (CAK) consisting of complex of CDK7/cyclin H/MAT1 [13, 14], and recent evidence

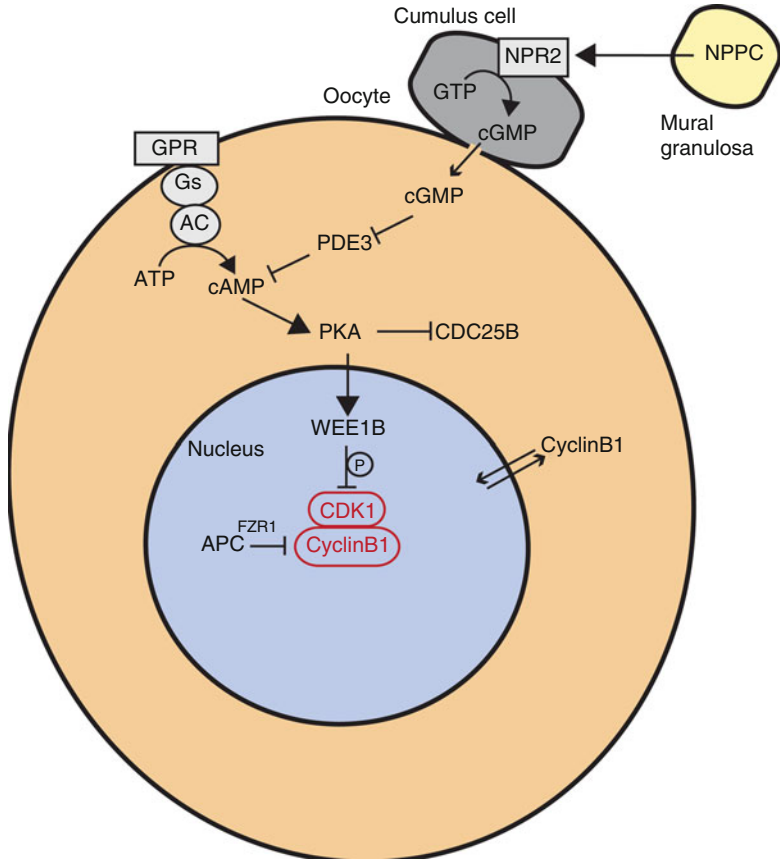

Fig. 13.2 Pathways involved in maintenance of prophase I arrest in the fully grown mammalian oocyte. A G-protein (Gs)-linked GPR membrane receptor on the oocyte membrane stimulates adenylate cyclase (*AC*) to produce cAMP which promotes the activity of PKA. Loss of cAMP is prevented by cumulus cell contribution of cGMP produced by guanylyl cyclase NPR2 in response NPPC produced by the mural granulosa cells. High PKA activity (1) stimulates WEE1B kinase activity which ensures inhibitory phosphorylation of CDK1 and (2) inhibits the activity of CDC25B, the phosphatase responsible for removing this inhibitory phosphorylation. APC^{FZR1} activity ensures the degradation of cyclin B1, the activating subunit of CDK1. Both WEE1B and APC^{FZR1} localize predominantly to the nucleus during prophase I arrest with cyclin B1 and CDC25B sequestered primarily to the cytoplasm

suggests this complex may also promote CDK1 phosphorylation in mammalian oocytes [15]. Inhibitory phosphorylation on threonine 14 and tyrosine 15 must also be removed for full CDK1 activation. This inhibitory phosphorylation occurs through the action of WEE1 and MYT kinases (WEE1B in oocytes) and is reversed by members of the CDC25 phosphatase family in mitotic cells, specifically CDC25B in oocytes [16–18].

Thus, the oocyte employs similar means of regulating CDK1 activity by phosphorylation as does the somatic cell; however, a unique feature of the mammalian oocyte is the requirement for gap-junction communication with the surrounding cumulus cells to maintain CDK1 in an inactive state. This cell-cell interaction promotes maintenance of high levels of cAMP that are essential for GV arrest [19–21]. The mature GV oocyte produces cAMP via a constitutively active G-protein-coupled receptor (GPR) on the oocyte membrane that stimulates adenylyl cyclase activity [22–25]. Although the oocyte also contains phosphodiesterase 3 (PDE3), which may

potentially hydrolyze cAMP, the surrounding cumulus cells produce and transfer cGMP to the oocyte thereby inhibiting PDE3 activity [26–30]. cGMP is produced in the cumulus cells by a guanylyl cyclase, the NPR2 receptor, in response to natriuretic peptide precursor C (NPPC) released by mural granulosa cells (Fig. 13.2) [29, 31]. The LH hormonal surge causes closure of oocyte-granulosa gap junctions so reducing the oocyte's supply of cGMP, resulting in increased turnover of cAMP [27]. Loss of cAMP reduces the activity of protein kinase A, which normally phosphorylates WEE1B maintaining its activation by phosphorylation as well as providing inhibitory phosphorylation of CDC25B [18, 32] (Fig. 13.2). Loss of PKA activity and a reversal in the activities of these two enzymes allow loss of CDK1 inhibitory phosphorylation and promotes its subsequent activation [33].

In addition to direct modification of CDK1 phosphorylation sites, the levels and availability of cyclin B1 are also important for regulating CDK1 activity in the mammalian oocyte in order to maintain prophase I arrest. Oscillatory degradation and resynthesis of cyclins drive the somatic cell through its cycle, and the oocyte utilizes these same processes to control levels of cyclin B1 throughout the period of prophase I arrest. In the fully grown GV oocyte that contains high cyclin B1 levels, ubiquitin-mediated proteasomal degradation of cyclin B1 becomes key to regulating CDK1 activity and is essential during the subsequent stages of meiosis I and II. Known for its role as the driver of anaphase progression in mitosis, the anaphase-promoting complex (APC) is a multimeric E3 ubiquitin ligase, responsible for the ubiquitylation of cyclin B1 and other substrates, which thereby targets them for degradation by the 26S proteasome. The activity and substrate specificity of the APC are regulated by its binding to one of two coactivator proteins that function in a reciprocal manner to coordinate the different phases of the cell cycle [10]. Anaphase progression in mitosis, and meiosis I and II, requires interaction of the APC with its coactivator CDC20 [12, 34]. In contrast, FZR1 (also known as CDH1) has a well-established role in mitotic exit and maintenance of G1/S phases of the mitotic cell cycle, but in the GV oocyte, FZR1 regulates APC activity during prophase I arrest [35, 36]. APC^{FZR1} activity in the GV oocyte promotes the degradation of cyclin B1, thereby providing an additional mechanism for maintenance of low CDK1 activity [37–39]. Interestingly, FZR1 levels also need to be regulated, to prevent too much or too little APC^{FZR1} activity, and so far, this appears to involve several proteins including Emi1 and CDC14B [40, 41].

A spatial aspect to CDK1 regulation is also important for GV arrest and involves both the cAMP/PKA phosphorylation and APC^{FZR1} ubiquitylation/degradation pathways. During GV arrest, WEE1B, components of the APC^{FZR1}, and 26S proteasome are located predominantly in the oocyte nucleus while cyclin B1 is retained in the cytoplasm [42, 43]. Although some cyclin B1 can enter the nucleus during GV arrest, nuclear APC^{FZR1} is thought to degrade it in this subcellular location, a conclusion supported by the observation that APC^{FZR1} activity is highest in the nucleus [42, 43]. Shortly before spontaneous meiotic resumption in vitro, WEE1B translocates from the nucleus to the cytoplasm while CDC25B and cyclin B1 enter the nucleus [42–44]. Enhanced CDK1 dephosphorylation by CDC25B and levels of cyclin B1 that overwhelm APC^{FZR1}-mediated degradation are therefore the likely events promoting the high CDK1 levels capable of precipitating meiotic resumption.

Regulation of Meiosis I

Unlike prophase arrest which is broken by hormonal cues and release from metaphase II arrest which is achieved by fertilization, the timing of meiosis I is governed by the oocyte itself. However, like prophase I and metaphase II arrest, regulation of CDK1 activity is essential for this dynamic period of oocyte maturation. Following GVB, increasing CDK1 activity promotes chromosome condensation and spindle formation, such that by early prometaphase the condensed homologous chromosomes (bivalents) find themselves among microtubules emanating from the newly forming spindle [12]. In meiosis I, unlike

any other cell division, sister kinetochores form a functional unit and are mono-orientated, attaching to the same pole of the spindle. However, the two sister chromatid pairs within the bivalent are attached to opposite spindle poles, i.e., the bivalent is said to be bioriented. By mid prometaphase of meiosis I, sister kinetochore pairs start to make attachments to the spindle microtubules, pulling on the bivalents and orientating them in parallel with the axis of the spindle [45]. Once bivalents are aligned on the metaphase spindle, anaphase and meiotic exit can proceed, which requires a decline in CDK1 activity and continued activity of its rival phosphatases [46]. This loss in CDK1 activity is achieved principally by degradation of cyclin B1 during meiosis I [47]. Of the two APC activators discussed previously, it is CDC20 that becomes the dominant driver of anaphase during meiosis I [48, 49].

To couple exit from meiosis I with separation of bivalents, the APC also promotes the activation of the protein, separase. Active separase cleaves the cohesin molecules tethering bivalents together permitting them to be pulled to opposite poles of the meiotic spindle by microtubules during anaphase [50]. Separase is kept inactive prior to anaphase both by CDK1 phosphorylation and by binding a chaperone protein called securin. Like cyclin B1, securin contains a degradation motif and is targeted by APC^{CDC20} for proteolysis [51]. Therefore, the APC is essential for anaphase progression by promoting the concerted degradation of both cyclin B1 to lower CDK1 activity and securin, to allow homolog separation and ultimately extrusion of the first polar body. In addition, for the completion of meiosis I, there is an another mechanism of regulating CDK1 activity that involves binding separase. This physical coming together of separase with CDK1 leads to mutual inhibition for each enzyme, with both separase and CDK1 activity being quenched. This interaction is essential in mouse oocytes in order to decrease CDK1 activity sufficiently to allow completion of meiosis I and polar body extrusion [52]. The oocyte's need for this interaction may reflect its large size and large amount of cyclin B1 [4], which may be in excess of what APC^{CDC20} can degrade by itself.

A factor beneficial to prevent the missegregation of chromosomes at anaphase onset is the correct attachment of all bivalents to the spindle microtubules [53]. The fidelity of chromosome separation in both mitosis and meiosis is regulated by a molecular checkpoint known as the spindle assembly checkpoint (SAC). This pathway delays mitosis/meiosis I until all sister kinetochores (mitosis) or sister kinetochore pairs (meiosis) are occupied by microtubules and is achieved by a biochemical signal originating from unoccupied kinetochores [54]. This signal is produced through a pathway involving numerous proteins and is responsible for temporarily preventing loss of CDK1 activity that would otherwise drive the cell into anaphase.

The SAC, which has been far better studied in mitosis than meiosis, is comprised of proteins from the MAD (mitosis arrest deficient) and BUB (budding uninhibited by benzimidazole) families of proteins, first identified as being crucial for implementing mitotic arrest in yeast [55, 56] as well as the kinases Mps1 and Auroras B or C [57, 58]. These SAC proteins interact on the kinetochore where the mitotic checkpoint complex (MCC) comprising of MAD2, BUBR1, BUB3, and CDC20 is catalyzed. The binding of this complex to the APC prevents cyclin B1 and securin destruction and may work by presenting CDC20 to the APC as a substrate instead of an activator, thus keeping levels of CDC20 in check [59]. The same proteins are responsible for establishing a SAC in oocytes as knockdown or inhibition of MAD2, BUBR1, BUB1, Auroras, or Mps1, all result in an accelerated first meiosis and increased aneuploidy [60–65].

In mitosis, the SAC is regarded as being sensitive because one vacant kinetochore can delay anaphase [54]. On completion of the final attachment, MCC production ceases, and freed CDC20 activates the APC leading to cyclin B1 and securin degradation. In meiosis, the SAC may not be so sensitive. It is thought that only a majority of kinetochores need be occupied by microtubules in order to start the progression toward anaphase [66]. Following final satisfaction of the SAC, another striking difference between meiosis I and mitosis is the timing of anaphase and cytokinesis relative to APC activation. In mitosis, this period lasts for a

matter of minutes. In mammalian oocytes however, the time between activation of the APC and anaphase can be measured in hours [65].

An intriguing feature of mammalian oocytes is the high incidence of aneuploidy – up to 30–40 % in humans. Why should oocytes have such high rates of aneuploidy relative to mitotic cells? Much research has focused on answering this question, with the subtle differences between the mitotic and meiotic SAC suggested to be the key. Collectively, the lower threshold of microtubule attachment required before SAC inactivation in oocytes and the uncoupling of APC activation and anaphase may make the oocyte more susceptible to aneuploidy. Many questions regarding SAC function in meiosis remain unanswered, such as whether the oocyte SAC responds to tension between kinetochores as well as microtubule attachment as it does in mitosis [67, 68].

Meiotic Arrest at Metaphase II

The process of meiosis I is completed in the hours preceding ovulation. As detailed above, the first polar body is extruded, and the ovulated oocyte, referred to here from now on as an egg, is newly arrested at metaphase of the second meiotic division (metII). Soon after ovulation, usually within a period of a few hours, the egg is penetrated by a fertilizing sperm, and this fusion event triggers initiation of embryonic development.

The key feature before fertilization is the continuous metII arrest, which must be maintained to ensure viable embryo development. This arrest is maintained by high CDK1 activity, and one unique aspect in the egg is that CDK1 levels are constantly high as opposed to mitotic progression where raised CDK1 activity lasts just tens of minutes. Masui coined the term "cytostatic factor" (CSF) to describe the activity which must maintain this arrest, and one can think of CSF as the tool used by the egg to keep CDK1 activity stable [69]. c-MOS, a MAP kinase kinase kinase, was initially described as being CSF, and most critically in mammals, this was demonstrated in a knockout mouse, whose eggs failed to maintain arrest at metII [70, 71].

The downstream targets of the MAPK pathway, in which c-MOS is the most upstream component, are only really well studied in *Xenopus* eggs. In this species, it is clear that p90RSK is the main target of MAPK activity [72–74]. However, in mice, knockout studies have demonstrated that none of the main p90RSK family members are the targets of CSF activity [75]. Instead, one recent paper has reported that mitogen- and stress-activated kinase 1 (MSK1) may play the same function [76] and its role remains to be further investigated.

Independent of the identity of the most downstream component of the MAPK signaling pathway, it is likely that the common target in *Xenopus* and mammalian eggs is EMI2, an APC inhibitor. EMI2 appears to be a meiosis-specific inhibitor of the APC, whose activity is first seen at the end of meiosis I [77–82]. Importantly, the relationship between the pathways involving c-MOS-MAPK and APC^{EMI2} has been established in *Xenopus*, by the finding that p90RSK-induced phosphorylation of EMI2 is needed to keep this APC inhibitor stable [83, 84]. When this phosphorylation event is prevented, then EMI2 is not sufficiently stable to maintain CSF arrest. Mouse eggs during their maturation also express EMI2, and this protein also appears to play an essential role in establishing and maintaining metII arrest [85, 86]. Given that p90RSK has no role to play in mouse metII arrest and that Xenopus EMI2 and mouse EMI2 do not play identical functions when expressed in mouse eggs [87], further studies are needed to examine how differently EMI2 is regulated in mammalian eggs compared to the better characterized frog.

At fertilization in mouse, sperm calcium signals activate the calcium-sensitive kinase calmodulin-dependent protein kinase II (CAMKII) [88, 89]. This signal also occurs in frog, where it has been established that CAMKII phosphorylates EMI2, which in so doing causes its degradation and thereby releasing the APC from its arrest [77–82]. Interestingly, in the mouse, this CAMKII signal also activates WEE1B, which contributes to switching off CDK1 activity, necessary for meiotic exit [90].

Finally, with respect to release of metII arrest, it is important to stress that the sperm may also have to "put its foot on the accelerator" as well as "releasing the brake." Thus far, we have focused on how the break is released, but in *Xenopus* eggs, it is clear that the calcium-activated phosphatase calcineurin also needs to be activated at fertilization to ensure the correct initiation of embryonic development [91, 92]. Calcineurin is not important for mammalian fertilization [87], but other processes may likely be activated in order to reverse the phosphorylation events induced by CDK1. In mitotic exit and possibly mouse eggs, this is achieved by the phosphatase 2A [93, 94].

Concluding Remarks

We have simplified the account of meiosis to CDK1. We focus on this kinase, not because it is the only important kinase in the two meiotic divisions, but rather because changes in its activity are the primary drivers of these processes. There is still much detail to be established on how many of the pathways that feed into its activation and inactivation are regulated, such as the control of the SAC in inhibiting APC activity in meiosis I. These signaling pathways are likely to lead to fundamental insights into oocyte biology that will be important within the IVF clinical context as well as more widely with respect to understanding cell-cycle control and its dysregulation in cancer.

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Cytoskeletal Correlates of Oocyte Meiotic Divisions

14

Marie-Hélène Verlhac and Manuel Breuer

Abstract

Meiosis in oocytes is demanding, requiring chromosomes to be evenly segregated between daughter cells while the cytoplasm should be unequally shared to benefit the oocyte. Indeed, oocytes are extremely large cells compared to most somatic cells and divide twice asymmetrically, giving rise to tiny abortive daughter cells, at least in species that do not reproduce by parthenogenesis. The asymmetry of oocyte meiotic divisions allows preservation of maternal mRNAs, proteins, and nutrients in the cytoplasm, accumulated during the growth phase of oogenesis. In mammals, this asymmetry supports early embryonic development before implantation of the blastocyst in the female reproductive tract. We will review in this chapter how mouse oocytes have resolved the difficult task of dividing asymmetrically at the level of the cell's cytoskeleton. First, chromosome alignment and subsequent segregation happens without centrosome-mediated spindle assembly; second, the positioning modules employed to ensure strong asymmetry based on the actin cytoskeleton.

Keywords

Asymmetric Divisions • Meiosis • Oocyte • Microtubules • F-actin

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Dual Consequences of Losing Canonical Centrosomes During Oogenesis

Asymmetric cell division generates cell diversity from a single mother cell. It is striking that all oocytes are big – often larger than their male counterpart, the spermatozoa – and are produced by asymmetric divisions in size. This suggests that sexual reproduction has imposed a unique asymmetric way of transmitting heredity to progeny. Interestingly, disposal of canonical centrosomes

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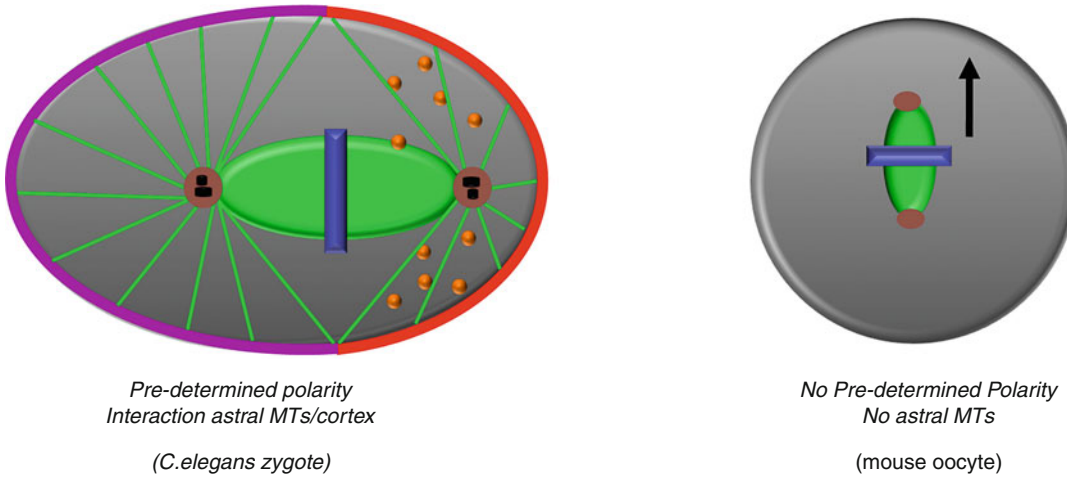

Fig. 14.1 An alternative mode of asymmetric division. (*Left*) In mitotic cells, centrosomes and associated long astral MTs determine not only the axis of spindle formation but also the position of the spindle in the cell via interactions between astral MTs and the cortex (as seen in *C. elegans* zygotes). (*Right*) Lack of canonical centrosomes imposes a strong F-actin-based spindle positioning, as no astral MTs connect spindle poles to the cell cortex. Furthermore, in mouse oocytes, the spindle axis is not predefined by the position of the two centrosomes facing each other before nuclear envelope breakdown. The *arrow* represents the direction of spindle migration towards the oocyte cortex

and their associated astral microtubules has been a frequent strategy used to prevent centering of the division spindle, allowing the oocyte to be cut into extremely unequal parts (Fig. 14.1). However, centrosome loss is not without cost: First it requires that meiotic spindles form in the absence of predefined poles, and second it imposes modes of spindle positioning that do not rely on astral microtubules, which are used in most somatic cells undergoing asymmetric divisions (Fig. 14.1). We will focus our review on the way mouse oocytes assemble and control the positioning of meiotic spindles in the absence of centrioles. We will not review what is known in other mammalian species (for a recent update on the subject, please see [1]) nor will we review the mechanisms controlling polar body extrusion (for review, see [2]).

Meiotic Spindles Without Centrioles

The vertebrate oocyte is naturally arrested in prophase I. Hormonal stimulation transduces into inter- and intracellular pathways so that the nuclear envelope, also referred to as the germinal vesicle (GV), breaks down (GVBD) to commence meiotic maturation. Similar to actin microfilaments (see below), the microtubule (MT) cytoskeleton has to undergo dramatic changes in its organization and dynamics. From a pseudo-interphasic MT network with low dynamics during prophase arrest, the oocyte has to form the meiotic spindle, a structure built of mostly highly dynamic MTs. The spindle must orchestrate the alignment and proper segregation of homologous chromosomes and sister chromatids in the two subsequent meiotic divisions. In the following, we will describe the mechanisms that drive the initial assembly of the spindle; afterwards, we will outline how the meiotic spindle controls the alignment and segregation of homologous chromosomes, with an emphasis on the control of spindle stability in the two meiotic divisions.

E Pluribus Unum: Organizing Discrete MTOCs into Robust Poles

The primary consequence of centrosome loss is the need for substitution by a center for MT organization. Even further strategies are required to form a meiotic spindle: Lack of centrosomes prevents the establishment of a long spindle axis prior to its assembly. Furthermore, an efficient probing

of the cytoplasm for kinetochores, chromosomal attachment sites, by microtubules initiated at centrosomes [3] will be missing; the process of "search and capture" should be highly inefficient in the large volume of the oocyte [4]. Kinetochore capture is a pivotal task for spindles during division to ensure faithful and timely chromosome repartition into the emerging cells afterwards. It therefore does not come as a surprise that mouse oocytes display a very long prometaphase I and also lack stable interactions of microtubules with the kinetochores for at least half of its duration [5, 6].

How does the oocyte build a spindle after meiosis resumption? Several small microtubule-organizing centers (MTOCs) have been known for a long time to exist and potentially control spindle assembly [7, 8], but it is only recently that the behavior of these MTOCs in early meiosis has been studied in vivo.

Controlling MT Assembly Around Chromosomes

Initially dispersed throughout the cytoplasm, the MTOCs cluster around the nuclear envelope prior to NEBD through interaction with the envelope and one another. Centrosomal proteins like γ-tubulin, pericentrin, or NEDD1/GCP-WD, a γ-TuRC-associated protein, localize to these structures, confirming their microtubule nucleating capacity, replacing centrosome function [8–12]. Indeed, knockdown of NEDD1 by an siRNA-mediated approach reduces γ-tubulin recruitment to spindle poles thus affecting microtubule assembly and spindle function [12]. After NEBD, the MTOCs gather in proximity of the condensed chromosomes. Concomitantly, the nuclear release of factors involved in spindle assembly strongly increases MT nucleation [13]. This is under the control of the small GTPase Ran. Switching from its inactive, GDP-bound state to an active, GTP-bound state in vicinity of chromatin, RanGTP has been shown to be at the heart of centrosome-independent spindle assembly during the last decade [14]. It is in its active form that Ran triggers the release of spindle assembly factors (SAF) specifically in the vicinity of chromosomes. This is achieved through building up a gradient of Ran, with a high concentration of active RanGTP declining with further distance from the cytoplasm [15]. The NEBD releases RanGTP into the cytoplasm, establishing the gradient around meiotic chromosomes throughout meiotic maturation as monitored by the FRET sensor Rango [16]. The discovery of a role for the RanGTP pathway in spindle assembly and the demonstration of its existence provided a nice explanation for earlier models of a long-range effect of chromatin on the stabilization of microtubules [17, 18]. Yet, this does not directly induce MT nucleation as postulated [19]: No microtubules emanating from chromosomes can be observed immediately after NEBD and RanGTP release [20]. Rather, it seems that in mouse oocytes, the early function of a Ran gradient is to control the availability of SAFs such as HURP and TPX2 (see below). Also, asymmetric growth of MTs toward the chromatin might be promoted via the gradient, as MT nucleation and stabilization factors are made available through RanGTP only in chromosome proximity ([21]; Breuer and Verlhac, unpublished observations, Fig. 14.2). Enucleated mouse oocytes are still capable of forming bipolar structures without chromatin, yet multipolar structures occur frequently and MTOC organization is not achieved in these chromatin-free oocytes [22, 23]. Thus, the RanGTP-based, gradient-driven pathway might restrict spindle assembly to chromosomes and increase the efficiency of microtubule organization through RanGTP-effector release.

Establishing a Bipolar Spindle in the Absence of a Predetermined Spindle Axis

Consequently, abolishing the Ran gradient does not prevent spindle formation, but significantly delays the kinetics of assembly in meiosis I [16]. An explanation for this delay lies in the process of spindle formation through sorting of MTOCs toward opposite poles. Subsequent to their accumulation around the nuclear envelope, the MTOCs will redistribute in a symmetric fashion around the chromosomes [20, 21]. The positioning of MTOCs relative to one another is likely promoted by plus- and minus-end-directed motor proteins. These motors exert pushing and pulling

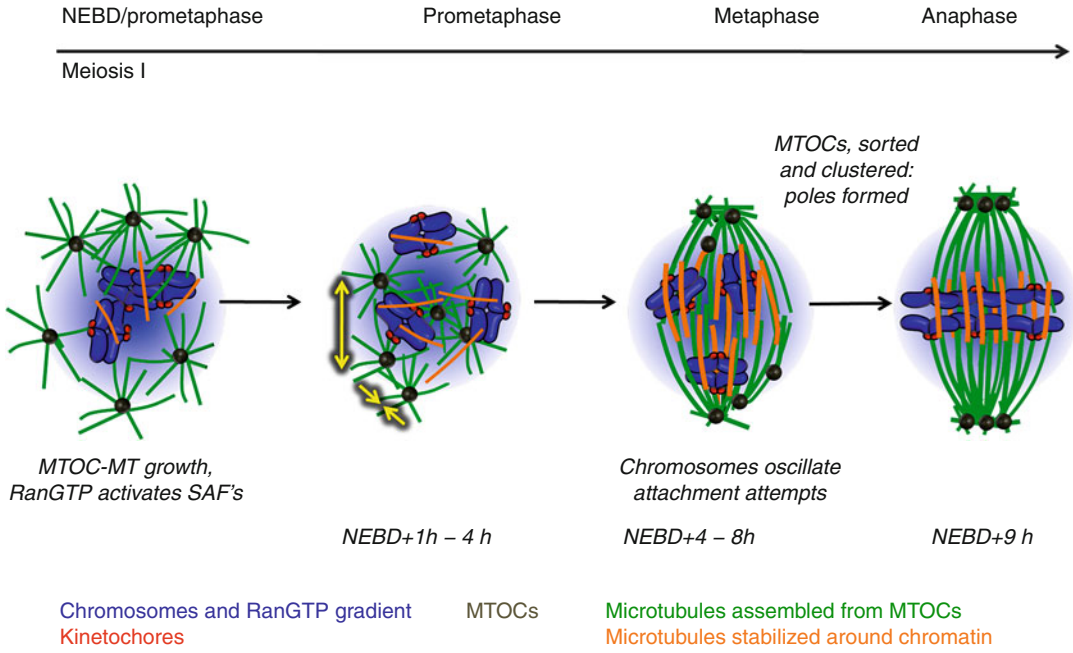

Fig. 14.2 Meiosis I spindle assembly in mouse oocytes. After NEBD, MTOCs (*dark green*) promote MT (*green*) growth, which is biased towards chromatin (*blue*) through release of SAFs via the RanGTP gradient (*light blue*). MTs stabilized via HURP, close to chromatin, displayed in orange. Initially dispersed MTOCs are sorted toward opposing poles through MT-based repulsion and attraction forces, mediated by SAFs (such as HURP) and motor proteins (Eg5). Transient MT-chromosome interactions at kinetochores (*red*) move the chromosomes at the surface of the MT zone. Chromosomes congress, the spindle acquires bipolar shape through pole ejection, and the amount of amphitelic attachments increases during prometaphase. Eventually, all MTOCs are sorted to the poles. Their clustering is maintained via an increase in MTs assembled at the poles and controlled by TPX2 activity. Once the first meiotic spindle is robust with fully organized poles, 8 h after NEBD, bivalents can segregate upon anaphase onset

forces on microtubules that emanate from neighboring and opposed MTOCs, resulting in attraction and repulsion of the centers, respectively (Fig. 14.2). After equal distribution, the MTOCs then coalesce towards opposite sides of the forming spindle, thereby setting ground for the future spindle poles. It is at this point that the long axis of the meiotic spindle becomes apparent. However, the completion of MTOC sorting to the poles is only achieved long after a bipolar structure is visible, indicating that MTOC organization determines robust pole formation in meiosis [21].

The RanGTP-regulated, microtubule-associated protein TPX2, essential for spindle assembly in many experimental systems [24, 25], is a crucial factor for meiosis I spindle assembly [26]. Accumulating at MTOCs and thus the forming spindle poles, it controls both the assembly of microtubules and the integrity of poles. The latter is achieved through phosphorylation of TACC3, known to stabilize centrosomal/MTOC-emanating microtubules [27]. TPX2 is an interactor and activator of the cell-cycle-regulated kinase Aurora A, which in turn is known to phosphorylate several mitotic targets for spindle assembly [28]. Thus, the interaction of TPX2 and Aurora A, which accumulates at MTOCs in its active form in early meiosis [29], promotes TACC3 phosphorylation, thereby establishing MTOC integrity and spindle pole formation. Knockdown of Aurora A leads to mislocalization of MTOCs in the meiosis I spindle [30]. Apart from TPX2's role in pole organization, it is also the major MT generation force in meiosis, as TPX2 depletion

strongly diminishes microtubule assembly from MTOCs around chromosomes in meiotic prometaphase [26]. Interestingly, the protein gradually accumulates during meiosis I and II in mouse oocytes, with very low levels at and after GVBD. Conversely, when high protein levels of TPX2 are introduced via overexpression, spindle assembly is highly compromised [26]. Thus, TPX2 levels have to be tightly regulated during meiotic maturation to ensure early spindle assembly. It is therefore reasonable to assume that RanGTP activity after NEBD is directed toward targets other than TPX2.

In fact, an immediate target of RanGTP in early prometaphase appears to be HURP. With its microtubule-stabilizing activity, it promotes the aforementioned process of MTOC organization in meiosis I. Active RanGTP is required for HURP localization, and HURP-deficient oocytes are perturbed in MTOC-sorting kinetics [21]. As the microtubules in the vicinity of chromosomes are missing in HURP-deficient oocytes, this suggests that activity of motor proteins and stabilization of these MTs are central to the process of sorting. Other molecules implicated in this process remain to be identified in meiosis. Good candidates are motor proteins like the plus-end-directed Eg5 or the minus-end-directed motor HSET/Ncd, which could enable the coalescence of neighboring MTOCs by pulling them towards one another. However, a study depleting HSET via antibody injection found no phenotypes in these early steps of meiosis [31]. Assuming the specificity of the depletion method, this points towards redundant mechanisms for minus-end-directed motor protein organization. As for Eg5, it is not only required for spindle pole ejection and thus spindle size maintenance in mouse oocytes [20, 32] but also to accumulate HURP in the central domain of the meiotic spindle [21]. These observations point to an interaction between Eg5 and HURP to organize and stabilize antiparallel microtubules in the spindle center; as both proteins accumulate in the chromosome vicinity, this could be equally required prior to bipolarization for MTOC sorting.

Resolving the exact mechanisms for MTOC sorting is not only an interesting open question for mammalian meiotic spindle assembly but also contributes to the understanding of cancer cell division. Recently, the first evidence for a shared pathway between mouse oocytes and U2OS cells was provided by depleting HURP from the latter. Cancer cells often harbor multiple centrosomes and ensure viability through centrosome clustering to prevent multipolar anaphase and thus deleterious segregation of chromosomes, compromising viability [33]. RNAi against HURP in these cells results in multipolar spindles in anaphase, demonstrating a conserved role for HURP in MTOC sorting for both systems [21].

Maintaining a Bipolar Spindle Structure

Despite the discovery of TPX2 and HURP as essential SAF targets in spindle assembly, it is clear that initial targeting of RanGTP targets to microtubules in the spindle should trigger the recruitment of multiple SAFs to the spindle. For example, HURP itself has been shown to complex with several Ran-regulated factors in *Xenopus* egg extracts [34]; also, the list of importin-regulated and RanGTP-released SAFs is long [14]. While the meiotic spindle forms based on the microtubule-MTOC dense ball, homologues individualize and congress. Movement of chromosomes in mitosis is mediated by microtubule dynamics. For example, microtubule poleward flux results from the depolymerization of MTs at spindle poles and assembly in the spindle midzone [35]. MT flux controls spindle length and chromosome movement in anaphase; in mouse oocytes, MT flux is mediated by Eg5 activity [32]. Further, pulling forces from kinetochore MTs from opposite poles help congressing chromosomes [36].

Intriguingly, in mouse oocytes, kinetochore fibers are largely absent until midway through meiosis I. Here, it seems that it is mostly lateral interactions of chromosomes and microtubule bundles in the forming spindle center that enable the orientation and congression of chromosomes [5, 6, 37]. Studies in *C. elegans* oocytes and cultured cells with invalidated kinetochores have supported evidence for the role of interpolar

microtubules to congress chromosomes [38, 39]. Support might come from the polar ejection force provided by chromokinesin motor proteins, applied on microtubules with the chromosomes as cargo, promoting their displacements [40, 41]. The chromokinesin Kid, however, is dispensable for chromosome individualization and congression in mouse oocytes [6, 42].

The microtubules in the spindle center in mouse oocytes are sorted and stabilized by Eg5 and HURP to provide robust bipolarity; in the absence of HURP, chromosome clumping is observed which blocks individualization and congression of bivalents [21]. Recently, detailed in vivo observations of kinetochores and chromosome behavior throughout meiosis I revealed that after congression, chromosomes "invade" the spindle from a belt-like configuration around the microtubule-MTOC ball towards the spindle equator [6]. With progression through meiosis I, the spindle elongates, chromosome biorientation is observed along with the formation of kinetochore fibers. Strikingly, this achievement of correct kinetochore-microtubule attachments is highly erroneous, needing several rounds of corrections before stable biorientation is achieved [6]. However, correct alignment and segregation is eventually achieved upon anaphase I onset. Interestingly, this ongoing correction mechanism does not seem to predestine oocytes for the observed high incidence of segregation errors: Even in the absence of MCAK, a microtubule depolymerizer known to correct improper MT attachments at kinetochores, homologues segregate properly [43, 44]. The late establishment of end-on attached kinetochores, following robust spindle bipolarity establishment, might thus prevent segregation errors rather than promote them. Such a mechanism, where the inhibition of stable attachments by a kinetochore protein complex enables chromosome orientation through lateral attachments, has been suggested in *C. elegans* [45]. Accordingly, MTOC sorting, which prevails throughout the entire length of prometaphase I in mouse oocytes, might be a limiting step. Indeed, an early capture of bivalents by unsorted MTOCs might promote faulty attachments and subsequently aneuploidy, as observed in cancer cells with extra centrosomes [46], and thus has to be strictly avoided until all MTOCs are clustered at poles.

Once the chromosomes are properly aligned on the metaphase plate, bivalents segregate into the small polar body and the remaining large oocyte. Subsequently, the oocyte enters meiosis II without an intervening S phase. The formation of the meiosis II spindle is rapid compared to meiosis I, with a bipolar structure assembled 1 h after polar body extrusion [16]. With its MTOCs sorted and coalesced at spindle poles and with robust kinetochore fibers, the meiosis II spindle resembles a mitotic one.

Intriguingly, the difference between meiosis I and II spindles seems to be reflected in the fundamental requirement for a RanGTP gradient in spindle assembly in meiosis I versus meiosis II. Inactivation of the gradient with both dominant-negative or constitutively active RanGTP in the oocyte cytoplasm compromises spindle assembly in MII oocytes, with the appearance of MT asters throughout the cytoplasm and the inability to segregate chromosomes upon anaphase II [16]. In parallel to RanGTP and its effectors, the MAPK kinase pathway has been shown to control MII spindle stability in the oocyte [47–50]. As it is not the microtubules per se that are more stable in metaphase-arrested meiosis II spindles [51], mechanisms to ensure spindle stability might prove useful in a cell awaiting fertilization for extended periods. Two MAPK kinase substrates, DOC1R and MISS1, have been shown to be meiosis-II-specific factors for spindle organization [52, 53]. Downregulation of both MAPK targets leads to spindle defects similar to those observed in Ran-mutant-injected oocytes. Currently, it is not known, however, which molecules are at the converging point where both pathways interact.

Positioning in the Absence of Astral Microtubules

The spindle forms in the location of the nucleus when it breaks down. In mouse oocytes, the nucleus resides in the vicinity of the cell center.

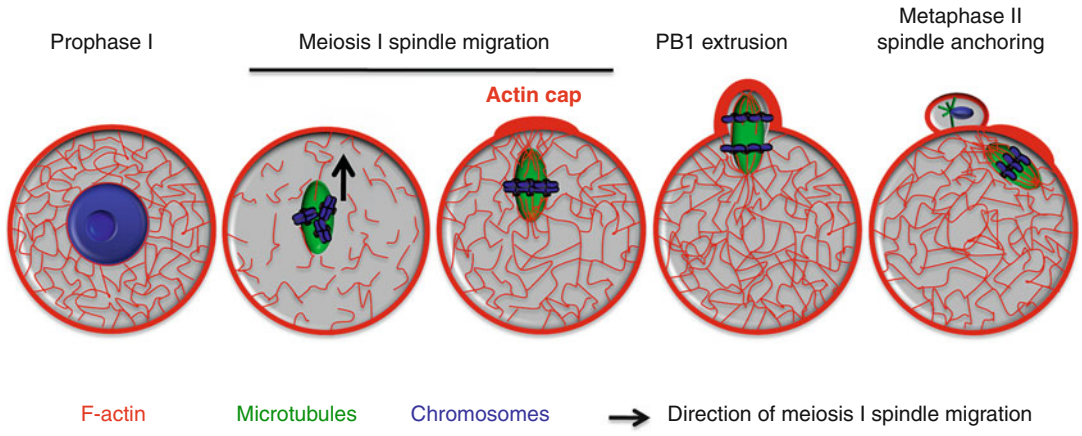

Fig. 14.3 The actin meshwork controls spindle positioning in meiosis. The dense actin meshwork in prophase loosens up after NEBD and reassembles during spindle migration to the cortex. Being highly dynamic, the meshwork interacts with the spindle and chromosomes to promote their displacement from the initially central position in the oocyte. A prominent actin cap forms at the cortex overlying the spindle at the site of polar body extrusion upon anaphase I onset. In meiosis II, this cap seems to promote anchoring of the spindle parallel to the oocyte cortex via activation of an actin flow

We will first describe the modality of spindle positioning and its geometry; then, we will review the mechanisms at play.

Geometry of the Mouse Oocyte

The first meiotic spindle reaches a size of approximately 30 mm long when it is fully functional and bipolar [16]. The diameter of a fully grown mouse oocyte is about 80 mm. It is striking that the first meiotic spindle starts migrating after spindle bipolarity and MTOC sorting are achieved in late meiosis I [49, 54]. Presumably, as a consequence of high cytoplasmic viscosity in the egg, the spindle always migrates along its long axis. The spindle forms in the center of this huge cell, with no connection to the cortex and no constraint on its long axis, which takes a random orientation (Fig. 14.3; [55]). Furthermore, by measuring the distance between the two spindle poles and the cortex, it was shown that the spindle moves towards the closest cortex. The spindle has to drift approximately 20 mm to reach this region of the cortex, doing so in about 2.5 h, therefore moving at a mean speed of 0.13 mm/min [55]. By flipping the spindle axis with a microneedle, after entry into meiosis I, it was confirmed that the spindle always migrates toward the closest cortex and, if artificially displaced, can change direction of migration to the newly imposed cortex [56]. The first polar body is extruded at anaphase I, while the spindle keeps moving, imposing a dynamic definition of the cleavage plane. Anaphase I is not triggered by the spindle reaching the cortex and occurs with normal kinetics even when the spindle does not move to the cortex [57]. However, one might expect a certain level of coordination to allow anaphase I and chromosome anchoring at the cortex to take place simultaneously.

Importance of F-actin in the Control of Spindle Positioning

It has been known for a long time that microtubules are not required for chromosomes to reach the cortex, contrary to microfilaments which are indispensable [58]. However, it is only recently that cytoplasmic filaments involved in the control of meiosis I spindle positioning could be visualized, thanks to a probe which specifically binds F-actin rather than G-actin [59]. In prophase I-arrested oocytes, a dense meshwork of dynamic cytoplasmic F-actin connects filaments from the cortex to filaments running along the nucleus

(Fig. 14.3; [60]). This dense meshwork is remodeled with microfilament destabilization at meiosis resumption, resembling a G2/M transition for microtubules: Both major cytoskeletal elements of the meiotic cell need thus to be reorganized potentially through modifications switching microtubule and actin properties from a pseudo-G2 state in prophase-I arrest to de novo dynamics in prometaphase after NEBD. The molecular pathways involved in the change of both actin and microtubule regulation during this transition in mouse oocytes remain to be elucidated; however, they are likely to be governed by the increased activity of cell-cycle-regulated kinases such as cdk1/cyclin B, polo-like kinase, and the Aurora – family of kinases.

Cytoplasmic F-actin reappears in meiosis I concomitantly with chromosome movement to the cortex [61]. Theses filaments are very dynamic; they seem to move towards spindle poles, and their dynamics promotes spindle migration [56, 61, 62]. Also, myosin II activity would allow contractility of the meshwork, thus favoring spindle movement [56]. Apart from a cytoplasmic mesh, F-actin is also observed surrounding the microtubule spindle, mostly outside the spindle and with few filaments inside [61]. The nature of the connection between actin and microtubules in mouse oocytes is not known; however, based on experiments performed in *Xenopus* oocytes, myosin X is an excellent candidate for such an interaction [63]. The "cage" of F-actin around the microtubule spindle is directly connected via straight filaments to the closest cortex and permits its anchoring at the cortex. Anchoring is important and when compromised does not allow the spindle to remain in an excentric location: This happens when a dominant-negative RacGTPase mutant is expressed in metaphase II mouse oocytes and induces spindle drifting towards the center of the cell [64].

It is noteworthy that the F-actin cage and the cytoplasmic mesh are not specific to meiosis or early embryos [65]. They have also been observed in somatic cells undergoing mitosis [66, 67]. In these cells, filaments form extremely dynamic clusters that follow external forces transmitted by retractions fibers from the substratum to the cell body to orient the spindle axis [66]. The use of an F-actin mesh to control spindle positioning, first characterized in yeast [68, 69], is hence a widespread mechanism – dominant when astral microtubules are absent, that is, in mouse oocytes (for reviews, see [70, 71]).

Mechanisms That Control Cytoplasmic F-actin Assembly

To Promote Meiosis I Spindle Migration

Actin filaments, like microtubules, are polarized polymers with a slow growing pointed end (– end) and a fast growing barbed end (+ end). The equilibrium between G- and F-actin in vivo is not favorable towards actin polymerization, but is enhanced by the presence and activity of nucleators. Formins as processive motors remain bound to the growing barbed ends. A formin homology (FH2) domain mediates this binding, and a proline-rich FH1 domain binds profilin/actin, both being essential for filament elongation (for reviews, see [72, 73]). Formins also present modules of regulation of their activity, such as GTPase binding or auto-inhibitory domains [74]. Formin-2, however, belongs to the subfamily of proteins not regulated by Rho GTPases nor containing any obvious C-terminal diaphanous autoregulatory domain (DAD; [75]). This nucleator is essential for spindle migration to the cortex: In its absence, in fmn2–/– oocytes, spindle migration as well as cytokinesis does not occur; female mice produce triploid gametes and are therefore sterile [57, 76]. The absence of spindle migration in this strain is due to the lack of cytoplasmic microfilaments in oocytes [56, 61]. Endogenous formin-2 associates with the cortex and is degraded at meiosis resumption and resynthesized later on in meiosis I, establishing a strong correlation between the cytoplasmic meshwork remodeling and formin-2 regulation [60]. Recent evidence has shown that the level of formin-2 needs to be tightly regulated to allow F-actin meshwork remodeling to support spindle migration to the cortex (Fig. 14.3; [60]).

There is a growing body of evidence that nucleators cooperate to assemble the highly

dynamic mesh in cells (for review, see [77]). The nucleator Spire is conserved across Metazoa and presents four WH2 domains, which are known to bind to four actin monomers at the pointed end, promoting filament nucleation [78]. Spire is a versatile nucleator, being capable of severing filaments but also of capping them at their barbed end, competing with profilin-/formin-mediated elongation [73, 79]. *Drosophila* oocytes mutant for Spire have dorsoventral and anteroposterior axis defects, similar to mutants for Cappuccino, a formin-type nucleator, and resemble cytochalasin-D-treated oocytes [80]. Spire and Cappuccino regulate the onset of ooplasmic streaming by organizing an isotropic mesh of actin filaments in the oocyte cytoplasm. The streaming per se is microtubule-based, yet actin assembly is required for its correct timing. Cappuccino and Spire harbor microtubule and microfilament cross-linking activity, essential for oocyte patterning [81, 82]. Recently, the cooperation between the two mammalian isoforms of Spire, Spire 1 and 2, and formin-2 was shown to be required for meiotic spindle movement to the cortex in mouse oocytes [83]. The phenotype of Spire 1 and 2 depletion by RNAi is very similar to the phenotype of fmn2−/− oocytes, absence of spindle migration and cytokinesis at anaphase I [83]. Furthermore, exogenous proteins co-localize at the plasma membrane, yet formin-2 cannot compensate for the depletion of Spire 1 and 2 and vice versa [83]. Spire and formins interact via the N-terminal noncatalytic C-lobe domain (KIND) of Spire [84] and a short sequence at the C terminus of formins [85]. The crystal structure of this interaction has been resolved and has shown that the KIND domain of Spire is indeed structurally similar to the C-lobe of a protein kinase, yet having no enzymatic activity, where the C-terminal domain of formin binds in an acidic cleft [86]. Paradoxically, the KIND/tail interaction blocks nucleation by formin [86] which is at odds with the in vivo evidence that suggest cooperation between these two nucleators. It will be important to determine in the future how Spire 1 and 2 cooperate in vivo with formin-2, especially in light of recent results which show that formin-2 gets degraded at meiosis resumption in mouse oocytes. It is nonetheless interesting that the cooperation between Spire and formin has been conserved through evolution for functional female gamete formation.

To Maintain the Meiosis II Spindle in Its Asymmetric Location

Other nucleators, such as the Arp2/3 complex, have also been involved in the control of asymmetric division of mouse oocytes, but their mode of action remains totally elusive [87]. However, a very recent study has shown that the Arp2/3 complex is essential to promote anchoring of the metaphase II (MII) spindle during the prolonged arrest experienced by vertebrate oocytes until fertilization [88]. Yi et al. [88] have discovered a novel mechanism, whereby the formation of an F-actin flow, originating from the cortex above the MII spindle, maintains the asymmetric location of the spindle at the cortex of the mouse egg. After the first meiotic division, the MII spindle forms parallel to the cortex and remains anchored into it. Chromosomes, via their RanGTP gradient, permit local activation of N-WASP, which in turn allows Arp2/3 complex localization in the actin cap [88]. A positive feedback loop is therefore established between chromosomes and the cortex to prevent drifting of the meiotic spindle from its initial location. Furthermore, a retrograde flow of F-actin is initiated at the actin cap, which in turns provokes cytoplasmic streaming inside the egg resulting in further pushing of the spindle toward the cortex [88, 89]. This dual-lock ensures that the spindle will stay in place and not fall off in the egg cytoplasm, a vital condition for successful development. Indeed, the loss of MII spindle anchoring is observed in aging oocytes known for their reduced developmental capacity [90]. Rac controls both meiosis I and II anchoring [64]; therefore it is reasonable to assume that first Rac regulates Arp2/3 activity both in MI and MII and second that similar cytoplasmic streamings promote spindle positioning and anchoring to the cortex in meiosis I. Cytoplasmic streamings, present in oocytes from species as diverse as ascidians, *Drosophila*, worm, and mouse [81, 82, 88, 91, 92], permit efficient translocation of mRNAs, proteins,

or cellular structures over long distances, a convenient characteristic in such large cells.

Conclusions

Considering the importance as well as the refined spatial and temporal regulation of F-actin meshes in the control of spindle positioning, further discoveries on the nature and mode of action of all nucleators at play will certainly be a major challenge for the years to come. The increasing data on how the meiotic spindle is organized progressively into a robust bipolar structure will help the identification of pathways implicated in the interaction of chromosomes, microtubules, and MTOCs to ensure faithful segregation of homologous chromosomes. The meiotic spindle exerts forces of different nature on chromosomes depending on their coordinates in the spindle; transmission of these forces on kinetochores is essential for efficient chromosome segregation [93]. Centrosome-free formation and positioning of meiotic spindles are certainly coupled. The F-actin spindle depends on the presence of the microtubule spindle, and conversely, the F-actin spindle might help the transmission of forces inside the microtubule spindle. This might be of particular importance for meiotic spindles with loose poles such as the ones present in mouse oocytes. F-actin-based anchoring of the first meiotic spindle to the cortex by one pole in meiosis I versus the two poles in meiosis II may also be at the heart of the mechanism responsible for the increased incidence of chromosome segregation errors in meiosis I [94].

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Organelle Rearrangement in the Maturing Oocyte

15

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Abstract

Mammalian oocytes acquire a series of competencies during follicular development that play critical roles at fertilization and subsequent stages of preimplantation embryonic development. Before the mammalian oocyte engages in the fertilization process, it must acquire an array of molecular and cellular assets defining its developmental potential. These properties specify competencies to complete meiosis and initiate mitosis. Meiotic maturation requires the acquisition both of nuclear and cytoplasmic competence and this complex mechanism involves most of the organelles, the cytoskeleton and molecules that are relocated from the nucleus to the cytoplasm. Messenger RNAs of maternal origin are accumulated in the oocyte throughout its growth in the ovary. These transcripts are then shuttled to specific sites of the ooplasm, where local translation is promoted. The nucleus as an organelle undergoes significant positional rearrangements during maturation. At the same time, the spindle changes its localization drifting from the cortical region to the centre of the oocyte and then back cortically. The endoplasmic reticulum undergoes significant changes during maturation in its distribution that reflect an enhanced ability to release Ca^{2+}. Energy derives from cellular ATP as a result of mitochondrial activity. Mitochondria are themselves subject to redistribution and changes in activity during transition from the germinal vesicle to the metaphase II stage, in a fashion that can profoundly influence the quality of the mature oocyte and the destiny of the ensuing embryo. The dynamics of Golgi membranes during meiosis is still partially unknown but generally, as it occurs in the bovine, involves movements from the centre of the cytoplasm to the cortical area. Afterwards, a second translocation occurs

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between germinal vesicle breakdown and metaphase I stages, repositioning the Golgi from the periphery to the central cytoplasmic area.

Keywords
Oocyte maturation • Oocyte development • Calcium oscillations • Endoplasmic reticulum • IP_3 receptor • Calreticulin • Cortical granules

Introduction

The human oocyte is certainly the most fascinating cell in our body for its peculiarities both in structure and in function. The egg acquires all its developmental potential during a long period of time compared to all other cells. Oogenesis begins early during embryonic-fetal life and continues well after birth through a process entailing first the production of numerous oogonia followed by a dramatic reduction in the pool of available oocytes that will be retained in the ovary. The oogonium is organized within the simultaneously growing female gonad generated in the fetus and becomes enclosed in the follicle where coordination of the forthcoming growth and maturation will take place. The gonad is generated from a single primordium of mesenchymal tissue following the invasion of three cell populations: primordial germ cells, epithelial cells, and mesonephric germ cells. The first will lead to oogonia, the second to the follicular structure, and the third give rise to the theca and vascular system of the gonad. Ovarian development ceases and waits for the organism to become hormonally competent to support complete oocyte maturation, allowing the egg to achieve the capability to undergo fertilization and give rise to an embryo able to develop and implant in the uterine cavity. The pool of oocytes that has the ability to perform this last step is small compared to the totality of germ cells, and the initial reduction of this gametic heritage is known under the name of follicular atresia [1]. This chapter will not explore oogenesis as the process of formation, migration, and distribution of germ cells within the ovary but will focus on issues involving the maturation of the oocyte within the follicle.

Mammalian oocytes acquire a series of competencies during follicular development that play critical roles at fertilization and subsequent stages of preimplantation embryonic development.

Before the mammalian oocyte engages in the fertilization process, it must acquire an array of molecular and cellular assets defining its developmental potential. These properties specify competencies to complete meiosis and initiate mitosis, support monospermic fertilization and egg activation, and ensure a timely transition from reliance of gene products of oocyte origin to those derived from the zygotic or embryonic genome [2].

Meiotic maturation requires the acquisition both of nuclear and cytoplasmic competence, and this complex mechanism involves most of the organelles, the cytoskeleton, and molecules that move from the nucleus to the cytoplasm.

Compared to somatic cells whose growth in volume is limited by the intervention of mitosis, the female gamete does not undergo cell division, and therefore the oocyte continues to expand to 100-fold, its volume reaching the diameter in the human species of 120 μm. Such an impressive growth aims to provide the egg with a sufficient amount of organelles and molecules that will support early stages of embryonic development up to the complete metabolic autonomy.

Meiotic maturation is a rather well-established process in mammals; however, some mechanism are not yet completely known in humans given the difficulties in obtaining oocytes for research purposes [3].

Transcriptional Activity and Macromolecular Trafficking

Early embryonic development is that unique period during life in which the individual is governed by the genetic information synthesized

from the genome of another individual (the mother) and stored during oogenesis as proteins or RNA in a cytoplasm of a single cell (the oocyte). In fact, oogenesis is characterized by intense synthesis of RNAs and proteins during oocyte growth and stored for long periods of time (months or years). Such a long storage requires that mRNAs have to be protected from the translational machinery and proteins have to be stored in a nonfunctional form. The recruitment of such information also requires specific mechanisms that are necessarily posttranscriptional for mRNAs and posttranslational for proteins. These mechanisms allow specific molecules to be available at the right time for appropriate function [4]. Messenger RNAs (mRNA) of maternal origin are accumulated in the oocyte throughout its growth in the ovary. These transcripts are then shuttled to specific sites of the ooplasm, where local translation is promoted. The transport of mRNAs, from their transcription site in the nucleus to their translation and degradation sites at subcellular regions of the cytoplasm, modulates gene expression and is a way to regulate local translation. This ensures appropriate concentrations of the encoded protein where needed. This process represents an essential step in the maturing oocyte and involves specific RNA-binding proteins that are able to recognize mRNAs as a target for transport, due to secondary structures, formed by the 3′untranslated region (3′UTR). Among the many regulatory elements identified so far, Staufen protein has been shown to allow RNA localization in female gametes of different species. Staufen is a double-stranded RNA-binding protein involved in mRNA transport and localization in Drosophila, while genetic and molecular studies have shown that the activity of the Staufen gene product is necessary for the proper localization of mRNAs in the cytoplasm of oocytes and in the neuroblasts. In mammals, Staufen displays a vesicular pattern of localization, especially in perinuclear region. Apart from ribosomal proteins, Staufen is the first RNA-binding protein to be associated with the rough endoplasmic reticulum. In 1999, Wickham and colleagues [5] demonstrated for the first time that mammalian Staufen (including human) is a double-stranded RNA and tubulin-binding protein. Their observations confirmed previous study in which mRNA colocalize with endoplasmic reticulum although in Xenopus and Drosophila this seems to occur predominantly with the smooth endoplasmic reticulum, demonstrating in those cases that translational activity is not the major function of these associations.

Germinal Vesicle and Spindle Asymmetry

Superimposed to gene expression regulatory mechanisms, the nucleus as an organelle undergoes significant positional rearrangements during maturation. During maturation at both MI and MII, the spindle is localized cortically just beneath the oolemma. This is obviously imposed by the necessity to direct a cleavage plane suitably near the cell surface in order to extrude a small polar body and minimize the loss from the oocyte environment of organelles (e.g., mitochondria) and regulative molecules (e.g., γ-tubulin) from which the development of the ensuing embryo will depend crucially [6]. The way in which spindle asymmetry is obtained has been extensively studied in the mouse. In this species, it has been repeatedly reported that, after recovery from large antral follicles and removal of cumulus cells, fully grown oocytes display the GV in a central or pericentric position and that GVBD occurs in the same position [7, 8]. This implies that the microtubular dynamics leading to the formation of the MI spindle also requires a process of repositioning from the center to the periphery. Once formed after breakdown of the GV, the MI spindle in fact appears to migrate centripetally in a pole-first orientation at a speed of 0.12 μm/min [9]. Cortical domains predetermined to host the spindle do not seem to exist because, after experimental displacement, the spindle is moved again by cytoskeletal forces toward the nearest cortical region [10]. Oocytes treated with nocodazole, a microtubule depolymerizing agent, are still able to support MI translocation [9], while treatments that depolymerize [9], stabilize [11], or interfere [11] with the regulation of actin filaments can inhibit spindle migration. Therefore, actin

undoubtedly plays an essential role in this process, although it is less clear how. In some studies, a dense meshwork of actin has been observed to envelope the migrating spindle. This actin array, initially homogeneous and uninterrupted, has been seen to change into a conformation involving a decrease in density at the front where the spindle migrates toward the cortex and an accumulation at the innermost side [11]. Such an asymmetric and specific actin distribution is suggestive of differential depolymerization and polymerization activities that in combination generate a centripetal pushing force, similar to that observed in a nonmammalian species. However, alternative possibilities have been proposed to explain spindle migration, based on the fact that in other studies a higher actin density has been observed not posteriorly but rather in front of the migrating spindle [10]. In one model, molecules of myosin II connected to the front pole would move toward the cortex on bundles of actin filaments, thereby exerting a pulling force on the entire spindle. In another hypothesis, antiparallel filaments of actin bridging the space between the front pole and the cortex would slide one on each other assisted by bi-oriented myosin II molecules in a fashion that would pull the spindle toward the cortex [7]. None of the above models have been unequivocally demonstrated, leaving the question of the mechanism of spindle migration still open.

Different lines of evidence question the paradigm that in mouse fully grown oocytes, the GV is centrally located and that the MI spindle is formed in the same position and only subsequently moved to the cortex during maturation [12]. While it is true that, after oocyte isolation from the follicular environment and removal of cumulus cells, the GV is often seen in a central position; direct observation of the oocyte within the follicle after fixation by conventional histological techniques or in a live sample by differential interference contrast indicates that in reality the GV is situated eccentrically. Cortical positioning of the GV appears to be the rule, rather than the exception in mammalian species, having been observed in a wide variety of primates (including human), ungulates, and other rodents [12]. The question then arises of why in the mouse the GV is arranged near the cortex in oocytes observed while still in their natural environment but appears localized centrally when oocytes are isolated and placed without their companion cumulus cells in an in vitro environment. A recent study may provide an answer to this discrepancy [13]. Confocal microscopy techniques have shown that in oocytes, matured in vivo intercellular contacts – in the form of actin transzonal projections (aTZPs) originating from adjacent cumulus cells – are maintained during at least the first 6 h from hCG treatment. In coincidence with this, the GV remains eccentrically localized until GVBD. This leads to a situation in which the MI spindle is formed near the cortex and is not repositioned in preparation for the first meiotic division. A similar pattern of GV and spindle arrangement is observed in vitro under conditions that are known to provide adequate support to the process of maturation. However, when maturation in vitro is carried out without appropriate growth factor and hormone support, aTZPs appear less preserved, while the GV increases in size, is displaced from the cortex, and moved toward a more central position. As a consequence, the MI spindle is formed centrally and grows to an abnormally large size. Reduction in aTZPs abundance, GV disanchoring from the cortex, and formation of the MI spindle in a central position is also observed in oocytes treated with latrunculin and actin depolymerizing agent. Collectively, these studies illustrate the following scenario. In vivo, or in vitro under conditions compatible with the normal unfolding of the maturation process, through their cellular projections, somatic cells influence the oocyte cortical domain in a fashion that preserves the GV in an eccentric position. This involves that GVBD and MI formation occur near the cortex, preventing the necessity of spindle relocation as a prerequisite for first polar body extrusion. Vice versa in vitro, in the absence of cumulus cells or simply under conditions that interfere with oocyte-cumulus cell contacts, the GV disengages from the cortex with the consequence that spindle repositioning is required before the first meiotic division takes places. GV disanchoring from the cortex, MI formation in a central position and displacement to a more peripheral position can have significant implications for oocyte competence. For example,

the abnormally increased size observed in MI spindles formed from centrally positioned GVs is associated with the emission of large first polar bodies, with predictable detrimental effects on embryo development [6]. Therefore, the kinetics of GV localization, GVBD, and MI spindle is crucial for a correct accomplishment of oocyte maturation, fertilization, and embryo development.

The cortical domain appears to have a significant role also in the maturation of human oocytes [14]. In immature oocytes recovered after controlled ovarian stimulation, the GV may be found in an eccentric or central position. Time-lapse microscopy has shown that, during culture, GVs localized eccentrically maintain cortical anchoring and exhibit angular displacements as large as 90° before undergoing GVBD. Interestingly, loss of anchoring and centripetal migration appears to be associated with failure to undergo GVBD. In vitro, immature oocytes displaying a central GV can also undergo GVBD but only after GV repositioning to the cortex. Thus, GVBD seems to occur when cortical anchoring is maintained or established.

Endoplasmic Reticulum and Inositol 1,4,5-Trisphosphate Receptors

As described elsewhere in this volume, in the mature oocyte, cytosolic oscillations in the concentration of free Ca^{2+} are fundamental to orchestrate the events (CG release, resumption of meiosis, pronuclear formation, recruitment of maternal RNAs) that collectively characterize the fertilization process. The ability to sustain intracellular Ca^{2+} oscillations is typical of and essential for MII oocytes but is not fully established in fully grown GV-stage oocytes [15]. Studies carried out in the mouse have clarified the cellular basis of such a difference between mature and immature oocytes. Ca^{2+} is stored in the cisternae of the endoplasmic reticulum (ER). At fertilization, its release is mediated by inositol 1,4,5-trisphosphate (IP_3), which is generated from cleavage of the oolemma-bound lipid phosphatidyl-1,4-trisphosphate. Interaction of IP_3 with its receptor situated in the membranes of the ER triggers the release of Ca^{2+} in the cytoplasm [16]. This is made possible by the fact that ER development and receptor sensitivity to IP_3 increase during maturation and are maximal in the mature oocyte. In GV-stage mouse oocyte, the ER is continuous with the nuclear envelope and forms aggregate preferentially distributed in the oocyte interior [17]. At GV breakdown, ER membranes organize around bundles of microtubules and envelope the newly formed MI spindle. Interestingly, in oocyte experimentally arrested at the GV stage, this ER rearrangement does not occur, suggesting dependence from cell cycle mechanisms. ER redistribution at GVBD is also prevented by treatment with inhibitors of microtubule polymerization or cytoplasmic dynein [18]. As maturation unfolds further, the ER cisternae and vesicles develop and form aggregates of 1–2 μm occupying also the cortical cytoplasm, although in a polarized fashion involving a preferential accumulation in the vegetal hemisphere, i.e., opposite to the MII spindle position [17]. This second phase of rearrangement is also dependent on cytoskeletal elements, as suggested by the fact that it does not occur in the presence of microfilaments' depolymerizing agents [18]. In coincidence with ER growth and redistribution, the IP_3 receptors increase in number and become localized in the oocytes cortex [19], area from which they are excluded in immature oocytes. These modifications of the ER and IP_3 receptor are believed to account for the augmented sensitivity of mature oocytes to Ca^{2+}-release mechanisms that regulate fertilization. In other mammalian species, the pattern of ER rearrangement during maturation may be different, as observed in the cow [20] and hamster [21] where elements of the ER are found in a cortical position in GV-stage oocytes and more homogeneously dispersed in small clusters in mature oocytes. Human oocytes display a pattern of changes in ER distribution and IP_3 abundance similar to the one of the mouse. In oocytes found at the GV stage after controlled ovarian stimulation, the ER is in most cases excluded from the cortical region and does not appear organized in cluster [22]. Unlike the mouse, though, ER accumulation is not visible around the GV. In in vivo matured oocytes, ER elements are associated in

clusters of 2–3 μm preferentially localized in the cortex but also discernible in more internal regions. Therefore, also in the human, the ER undergoes significant changes during maturation in its distribution that reflect an enhanced ability to release Ca^{2+}. These changes can be achieved in vitro. In fact, GV-stage oocytes matured in vitro display an array of cortical and more internal clusters similar to those typical of in vivo matured oocytes. Conversely, in vitro maturation seems to affect the expression of the IP_3 receptor. In fact, in oocytes matured in vitro from GV-stage oocytes recovered from stimulated cycles, the amount of this protein is lower in comparison to oocytes matured in vivo. This may cause a reduced sensitivity of in vitro matured oocytes to Ca^{2+}-releasing mechanisms, as suggested by the observation that, following microinjection of IP_3, the rise in intracellular free Ca^{2+} in in vitro matured oocytes is comparable to the one of GV-stage, but not in vivo matured, oocytes. Caution should be exercised, though, in the interpretation of experiments of this kind, in which in vitro maturation (IVM) is carried out in the absence of cumulus cells, i.e., in rather nonphysiological conditions.

Mitochondria

All the above-described cellular rearrangements occurring during oocyte maturation are energetically demanding. Energy derives from cellular ATP as a result of mitochondrial activity. Mitochondria are themselves subject to redistribution and changes in activity during transition from the GV to the MII stage, in a fashion that can profoundly influence the quality of the mature oocyte and the destiny of the ensuing embryo. In fact, compromised oocyte quality is associated to aberrant mitochondrial rearrangement and low ATP levels [23, 24]. Recent studies in the mouse have unraveled the dynamics of these mitochondrial changes. During in vitro maturation, mitochondrial ATP undergoes three distinct phases of increase in ATP production, separated by two shorter phases in which ATP returns to basal levels [25]. These phases of augmented activity correspond to GVBD, spindle migration, and the transition between MI and MII. From previous observations, it is known that on large scale an accumulation of mitochondria surrounds the nucleus at the time of GVBD [26], accompanies the spindle during migration, and is present at the side of polar body emission. However, these rearrangements do not seem to be functional to or associated with the above-mentioned burst in ATP production, because when their formation is prevented by inhibitors of microtubule polymerization, ATP changes still occur [25]. On the contrary, formation of mitochondrial clusters on a smaller scale appears to be a prerequisite for an increase in ATP production. Formation of these small clusters is temporally coordinated with the burst of ATP observed at GVBD and the successive maturation steps. Mitochondrial clustering seems to be driven by the actin microfilaments network. This cytoskeletal array, in fact, is dissolved and reformed in coincidence with the formation of small mitochondrial clusters, and its disruption with cytochalasin B, which causes actin depolymerization, prevents the formation of mitochondrial clusters and the increase in ATP production [25]. Therefore, in the mouse, the cytoskeleton, and in particular the microfilament network, appear to direct mitochondrial redistribution on a small scale and ATP production in support of GVBD, spindle translocation, and first polar body emission. However, little is known why mitochondrial clustering is required for an increase in ATP generation. Data indicating a strict correlation between mitochondrial distribution patterns and developmental ability have been obtained with porcine oocytes. In the pig, mitochondria are restricted to the periphery of GV oocytes but migrate during maturation to the inner region of the cell both in vitro [27, 28] and in vivo [29]. Even though there is paucity of information about the correlation between this phenomenon and subsequent oocyte development, it has been suggested that relocation of mitochondria occurs in oocytes with high developmental competence [30].

The Golgi Apparatus

The Golgi is composed of stacks of membrane-bound structures known as “cisternae,” and this apparatus is specialized in modifying, sorting, and

packaging macromolecules for cell secretion or intracellular needing. Although the role and function of the Golgi is rather well established during mitosis, the dynamic of Golgi membranes during meiosis is still partially unknown. Experiments conducted in rodents have shown that in GV-stage oocytes the Golgi consists of a series of structures dispersed in the ooplasm but considerably more concentrated in the interior than at the cortex. During in vitro maturation, the large Golgi apparatus structures fragment at GVBD and disperse homogenously throughout the ooplasm, remaining in a fragmented state at the MII stage. Although protein secretion is blocked during meiotic maturation, Moreno et al. [31] have shown that brefeldin A (a drug that inhibits protein secretion by blocking membrane trafficking from the ER to the Golgi apparatus) is able to reversibly inhibit the IVM of mouse oocytes and that this block occurs at the same stage at which it is arrested by protein synthesis inhibitors, suggesting that, in addition to protein synthesis, progression of murine oocyte maturation possibly also requires functional membrane trafficking sometime after GVBD, resulting in either the modification of proteins at the Golgi level, or the delivery of these proteins to appropriate (post-Golgi) sites. In non-rodent mammalian eggs during meiotic maturation and fertilization, the Golgi apparatus behaves in a very peculiar way because the centrosome is absent until sperm entry. Experiments conducted in the bovine, which represents a more appropriate model for the human in this respect, demonstrated that the absence of a maternal centrosome prevents Golgi association with the meiotic spindle. The absence of the Golgi in the vicinity of the meiotic spindle reduces the possibility of the segregation of this apparatus in the polar bodies, and once fertilization is started, nuclear trafficking and cytokinesis do not depend from a functional Golgi [20]. In 2012, Racedo and colleagues [32] demonstrated that, in in vitro matured bovine oocytes, cytoplasmic maturation is accompanied by a series of dynamic changes of the Golgi involving an active microtubular function. Using different specific inhibitors, these authors demonstrated that prior to GVBD, the Golgi moves from the center of the cytoplasm to the cortical area. Afterward, a second translocation occurs between GVBD and MI, repositioning the Golgi from the periphery to the central cytoplasmic area.

Cortical Granules

Cortical granules (CG (cortical granules)) are membrane-bound organelles located in the sub-oolemmal cortex of the mature oocyte having a crucial role in the process of fertilization. In their mature form, CG are secretory vesicles of 0.2–0.6 μm in diameter. They are unique to the female germ cell, are not renewed after release, and are functional to the modification of the zona pellucida. Upon oocyte-sperm fusion and triggering of the intracellular Ca^{2+} signaling mechanism that coordinates the diverse activation events, they fuse with the oolemma and release their enzymatic contents in the perivitelline space. The released CG proteins modify by proteolytic cleavage one of the zona pellucida protein (ZP2) in a form (ZP2f) that is incompatible with the binding of additional sperm and penetration through the zona pellucida [33], thereby preventing polyspermic fertilization (for a more specific description of mechanisms of block of polyspermy, see also the Chap. 20 in this volume). Their sub-oolemmal localization is typical of the mature metaphase II oocyte, but in fact during oogenesis, their formation and distribution involves other cellular districts. In the diverse mammalian species, CG start to be generated at different stages of folliculogenesis. In rodents, they can be observed already in primary follicles [34], whereas in the monkey [35] and human [36], they are discernible only from the secondary follicle stage. During the growth phase of oogenesis, at early stages of folliculogenesis, the Golgi complex is situated in internal regions of the oocyte cytoplasm and subsequently proliferates, becomes hypertrophic, and finally produces small vesicles that progressively acquire a more peripheral or cortical position. These vesicles coalesce, forming mature Golgi complexes in a continuous process during oogenesis that extends to the preovulatory period [37]. During the maturation phase, initiated by the resumption of meiosis and completed by the achievement of the metaphase

II stage, Golgi complexes are subject to redistribution assisted by cytoskeletal infrastructures. In particular, the peripheral relocation of CG during maturation appears to occur with the intervention of microfilaments [38]. In mature oocytes of almost all mammals, CG are homogeneously distributed cortically just beneath the oolemma. However, in murine species, fewer – if any – granules are found in the regions adjacent the metaphase I (MI) and metaphase II (MII) spindles [39, 40]. How such a differential distribution is generated is not unequivocally established, but studies in the mouse and the hamster indicate that a perispindle CG-free area (CGFA) may result from displacement and redistribution at MI (as suggested by an increase in CG density in the area surrounding the CGFA) or precocious and targeted exocytosis occurring during the MI–MII transition [41–43]. Even less understood is the reason why a CGFA is established in these species. It is possible that limited release of CG in the cortex near the MII spindle modifies the zona pellucida or the oolemma in a way that impedes sperm–oocyte fusion in the immediate vicinity of the MII spindle, thereby assuring that decondensation of the sperm head occurs distal to the spindle so as not to affect the integrity or function of maternal chromatin as it undergoes remodeling. It is known that sperm penetration in the mouse is less likely to occur in regions of the cortex where the MII spindle is located [39].

Conclusions

During the maturation process, initiated by the resumption of meiosis and completed by the achievement of the metaphase II stage, organelles rearrange and redistribute to subserve the changing functional requirements of the oocyte. Some events are common to different species, while they are typical of specific animals such as nonrodent. Even the nuclear compartment is involved in this progression because the transport of mRNAs, from their transcription site in the nucleus to their translation sites in the cytoplasm, modulates gene expression and ensures appropriate concentrations of the encoded protein where it is needed, representing an essential step in the maturing oocyte. The coordination of these events during oocyte maturation is mediated by the cytoskeleton regulating the distribution of the different structures at the appropriate time and place to assure the completion of meiosis.

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Origins of Oocyte Aneuploidy

16

Liborio Stuppia

Abstract

The low reproductive potential of the human species is mainly caused by aneuploidies affecting embryo o fetal development. Although some of these aneuploidies may be paternally inherited or generated mitotically during preimplantation development, the vast majority of aneuploid karyotypes are generated at fertilization as an effect of meiotic errors occurring during the oocyte life cycle. Formation of an aneuploid oocyte derives from chromosome non-disjunction or premature segregation of sister chromatids at meiosis I or II. Less clear is why aneuploidy occurs. Advanced maternal age is strongly positively associated with the prevalence of aneuploidies, including Down syndrome, in spontaneously aborted fetuses and newborns. However, the links that connect maternal age and the cellular mechanisms that are involved in chromosome mal-segregation remain unknown. Factors that may play a role in the generation of aneuploidies are diverse. For example, number and position relative to the centromere of chiasmata formed in the process of recombination during fetal life influence the regularity of chromosome segregation during adult life. Alterations in the profile of the hormonal milieu are also suspected to cause oocyte aneuploidy. Environmental agents and certain lifestyles are believed to be additional factors that expose oocytes to an increased risk of chromosome mal-segregation. Better understanding of the nature and action of these factors could offer future opportunities for preventing at least part of the aneuploidies occurring in the female germ cell.

Keywords

Oocyte • Nondisjunction • Aneuploidy • Advanced maternal age

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In the human species, the reproductive rate per cycle is characterized by a relatively low success as compared to other mammals, since less than half of all natural conceptions lead to a live birth [1]. The most common genetic factor impairing human reproduction is represented by aneuploidy, that is, the presence of a modification of the normal diploid karyotype due to the gain or loss of one or more chromosomes (trisomy, tetrasomy, monosomy). About 5 % of all clinically recognized pregnancies are characterized by the presence of a trisomy or a monosomy, and it has been estimated that 0.3 % of newborns, 4 % of stillbirths, and more than 35 % of all spontaneous abortions are aneuploid in humans [2]. Thus, in the majority of cases, miscarriage represents the most likely outcome for embryos affected by such abnormalities [3]. Only a few trisomies (viz., those involving chromosome 13, 18, 21, and the sex chromosomes) and the 45, X monosomy can potentially survive to term, resulting in the birth of babies affected by congenital defects and/or mental retardation (Table 16.1). The main mechanism leading to the presence of aneuploidies in pregnancies and miscarriages is represented by chromosome segregation errors occurring during oogenesis, with the only exception of the 47, XYY condition derived from a paternal nondisjunction. In fact, between 1 and 4 % of sperms versus as many as 20 % of human oocytes have been estimated to be aneuploid [2]. In order to better understand the causes of the preferential involvement of female gametogenesis in the origin of aneuploidies, it is important to analyze the peculiarities of this process. Female meiosis consists of two divisions, meiosis I (MI) and II (MII), and of two different stages where the maturing oocyte undergoes a temporary arrest. Meiosis starts in the human fetal ovary at 11–12 weeks of gestation. During MI, oocytes enter prophase and the homologous chromosomes undergo pairing. An exchange of material between the chromatids of different homologues occurs via the formation of chiasmata in a process known as "recombination" or "crossing over." After recombination, the oocyte progresses to diplotene of prophase and enters a protracted arrest stage during which the cell cycle is arrested until ovulation, occurring several (10–50) years later. During this period, oocytes become surrounded by somatic cells (pregranulosa cells), forming primordial follicles. In the sexually mature females, in response to LH surge, the oocyte resumes meiosis; chromosomes condense and during anaphase, at the end of MI, the separations of the bivalents, which move to the opposite poles of the meiotic spindle, occur. Twenty-three chromosomes (each composed by two chromatids) enter the polar body (PB), while the remaining 23 are maintained within the oocyte. After MI, a second meiotic spindle forms immediately, the remaining chromosomes align at the spindle equator, and the cell cycle stops again until fertilization, when fusion of the sperm and egg plasma membranes triggers the resumption and completion of MII. Sister chromatids, which have been held together until this step, are separated passing one into the second PB and the other remaining in the oocyte. Thus, female meiosis takes a very long period to be completed, since this process starts during fetal life and reaches the conclusion until years later with the fertilization of an ovulated mature oocyte.

Table 16.1 Prevalence of the most common aneuploidies in newborns

Aneuploidy	Prevalence
Trisomy 21 (Down syndrome)	1:700
Trisomy 18 (Edwards syndrome)	1:7,000
Trisomy 13 (Patau syndrome)	1:20,000
47, XXY (Klinefelter syndrome)	1:900 males
47, XYY	1/1,000 males
47, XXX	1/1,200 females
45, X (Turner syndrome)	1/2,500 females

Alterations during the above-described process can lead to the presence of an aneuploidy through two different mechanisms: the first one is represented by the presence of a nondisjunction of entire chromosomes occurring during either MI or MII divisions, while the second one involves the premature division of a chromosome into its two constituent chromatids (premature separation of sister chromatids, PSSC), with a subsequent random segregation, upon completion of the first and/or second meiotic

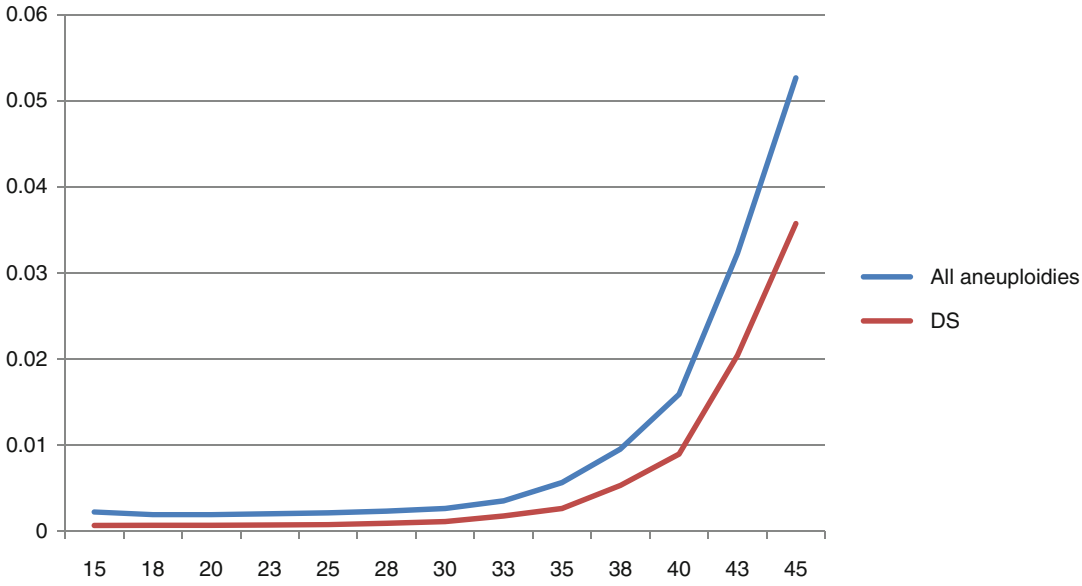

Fig. 16.1 Prevalence of all aneuploidies and DS in relation to maternal age

division [4]. Nondisjunction, and in particular maternal nondisjunction at MI, represents the main mechanisms leading to an aneuploidy. For most chromosomes, maternal MI errors are more common than maternal MII errors, with the exception of trisomy 18 which in more than 50 % of cases is caused by maternal MII nondisjunction [2, 3]. In details, about 100 % of trisomy 16 cases, 90 % of trisomy 22 cases, 75 % of trisomy 15 cases, and 65 % of trisomy 21 cases derive from an error during female meiosis I [2]. The second mechanism leading to aneuploidy, that is, PSSC [4], may be balanced or unbalanced, and in the unbalanced situation, the oocyte can either retain an extra copy or lack a copy of a single chromatid. Thus, PSSC can lead to an embryo aneuploidy in 50 % of cases, while nondisjunction produces 100 % aneuploidy embryos [5].

Advanced Maternal Age and Aneuploidies

Despite the large number of studies pointed to the identification of specific risk factors for nondisjunction, so far, the only striking evidence is represented by the association between advanced maternal age and risk of aneuploidy. In fact, a direct association between advanced maternal age and decline in reproductive fitness has been largely demonstrated, and this condition is strongly related to the relationship between advancing maternal age and increasing aneuploidy rates. Data from literature report that, while for women aging less than 25 years the expected oocyte aneuploidy rate is 5 %, an increase to 50 % is observed in the oocytes of women over the age of 40 years [3]. As a consequence, also, the prevalence of newborns affected by a chromosomal disease due the presence of an aneuploidy, in particular by Down syndrome (DS), is directly related to the advanced maternal age (Fig. 16.1). For this reason, since the 1970s, pregnant women older than 35 years of age have been offered prenatal diagnosis for fetal trisomy 21, and maternal age is evaluated in the risk calculation during the screening for DS carried out in the first or second trimester of pregnancy by using noninvasive approaches [5]. Not only the risk of nondisjunction is increased in cases of advanced maternal age. In fact, it has been reported that women aging is associated also to an increased frequency of PSSC, and it has been suggested that these mechanisms could be even more influenced by maternal aging than

nondisjunction [6]. Due to the increasing tendency of women in the last years to delay having children until their late thirties or early forties a general increase in the incidence of aneuploid syndromes is expected. In fact, an increasing prevalence of DS at birth over time has been observed in a study evidencing that from 1979 to 2003 the prevalence of this condition at birth across 10 US regions increased from 9.0 to 11.8 per 10,000 live births, representing an average increase of 0.9 % per year. This trend over time paralleled the increasing proportion of births to older mothers (aged ≥35 years) in these regions [7]. Another example of the relationship between advanced maternal age and decline in the reproductive fitness is provided by the evidence that in vitro fertilization (IVF) success rates reduce dramatically with advancing maternal age [8]. All these evidences suggest that aneuploidy of female origin will likely become an increasingly significant health issue in the coming years, due to the progressive increase in the age of women at the time of conception. However, it has been evidenced that also some women under 30 years of age have a high risk of nondisjunction and that trisomy recurrence is not only related to maternal age. In these cases, the presence of gonadal mosaicisms can explain the presence of homotrisomy (trisomy of the same chromosome), as demonstrated by studies carried out by performing cytogenetic investigation on fibroblasts and lymphocytes of parents with recurrent fetal aneuploidies [9]. As a matter of fact, germinal mosaicism is estimated to affect 6.5 % of young couples with a DS child [10]. However, germinal mosaicism may explain recurrence of homotrisomy, but not of heterotrisomy (trisomy of different chromosomes). Thus, other causes than the presence of germinal mosaicism must be identified in order to explain the presence of aneuploidies in young women.

FSH and Aneuploidies

FSH levels and FSH hyperstimulation have been hypothesized to represent possible causes of increased risk of oocyte aneuploidy [5]. It has been demonstrated in a mouse model that repeated ovarian stimulation has a progressive and significantly increased impact on spindle organization in metaphase II oocytes [11]. Moreover, studies carried out in mouse cumulus-oocyte complexes cultured in increasing concentrations of FSH have disclosed that exposure to high FSH levels accelerates nuclear maturation and induces chromosomal abnormalities [12]. In human, a baseline serum FSH increase has been observed in women with previous fetal aneuploidy obtained after natural conception or controlled ovarian hyperstimulation, suggesting that FSH levels could represent a predictor of fetal aneuploidy [13]. This is confirmed by the observation that menopause occurs at an earlier age in women with previous trisomic pregnancies compared to women with normal pregnancies, suggesting a predisposition to aneuploidy in women with premature ovarian failure (POF) [14].

Also, FSH hyperstimulation has been suggested to play a role in the risk of aneuploidies, since it has been demonstrating that aggressive hormonal stimulation produces higher aneuploidy rates in embryos derived from donor oocytes [5]. Thus, FSH appears to produce a relevant impact on oocyte aneuploidy, as confirmed also by "in vitro" investigations showing increased FSH levels in follicular fluid of oocytes with aneuploidy and PSSC [15]. This is in agreement with the fact that high-concentration FSH in IVF medium significantly increases first meiotic division error [16]. The mechanisms linking FSH levels and oocyte aneuploidy are still unknown but appear to play a role mainly during oocyte growth [5].

Synapsis and Recombination

Synapsis and recombination represent crucial factors in the determination of the risk of aneuploidies. In fact, it has been demonstrated that homologous chromosomes are at high risk of abnormal segregation either due to a failure in the recombination process or when the formation of an adequate number of chiasmata is hampered [3, 10]. Also, the position of chiasmata in relation to

the centromere (too proximal or too distal) could represent a risk factor for abnormal segregation of homologues. In this view, a detailed examination of the mechanisms underlying synapsis and recombination can provide useful information for a better understanding of the relationship between these processes and aneuploidy. The prophase events of synapsis and recombination occurring in the fetal ovary play a crucial role for the meiotic progression, as demonstrated by the evidence that chromosomal aberrations hampering the formations of synapsis cause the loss of a significant number of meiocytes in both male and female gametogenesis [17]. A further evidence is provided by studies carried out in mice carriers of targeted disruptions of genes involved in the regulation of the meiotic process. In fact, null mutants for genes regulating the formation of synaptonemal complex or involved in the recombination pathway show meiotic disruption or arrest [18–21]. The relationship between the loss of function of these genes and the disruption of gametogenesis is likely related to the presence of a checkpoint control mechanism acting at pachytene able to detect synaptic and recombination defects, as demonstrated by the transcriptional silencing of the chromosomal regions that remain unsynapsed at pachytene [22]. The timing of chromosome attachment and loss of cohesion plays a crucial role for chromosome segregation at meiosis. In fact, the cohesion between sister chromatids allows physical attachment by chiasmata of meiotic homologous chromosomes and is necessary for generating tension across centromeres when spindle microtubules have made bipolar attachment to homologues. During meiosis I, the paired homologues in the bivalents are held together and allowed to align on the meiosis-I spindle by chiasmata, which are resolved at anaphase I by the loss of cohesion between the arms of sister chromatids in the homologous chromosomes. However, cohesion is still maintained at centromeres between sister chromatids in order to prevent premature chromatid separation. The cohesion between sister chromatids is provided by the meiosis-specific cohesin complex, consisting of four core subunits, of which two (Smc1ß and Smc3) belonging to the family of structural maintenance of chromosomes (SMC), the third represented by the kleisin family protein Scc1/Rad21 (in some complex replaced by the cohesin subunit Rec8), and the last one consisting of the accessory protein Scc3, showing two isoforms [23]. Meiosis-specific cohesins, unlike their mitotic counterpart, are able to induce a sequential loss of cohesion between arms and centromeres of sister chromatids at meiosis I and meiosis II, respectively. This function is probably performed by means of the binding of cohesion complex around sister chromatids with a ring structure [24]. This structure is opened at the end of metaphase and at the onset of anaphase by a specific endopeptidase (separase) able to cleave Rec8 in meiosis, allowing chromatids to separate from each other [25]. During meiosis I, centromeric cohesion is provided by the centromeric protein shugoshin, which is also involved in mediating attachment of kinetochores of sister chromatids to only one spindle pole [26].

It has been suggested that the checkpoint control system during female meiosis, and in particular during meiosis I, could be more prone to errors, thus leading to an increased risk of nondisjunction and aneuploidies in oocytes [27]. This increased susceptibility to a loss of the control of cell cycle in oocytes could be at least in part due to the large volume of these cells and to a relative low expression of checkpoint proteins, since the time required to recruit the appropriate amount of checkpoint components to the spindle and kinetochores of meiotic chromosomes is crucial for providing the cell with a correct organization of this control mechanism [23]. This could also explain the higher meiotic error rate in female as compared with male germ cells, which are smaller and connected by cytoplasmic bridges allowing the passage of molecules from one cell to another. The above-described scenari could also explain the increased incidence of aneuploidy in aging oocytes. In fact, it has been demonstrated in animal models that aged mammalian oocytes show a deficiency of some major component of spindle assembly checkpoint which could be responsible for the increased susceptibility to aneuploidy [28]. Also, an abnormal meiotic spindle morphology, which is again typical of aged oocytes, has been related to the increased risk of aneuploidy. In this

view, it must be stressed that mutations in gene encoding for proteins involved in (1) the morphology of the meiotic spindle, (2) the motor protein activity, and (3) the regulation of the sequence of meiotic events affecting chromosome behavior and spindle integrity have been reported to cause meiotic aneuploidy [23].

The Role of Mitochondria

Another possible cause of the relationship between advanced maternal age and aneuploidy could be identified in the presence of a mitochondrial dysfunction affecting the correct spindle function. This relation could be mediated by the presence of a reduced availability of high-energy substrates and changes in mitochondrial redox potential, inducing alteration in cellular homeostasis, regulation of intracellular pH, and activity of motor proteins, which in turn could represent risk factors for meiotic nondisjunction in aged oocytes [29]. In addition, it has been suggested that the generation of oxidative stress in oocytes may contribute to the age-related increase in the rate of meiotic division errors and aneuploidies [30]. This hypothesis has been confirmed by the demonstration that antioxidants can protect meiotic divisions from alterations occurring either due to the exposure to oxidative stress or during maternal aging [31]. Studies carried out in order to verify if a mitochondrial dysfunction can affect formation of meiotic apparatus by causing instability of oocyte spindles have demonstrated that the decrease in mitochondria-derived ATP induced by oxidative stress causes a disassembly of mouse MII oocyte spindles [30]. Since maternal aging is associated with increased oxidative stress in human oocytes, these results have provided a new insight in the study of the relationship between advanced maternal age and increased risk of nondisjunction.

Environment and Lifestyle

The susceptibility of aged oocytes to undergo nondisjunction due to a disturbance in spindle function may increase in the presence of an exposure to environmental factors such as aneugenic (aneuploidy-producing) chemicals. This strongly suggests that also lifestyle could play a role in the increased risk for aneuploidies. The effect of environmental agents on the risk of nondisjunction can be observed at different levels. A first level is represented by the environmental effects on the reduction of the oocyte pool, which has been associated with oocyte aneuploidy. In fact, it has been demonstrated that environmental contaminants and lifestyle characteristics could play a role in the alteration of the oocyte pool. In fact, studies carried out in animal models have demonstrated that environmental contaminants during female fetal development could inhibit oocyte nest breakdown and primordial follicle assembly [32]. In the human, an increased risk of premature ovarian failure has been observed in hairdressers, a group of female workers who are exposed to chemicals that cause reproductive abnormalities in animal models. In fact, Caucasian hairdressers of 40–55 years of age showed a fivefold increased risk of POF as compared with nonhairdressers [33]. Smoking can be considered as another risk factor, since it has been demonstrated that cigarette smoke exposure induces in mice a significant reduction in the number of primordial follicles, but not growing or antral follicles [34]. Very recently, it has been also demonstrated that benzo[a]pyrene (B[a]P) represents an important toxic component of cigarette smoke that adversely affects follicular development and survival in mice [35]. In human, these data are confirmed by epidemiological and experimental results showing that each stage of reproductive function, including folliculogenesis, is a target for cigarette smoke components, whose effects are dose-dependent and may be influenced by the presence of other toxic substances and hormonal status [36]. Environmental agents can also increase the risk of aneuploidies by affecting the recombination process. In fact, it has been reported that exposure of pregnant female mice to bisphenol A, an estrogenic compound widely used in the production of polycarbonate plastics and epoxy resins, increases the frequency of synaptic defects and alters crossover placement, leading to a dramatic increase in oocyte aneuploidy in adult mice

exposed in utero [37, 38]. In the human, bisphenol A is associated with recurrent miscarriage [39], as evidenced by the presence of higher serum bisphenol A levels in patients with a history of three or more consecutive first-trimester miscarriages as compared to healthy women with no history of live birth and infertility.

The evidence that some environmental agents can increase the genetic risk of aneuploidies has suggested the possibility that some benefit could be gained by modifications in the lifestyle and/or treatment with specific drugs. Studies carried out using mice as an animal model have demonstrated that chronic administration of pharmacological doses of antioxidants during the juvenile period and throughout adult reproductive life can improve oocyte quality in aging females [31]. However, significant long-term negative effects on ovarian and uterine function have been observed, hampering the translation in the clinical setting of chronic antioxidant therapy. Very recently, it has been hypothesized that restricted caloric intake without malnutrition, known to be able to extend life span and attenuate severity of aging-related health complications in many species, could influence oocyte quality in female mice [40]. Based on this assumption, the effect of caloric restriction on oocyte quality has been investigated in mice, demonstrating that adult females maintained under 40 % caloric restriction did not exhibit the expected aging-related increases in oocyte aneuploidy, chromosomal misalignment on the metaphase plate, meiotic spindle abnormalities, or mitochondrial dysfunction, all representing typical markers of aged oocytes [40]. These results demonstrate that oocyte aneuploidy and spindle defects are not inevitable consequences of the aging process and that prevention strategies can be used in order to avoid the negative impact of aging on germline chromosomal segregation during meiosis.

Conclusions and Perspectives

In recent years, the tendency to delay childbirth to advanced maternal ages represents a common feature in all the industrialized societies. Due to the correlation between advanced maternal age and increased risk of aneuploidy, this condition will probably represent a major health problem in the next future, because of the consequent increase in the number of miscarriages or defects at birth and to the reduced fertility rate. As a consequence, a prompt increase in our knowledge of the major aspects of this process, such as cell cycle regulation and checkpoint controls in oogenesis, is required, in order to set up specific prevention protocols. Such preventive measures will probably imply changes in lifestyle and reduced exposure to environmental agents able to increase the risk of oocyte aneuploidy. Into the effort to obtain novel and useful information about the biological bases of oocyte aneuploidy, novel methodologies will play a key role, allowing the study of oocytes not only at the genomic but also at the transcriptomic and proteomic levels. Interesting data in this field have been provided by studies investigating the oocyte transcriptome and its association with aneuploidy. In fact, it has been shown that expression profiling obtained from first polar body removed from oocytes in which the presence of an aneuploidy had been detected by comparative genomic hybridization (CGH) was characterized by the presence of 327 genes showing statistically significant differences in transcript levels [41]. Interestingly, some of these genes encode for proteins involved in spindle assembly and chromosome alignment, confirming the association between these functions and the increased risk of aneuploidy. In addition, processes potentially affected by differentially expressed mRNA in aneuploid oocytes also include chromatin packaging and remodeling, cellular metabolism, DNA replication, and apoptosis. This suggests that chromosome malsegregation could represent only one of a series of problems affecting oocytes at risk of aneuploidy and that these oocytes are actually abnormal for several reasons. In another study, expression profiles of MII oocytes donated by younger (<36 years) or older (37–39) women undergoing controlled ovarian stimulation were compared, evidencing 342 genes showing a significantly different expression level between the two age groups. Among these, several genes

were involved in cell cycle regulation, chromosome alignment, sister chromatid separation, oxidative stress, and ubiquitination [42]. Thus, also, these results demonstrate that aging in oocytes is associated to the alteration of the activity of a number of genes playing key roles in the process of chromosome disjunction. These results strongly suggest that in the next years, novel and relevant information will be obtained about the molecular basis of aneuploidy, allowing to identify strategies able to prevent the increased rate of miscarriages and birth of children affected by malformations and/or mental retardation related to the advanced maternal age at conception.

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Part V

Oocyte Developmental Potential

Cumulus Cell Gene Expression as a Marker of Oocyte Quality

17

Mourad Assidi and Marc-André Sirard

Abstract

Cumulus cells (CCs) are a highly specialized cell type that surround the mammalian oocyte from antrum formation to the early stages of embryo development in the oviduct. During this period of close vicinity, CCs maintain paracrine and cell-to-cell communications with the oocyte. The increasing use of CCs to predict oocyte quality requires a growing understanding of their involvement in oocyte developmental competence acquisition. This chapter highlights the current knowledge about CC differentiation and communications with the oocyte. Special focus is given to the molecular biomarkers differentially expressed in CCs that reflect higher oocyte quality and therefore are associated with successful embryo development and/or implantation. The biological, signaling, and molecular functions and/or pathways of CCs during oocyte maturation, ovulation, fertilization, and early embryo development are also discussed. Using recent findings in other tissues/species, some hypotheses about the processes whereby CCs exert their functions are suggested. Further characterization will be required to refine these biomarkers in order to improve both animal and human ART.

Keywords

Cumulus • Oocyte • Gene expression • Biomarkers • Competence • IVF

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Introduction

The mammalian ovary is an organ that ensures the reproductive function, including both endocrine and germ cell production activities. This crucial function for the species' perpetuation involves many endocrine factors that regulate and synchronize the hypothalamic-pituitary-ovarian-endometrial axis and allow the ovulation of mature oocyte(s) able to achieve subsequent fertilization and embryo development. Significant progress has been made over the years to understand the ovarian function and to provide tools to control and/or improve its regulation. In this context, assisted reproductive technologies (ART) are providing potential techniques and strategies to improve the livestock genetics and productivity. In human and with the quick rise of the infertility incidence, ART are nowadays used worldwide to help infertile couples to procreate. Despite valuable ART advantages, the subsequent challenge is how to improve the embryo outcomes, both qualitatively and quantitatively. Oocyte quality has been recognized as the major limiting step and is therefore the target of numerous studies aimed at elucidating its intricate process [1–5].

Bidirectional interplay between the oocyte and its somatic neighborhood, mainly cumulus cells (CCs), appears to be indispensable to oocyte competence acquisition. Their early removal significantly affects the blastocyst rates [6]. Moreover, the properties and functions of CCs are to some extent regulated by the oocyte and reflect the degree of maturation of the oocyte [7, 8]. Therefore, CCs are thought to be a suitable target of potential markers that may reflect oocyte quality.

In this chapter, we focus on CCs and their involvement in oocyte quality in mammalian species. Using recent work about CCs both in our laboratory and elsewhere, we attempt to shed light on the CCs' contribution to oocyte quality achievement and how this somatic compartment may be a mine of several markers that reflect oocyte quality.

Oocyte Competence: Definition and Levels

What Is "Oocyte Competence"?

Oocyte competence is the ability of the oocyte to complete successful maturation, to be fertilized, and to produce a good-quality, transferable blastocyst able to give viable and healthy progeny. It has been established that maturation is the crucial step in competence acquisition [1, 2, 9, 10]. In order to assess oocyte quality at this phase, it is important to consider the oocyte maturation process at its three levels: nuclear, cytoplasmic, and molecular. An optimal maturation at these three aforementioned levels is of considerable importance to the global successful competence. These processes are concomitant and interdependent.

Levels of Oocyte Competence

Morphological Competence

Morphological parameters have been used to select good-quality oocytes in mammalian species since Leibfried and First [11]. Many studies focused on some morphological features associated with the oocyte (ooplasm transparency, diameter), the cumulus cells (number of layers, compactness), the follicle (follicle size, follicular vascularization), and/or even the early embryo (fragmentation, number of blastomeres, multinucleation) to predict developmental and implantation competence [12–17]. Positive correlations between some of these morphological indicators and oocyte developmental competence were reported in a previous study in our laboratory using the bovine model [18]. Interestingly, this study showed that early atretic COC (slight apoptosis in cumulus cells' outer layers) appeared to give the best-quality oocytes.

Nuclear Competence

Nuclear maturation refers to the progression of the oocyte nucleus from the germinal vesicle stage

(GV), and through the GV breakdown (GVBD), the association of the homologous chromosomes with the first polar body extrusion, until the second meiotic arrest at metaphase II (MII). The second meiotic resumption is induced by the male pronucleus and leads to the fulfillment of meiosis and the second polar body extrusion [19, 20]. This meiotic progression is very exceptional in terms of its duration as well as its multiple arrests. In fact, the oocyte is the only cell that undergoes the long meiotic division marked by a first locking prophase I (GV) which can last for years, a second arrest in MII after ovulation, and yields only one egg (mature oocyte) that inherits almost the whole cytoplasm of the mother oocyte by means of unequal meiotic division.

Cytoplasmic Competence

Cytoplasmic maturation includes all the ultrastructural modifications that occur in the ooplasm during prematuration (a few days before the LH surge) and final maturation of the oocyte to allow successful fertilization, cleavage, and early embryo development [1, 19, 21]. Since no direct measure of competence level is available, this maturation can be retroactively assessed through the early embryo development outcome. This is probably the reason why meiotic maturation is commonly used as a reference to cytoplasmic maturation. Conceptually, this maturation begins in the days prior to ovulation and is characterized by a decrease in transcriptional activity and changes in nucleolar morphology. However, some later criteria such as cellular organelle reorganization, perivitelline space formation, and first polar body emission might also be used to predict the progression of the cytoplasmic maturation of the oocyte [19]. At final maturation (MII), there is an important decrease in Golgi membranes and a peripheral migration of the cortical granules prior to fertilization to prevent polyspermy [22–24].

Molecular Competence

During oocyte maturation, many molecules that may contribute to oocyte fertilization and early embryo development are produced and accumulated. Molecular maturation includes all the molecular processes that occur in both the nucleus and ooplasm during oocyte maturation. Although still poorly defined, this accumulation of proteins and RNA is crucial to assess the molecular maturation status of the oocyte [10]. Due to the limits of the morphological criteria and despite similar visible properties (of oocytes and/or COC), some oocytes are more competent than others. Although invisible, these molecular events are associated with the oocyte reaching its final size [22, 25]. Among the main events involved at this level are the RNA transcription and protein synthesis that allow the oocyte to suitably support early embryo progression until embryo genome activation [26]. In fact, protein synthesis is essential for the resumption of meiosis in cattle [27]. Similar to most mammalian species, a period of mRNA synthesis is observed during maturation of the bovine oocyte at the GV stage, which decreases sharply when the oocyte reaches the GVBD stage [24, 28]. This active transcription machinery at diplotene stage (GV) is promoted by a permissive structure of chromatin to transcription factors. Despite its very small fraction compared to total RNA, oocyte mRNAs have a poly(A) tail of variable length and are bound by proteins (RNA-binding proteins), which protect them from both translation and degradation machineries in a well-established posttranscriptional regulation [29–31]. Metabolic activity within the oocyte is also a main molecular process characterized by a significant increase in pyruvate, glutamine, and lactate consumption, which seems to have a direct effect on nuclear maturation and competence development of the oocyte [32–35].

Cumulus Cell Behavior During Folliculogenesis

Origin of Cumulus Cells

In addition to its endocrine role, the ovarian follicle ensures germ cell line housing by

providing them with a suitable environment for their multiplication and storage from the prenatal life until the germ cell is ovulated. Granulosa cells (GCs) are a pseudostratified internal epithelium that undergoes deep morphological and physiological differentiation from the secondary follicle stage until ovulation and luteinization [36]. It is widely accepted that GCs are derived from the mesonephric precursor cells [37]. However, this assumption was challenged by a recent study suggesting a mesothelium origin for sheep GCs [38]. This suggests that GCs might also derive from the presumptive surface epithelium of the ovary. Since the cumulus cell line derives later from GCs, they therefore have the same embryonic origin.

CCs are also the result of the differentiation of preantral GCs into two separated antrum cell subtypes: mural granulosa cells (MGCs) localized close to the basal lamina of the ovarian follicle and CCs that surround the oocyte. The squamous pregranulosa cells of the primordial follicle become cuboidal and start to actively proliferate until the secondary follicle stage. From secondary follicle, GCs become FSH responsive in most mammal species [39]. Both oocyte and follicular growth first occur in a linear fashion and positively correlate until the oocyte is close to its maximal size. Thereafter, the growth of the ovarian follicle becomes faster with quick cell proliferation (GC mitosis) and formation of islets filled with follicular fluid (FF) within the GC intercellular space [37]. The fusion of FF pockets leads to the formation of the antrum cavity, synonym of tertiary follicle. At this stage, the cumulus-oocyte complex (COC) occupies a more peripheral position compared to the rest of the follicle, possibly to prepare for subsequent ovulation [5, 40]. The final differentiation of GCs is marked by a centripetal gradient increasing meiotic index in follicles of cyclic mouse [41]. Interestingly, this gradient is deeply affected following surgical oocytectomy [42] supporting an important role for the oocyte in the centripetal gradient of GC differentiation. While the GCs of rat preovulatory follicles contain well-developed mitochondria, smooth RE, and lipid droplets, the CCs show large rough RE with/without lipid droplets [43]. CCs therefore show several ultrastructural and molecular signs of differentiation and high specialization that might be associated with specific functions. Despite the presence of the LH receptor (LHR) transcripts in both cumulus and granulosa [44–46], immunoassays using anti-LHR in pig ovary revealed the absence of LHR protein at the surface of both cumulus cells and oocyte compared to mural GC [47].

While MGCs represent the major part of the somatic compartment, the neo-formed CCs continue to differentiate and to be influenced by oocyte-derived factors [42, 48–51]. The innermost layers of CCs, known as the corona radiata, maintain intimate contact with the zona pellucida (ZP) and give specific support to oocyte maturation. During the subsequent ovulation, most CCs and follicular fluid (FF) will accompany the oocyte to the oviduct while the MGCs and theca cells will contribute to corpus luteum formation.

It has long been established that somatic cells are essential to properly support oocyte maturation, meiosis resumption, and competence acquisition. More persuasive data obtained during the last few years documented crucial roles for the oocyte in folliculogenesis by promoting follicular cell proliferation and differentiation. The TGF-β family was suggested as one of the main players triggering these effects [52, 53]. In fact, the TGF-β family proteins (mainly GDF9 and BMP15) are important oocyte-derived paracrine factors that have been shown to drive the final differentiation of CCs prior to ovulation. These factors were reported to allow CCs to acquire the ability to expand and to express key genes needed for final maturation and ovulation [54]. While the main distinctive morphological change of CCs is mucification, transcriptomic studies showed that MGCs and CCs differentially respond to FSH by the expression of different gene sets [55–57]. Recently, significant differences in gene expression patterns between the two somatic cell types were demonstrated in women undergoing IVF or ICSI [58]. These major functional differences between MGCs and CCs are not only due to the oocyte effect but also to intrinsic molecular pathways that allow differential gene expression and

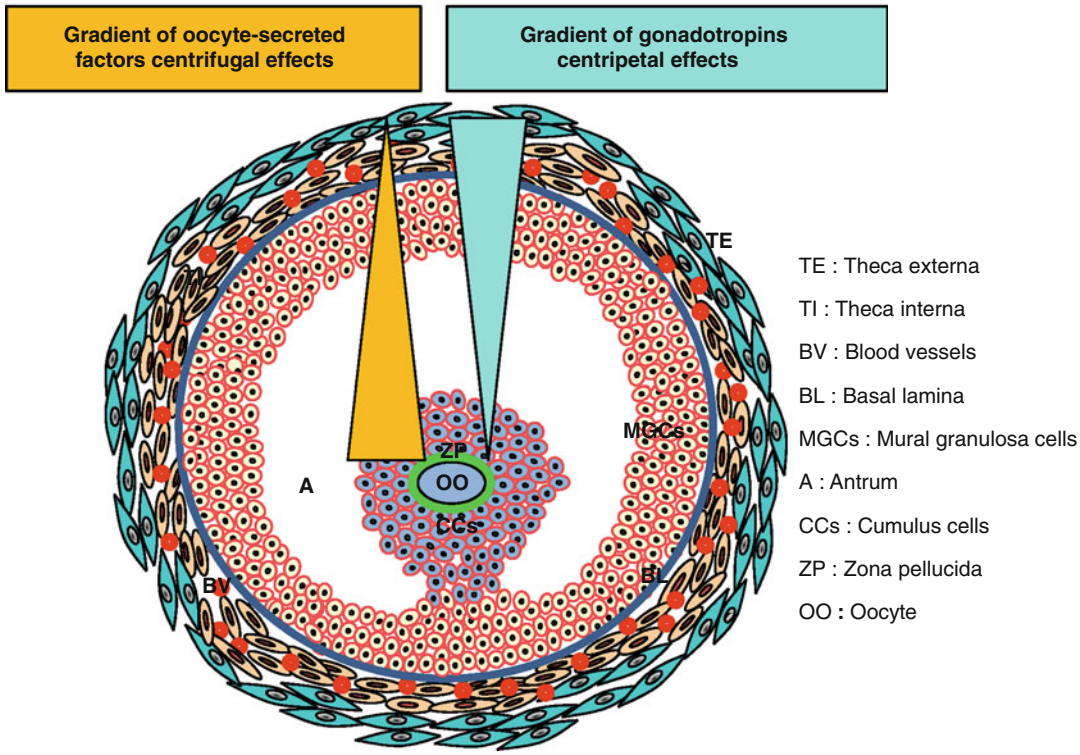

Fig. 17.1 Schematic illustration of the two main gradients that drive CC differentiation and their reciprocal communications with the oocyte: the gonadotropins centripetal signal versus the oocyte-derived paracrine factors

therefore distinctive functions between the two somatic compartments [59]. These findings were also confirmed by a comparative proteomic approach between CCs and GCs using two-dimensional PAGE (2D-PAGE). GCs cocultured with the oocyte showed a reduction of both synthesized proteins patterns and developmental competence compared to CCs matured in COCs in vitro. Authors suggested that CCs would be more helpful to oocyte competence acquisition than GCs [60].

Looking at the effect of both gonadotropins and oocyte-derived paracrine factors on follicular cells, a gradient was observed given the unique characteristics of CCs such as repression of LH-R [61–63] and the ability of progesterone secretion [64–67]. Two different gradients could be described. Based on the sphere-like structure of the mammalian ovarian follicle, centrifugal effects would refer to the influence of oocyte-derived factors, and centripetal effects would describe the gonadotropins actions. The gonadotropins effect predominates in theca and MGCs and decreases progressively closer to the oocyte. However, the oocyte-derived paracrine factors are more efficient in CCs and seem to have less influence thereafter [68] (Fig. 17.1). These two opposite gradients are key regulators of follicular cell differentiation and specialization mainly observed at the preovulatory stage. Further exploration of the differential gene expression between GCs and CCs should reveal additional functional differences.

Cumulus Cell Phenotype as an Indicator of Follicular Stage

Cumulus cells are an important parameter to determine the follicular stage. Their absence is associated with a preantral stage. Antral or tertiary follicles

are marked by the formation of a follicular fluid-filled antrum and the differentiation of granulosa cells into MGCs and CCs. These events are mainly induced by FSH stimulation [22, 69]. At the antral stage, the zona pellucida (ZP), which is a filamentous glycoprotein-based matrix secreted around the oocyte, reaches its final differentiation, and the CCs cover the COC [70, 71]. Just prior to ovulation and following the LH surge, CCs respond by volumetric enlargement of the extracellular space (CC expansion), which is required for the oocyte's final maturation, ovulation, and fertilization [7, 72]. In vivo, this mucification process is distinctive of the follicular preovulatory stage and oocyte meiosis resumption [73, 74].

Cumulus Cell Gene Expression

Importance of Cumulus Cell Gene Expression in Oocyte Developmental Competence

Given that the morphological appearance of the oocyte and embryo does not accurately predict oocyte quality and therefore the health of the embryo [75], studies of CC gene expression both in vivo and in vitro could potentially lead to the elucidation of signaling pathways involved in the intricate cross talk between the oocyte and its cumulus, thus allowing a better understanding of some disturbances in oocyte maturation associated with reduced fertility. Since we believe that oocyte competence is a manifestation of the molecular memory of both the oocyte and its surrounding CCs [76], it makes sense to focus on gene transcription in CCs when investigating oocyte developmental competence. In fact, CC support to their oocyte maturation during the antral stage was shown to require both oocyte factors (such as GDF9 and BMP-15) [49, 77] and gonadotropins (FSH and/or LH) [9, 78, 79]. Additionally, gonadotropin signal transduction occurs in the somatic compartment and requires *de novo* mRNA synthesis within cumulus cells [80]. Therefore, the investigation of CC gene expression and the related intrafollicular signaling were explored in several mammalian species including mouse [81], pig [82], cow [1, 83], and human [84–88]. These findings confirm the assumption that CC gene expression is an important process reported to influence oocyte quality [87, 89, 90] and are in line with previous studies showing that inhibition of transcription in the cumulus-oocyte complex (COC) impaired both oocyte maturation and fertilization [91, 92]. Therefore, CC gene expression might be considered a prerequisite to oocyte maturation and early development. Moreover, gene expression patterns in CCs were shown to be influenced by oocyte-secreted paracrine factors, mainly from the TGF family [8, 52, 93, 94]. Additionally, CC-oocyte communication is believed to play important roles in supporting oocyte maturation [95]. Removal of the cumulus cells before IVM or blockade of the gap junction was associated with inhibition of oocyte maturation [96] as well as a significant reduction of oocyte competence expressed in terms of blastocyst rate in the bovine species [97].

Taken together, these findings support a key role for cumulus gene expression and their communication with the oocyte in the competence acquisition process. Consequently, it is expected that CCs of competent oocytes have a specific gene expression pattern that reflect successful maturation and subsequent embryo development [77]. These findings also highlight the importance of cumulus cell gene expression as a crucial factor of oocyte quality.

Main Molecular and/or Biological Functions Regulated by Over-Expressed Genes in CCs

In order to improve our understanding of the main molecular and/or biological roles induced by genes expressed in CCs at the preovulatory stages, a summary table is provided (Table 17.1). These gene lists were combined based on relevant studies in three main mammalian species: human, cattle, and mouse. This summary is useful to highlight the main potential roles driven by CCs at this stage and to shed light on the molecular involvement of CCs in the oocyte developmental competence process.

Cumulus Cell Gene Expression and Associated Functions

The CC gene expression profiles were studied in many mammalian species including mouse [100],

Table 17.1 A compilation of some overexpressed genes at the preovulatory stage in human, bovine, and/or murine CCs according to their biological and/or molecular functions

Biological/molecular functions	List of overexpressed genes(only official gene symbols are indicated)
Gene expression and cell differentiation	BAMBI, PGR, AP1, HIST1H4C, RPL9, THOC2, NRIP1, NFIB
Cell growth and survival	FGF2, SERPIN2, UTMP, CTSZ, CTSL, BARD1, RBL2, RBBP7, BUB3, and BUB1B
Lipid metabolism	SCD1, SCD2, and SCD5
ECM formation and stabilization	EREG, THBS1, TNFAIP6, HAS2,PLOD2, PTX3, and CD9
Immune and inflammatory-like factors	CD58, IL1, FTH1, THBS1, DNAJB6, IFNA, TGFB, and TNF ALCAM, PDCD1, CD34, CD52, CXCR4, ADAMTS1, ADAMTS4, and RUNX1
Steroidogenesis	HSD3B2, INHBA, PGR, HPGD, DHCR24, PTGS2, EP2, and STAR
Intercellular signaling and cell cycle	TNFAIP6, EREG, AREG, BTC, INHBA, PGR, GJA1, UBQLN1, PKN2, CALU, CALM1, GREM1, WNT4, CSPG2, LHCGR, BMPR2, and PCK1
Neuronal-like factors and vesicle trafficking	SNAP25, THBS1, FGF2, MYO1D, SYNPO, NRP1, CHGB, SYT11, HSPD1, MBP, TNC, and NTS
Apoptosis and catabolic process	UBQLN1, CASP9, TOM1, DPP8, and BCL2L11

References [40, 84, 87, 88, 98–102]

pig [82], cow [103–105], and human [87, 88, 99, 106]. The analysis of these genome-wide studies (conducted in different contexts, e.g., in vivo vs in vitro and at different time points, e.g., GV, GVBD, or the ovulatory stage) has provided valuable insight on the molecular functions occurring in CCs. It can clearly be concluded that CC gene expression patterns may vary from one follicular stage to another or between mammalian species. This may affect most of the subsequent CC signaling and metabolic cascades and consequently their functions. In addition to highlighting species similarities and differences in terms of follicular development and oocyte molecular maturation pathways, animal models are also a valuable way to address the human reproduction challenges where availability of tissues at different time points is highly restricted [73, 84, 102, 104]. Since CCs contribute to successful embryonic development through a meticulous and time-specific expression program of appropriate genes, identification of these differentially expressed genes, analysis of their pattern of expression, and associated molecular functions are powerful tools to gain information about relevant processes associated with oocyte competence. In this chapter, we will focus on CC transcriptomics using a functional genomic approach. Therefore, the gene patterns expressed in CCs will be analyzed and categorized according to their molecular and/or physiological processes.

Cumulus Cells Mucification

Before the onset of the gonadotropin preovulatory surge, CCs of a preovulatory follicle form a compact mass that enclose the oocyte and maintain cellcell communication both between each other and with the oocyte. The process of cumulus mucification, also called expansion, describes the dramatic morpho-structural changes induced in vivo by the LH surge that lead to the synthesis and the deposit of a muco-elastic matrix in the intercellular space between cumulus cells. This extracellular matrix (ECM) is formed following intensive secretion of an enriched network of glycoproteins, proteoglycans, and hyaluronic acid (HA). The expansion of this cell mass in the immediate vicinity of the oocyte is a crucial step that lies between the LH surge and ovulation. In vivo, CC expansion is induced by LH or by HCG in PMSG-primed animals [107, 108]. It is a

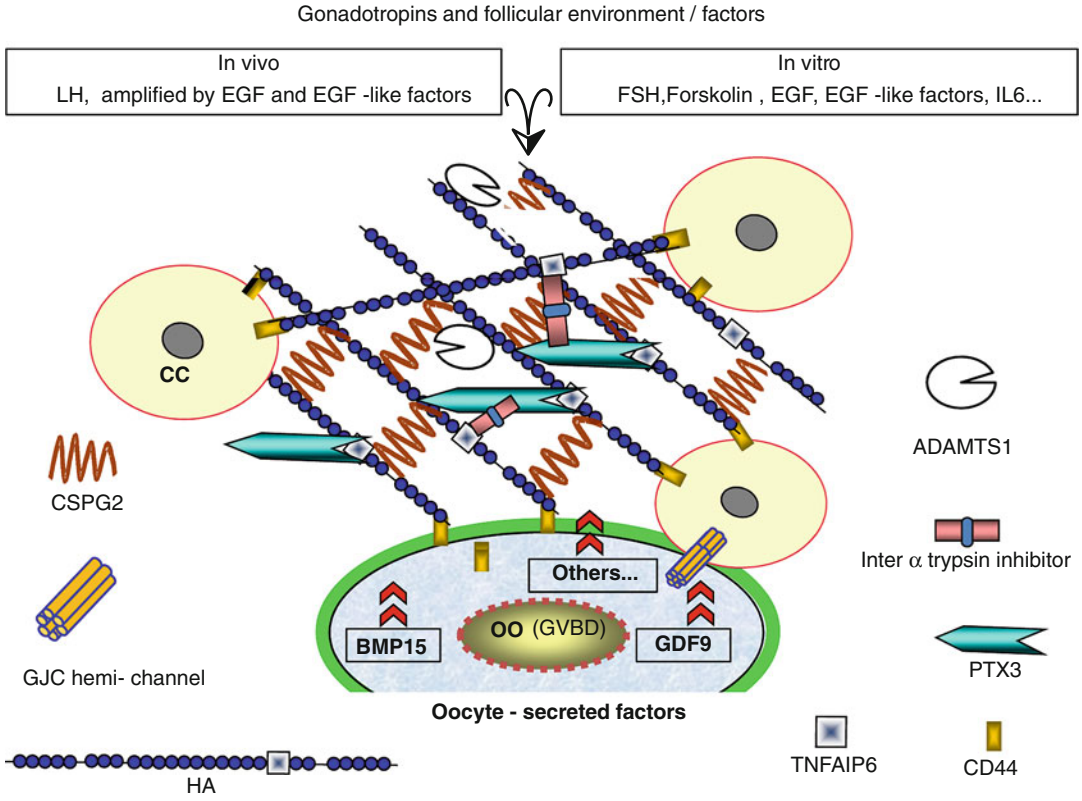

Fig. 17.2 Schematic representation of the main factors involved in ECM structure following CC expansion in vivo and in vitro

crucial step required for oocyte maturation and ovulation [7, 72]. However, CC expansion may be induced by many factors in vitro such as FSH, members of the EGF superfamily (EGF, AR, BTC, and EREG), IL6, and the adenylyl cyclase activator forskolin [79, 109–113].

After exposing preovulatory follicles to the LH surge, cumulus mucification starts with rapid expression of HAS2 and intensive synthesis and secretion of HA-rich matrix leading to CCs distancing. FSH stimulation of mouse COC in vitro increased HA biosynthesis around 20-fold within 3–12h [114]. The serum-derived factor inter-α-trypsin inhibitor (IαI) is another component of the ECM that was reported to form protein complexes and link them covalently to HA. It has an HA retention capacity, which is considered an essential step in ECM stabilization [115]. HA, which represents the backbone of this matrix, binds with high affinity the TNFAIP6 module domain [116]. Similar domains that recognize HA were also reported in other extracellular matrix proteins, mainly versican (CSPG2) and CD44. HA interaction with its ubiquitous receptor CD44 is involved in mediating a wide range of biological functions, notably its anchoring to the surface of cells, and therefore the whole ECM. This interaction is modulated by TNFAIP6 [117, 118]. In addition to IαI, matrix stabilization is also promoted by an immune cell-related gene, pentraxin 3 (PTX3). This multifunctional protein plays an essential role in ECM assembly by possible covalent link with TNFAIP6 [119]. CC mucification is also under the oocyte governance through oocyte-secreted factors, mainly GDF9 and BMP15 [40, 50, 120]. In fact, these TGFβ factors are crucial for successful ovulation by triggering the expression of hyaluronan synthase 2 (HAS2), pentraxin 3 (PTX3), and tumor necrosis factor-induced factor 6 (TNFAIP6) required for the formation and stabilization of the COC matrix (Fig. 17.2) [121]. Laminin, type IV

collagen, and their actin-linked membrane receptors (integrin α-6 and β-1) are also overexpressed during cumulus mucification and are involved in ECM structure [122]. Other proteases are also present, such as matrix-associated factor ADAMTS1 (a disintegrin and metalloproteinase with thrombospondin-like repeats 1). ADAMTS1 binds to HA and can specifically cleave versican (CSPG2) [123]. Cumulus expansion seems to be achieved and amplified through the EGF-like factors. Phenotypes of impaired ECM structure and ovulation failure were reported in each one of the three TNFAIP6-, PTX3-, and ADAMTS1-null female mice ([40, 124, 125] for reviews).

Using microinjection of fluorescent dyes and immunocytochemistry, Sutovsky and collaborators investigated cytoskeletal component distribution during bovine CC mucification. They reported that despite expansion, CCs remain joined via the same number of gap junctions (GJC) during the entire culture time. They also observed that there is remaining GJC between CCs and the oocyte even after 24h in culture [126, 127]. This decrease in GJC during in vitro culture was also reported elsewhere [128]. Concerning the importance of these cellcell junctions, previous reports in our laboratory have highlighted the importance of this functional coupling for bovine oocyte developmental competence acquisition during IVM [6]. Moreover, cumulus expansion is preceded by particular dynamics of the cytoskeleton concomitant with extension and elongation of newly formed cytoplasmic projections with CCs. Therefore, CC contacts between each other and with the oocyte are maintained despite distension. Incubation with cytochalasin B, a microfilament disruptor, inhibits these CC cytoplasmic extensions as well as subsequent cumulus expansion. Actin filament (F-actin) is therefore a mediator of the gonadotropin-induced CC mucification [122].

Cell to Cell Communication

Several genes involved in cellcell communication are expressed in CCs and contribute to the cumulus-oocyte dialog. Many types of junctions and communication gateways are found between the plasma membranes of CCs and oocyte, mainly the gap junctions (GJC). GJC are recognized as specialized junctions involved in small cellcell molecules exchange. They are found at all the follicular stages in mammals [129]. They are believed to be involved in the synchronization of the cytoplasmic and nuclear maturation of the oocyte [96, 130]. The main connexins reported in the follicle are Cx43 (GJA1), Cx37 (GJA4), and Cx32 (GJB1) [126, 131, 132]. GJA4-null mice are unable to overcome the preantral stage [131]. Consequently, these animals are anovulatory and infertile. Moreover, the oocyte is even unable to reach meiotic competency [130]. Follicular development using an organ culture technique of GJA1-null mice also blocks at the primary follicle stage [132]. Gonadotropins are directly involved in the expression and regulation of GJC in both CCs and oocyte. In fact, GJC are overexpressed and increase in number and size in the rat preovulatory stages following FSH and/or estrogen actions [133]. Inhibition of GJC in bovine COCs during IVM decreases oocyte developmental competence after IVF [6]. This intercellular coupling seems to be involved in transmission of the FSH signal transduction from the somatic compartment to the oocyte. Moreover, the GJA1 connexins are overexpressed in the cattle oocyte following the LH surge [126]. LH also induces the phosphorylation of GJB1 in the rat ovary which probably promotes the specific closure of these junctions [134]. In contrast, FSH induces GJB1 overexpression in rat granulosa cell lines [135]. Intact CC communication (i.e., functional GJC) is also reported to be crucial in the improvement of oocyte competence in vitro. Coculture of CCs with denuded bovine oocytes did not restore their developmental potential [136].

Cumulus Cell Metabolism

As an interface between the oocyte and its environment, CCs are the site of well-orchestrated sequences that ensure achievement of oocyte competence. The main visible transformation of CCs is the secretion and the arrangement of the

ECM. This process of mucification is the result of many coordinated cascades of gene expression, protein synthesis, and macromolecule deposition in the intercellular space [5, 84, 124, 137]. These CC activities are influenced by the oocyte and are required for its maturation and competence acquisition [52]. Several already established metabolic processes take place in CCs. In this context, glycolysis is a main metabolic CC function that has been shown to be promoted by the oocyte [93]. Six key enzymes involved in the glycolysis pathway, including enolase (Eno1), pyruvate kinase (Pkm2), and lactate dehydrogenase (Ldh1), were reported to be differentially expressed in mice CCs compared to MGCs. This glycolytic pathway in CCs is deeply affected by oocyte removal [138]. Moreover, CC glycolysis was reported to be a crucial nutritional need for the oocyte, known to metabolize glucose poorly. The CCs process glucose to pyruvate (both in vivo and in vitro), which provides energetic support for the oocyte during its growth and meiosis resumption in mice, pigs, and cows [139–143].

Amino acids are also required for the oocyte to pursue its maturation. In fact, the oocyte is unable to use some amino acids even when available in the culture media. This is probably due to poor transport capacity through its membrane [138, 144]. Additionally, specific amino acid transporters are differentially overexpressed in CCs of antral follicles [145]. These specific transporters allow the uptake of selected amino acids such as L-alanine and L-histidine by CCs and their subsequent transfer to the oocyte through GJC [144, 146].

Cholesterol production is another metabolic process that has been reported in CCs. It is reduced in denuded oocytes due to limitation in cholesterol biosynthesis [94]. Assuming reduced cholesterol concentrations in FF [147], CCs may overexpress key enzymes involved in cholesterol biosynthesis and transfer them to the oocyte through the raft structures. This metabolic activity is also influenced by the oocyte mainly through the FGF factors BMP15 and GDF9 [94]. The oocyte cholesterol stored in the rafts was recently shown to be essential not only in maturation but during the early embryo development stages [148].

Steroidogenesis is another metabolic function ensured by CCs. Steroid hormones, mainly progesterone and estradiol, were reported to be produced in vitro by bovine CCs [149]. These steroids are beneficial to porcine and bovine oocyte maturation in vitro [150, 151] as well as human oocyte competence [152, 153]. Moreover, the oocyte looks to prevent CC luteinization, therefore promoting their steroid production [56, 77, 154, 155]. Interestingly, the association between successful oocyte maturation and the overexpression of many steroidogenesis-related genes in their CCs reported in microarray studies [82, 156] are a further confirmation of the importance of this metabolic process.

Cell Signaling and Signal Transduction

In order to support oocyte maturation and fertilization, the CCs are the site of action of several signaling pathways. PKA was the first kinase identified downstream of the gonadotropins stimulation in mammalian CCs. It is involved in ECM formation and stabilization as well as oocyte meiotic maturation [79, 157–159]. It is a linear pathway (GPCR [G-protein-coupled receptor]/AC/cAMP/PKA) which induces the phosphorylation of some key factors such as p38MAPK, ERK1/2, and CREB (cAMP-regulatory element binding protein) in follicular somatic cells [160, 161]. Additionally, the catalytic subunits of PKA could also translocate to the CC nucleus and activate some transcription factors leading to the expression of several key genes including HAS2, TNFAIP6, CYP19A1, and EGF-like factors [98, 104, 111, 125, 157, 161–164]. Cyclic AMP (cAMP) has an established role in oocyte meiosis resumption (reviewed in [164, 165]). It can enter from CCs to the oocyte via gap junctions and block spontaneous meiosis resumption [166]. Phosphodiesterase 3A (PDE3A) inhibition in the oocyte was also reported to support the high endogenous levels of cAMP [167]. Following the gonadotropin surge, meiosis resumption is associated with a decrease in cAMP due to the disruption of gap junctions [128, 168]. The induction (end of inhibition) of phosphodiesterase (PDE3A in oocyte

and/or PDE4B, PDE8 in CCs) activity [164, 165, 167, 169, 170] and/or blockade of cGMP entry [171, 172] are also involved in this process.

Gonadotropins are also known to act in a PKA-independent manner to phosphorylate PKB/Akt via the phosphatidylinositol-dependent 3-kinase (PI3K) in rat granulosa [173]. Phosphorylation of PKB/Akt in mouse CCs promoted the oocyte developmental potential in vitro [174]. This PKB action in the oocyte was shown to occur via cyclin-dependent kinase 1 (CDK1) and PDE activation and/or PKA (via the dephosphorylation of CDK1 by the CDC25 phosphatase) which leads to maturation promoting factor (MPF – heterodimer of CDK1 and cyclin B1) activation (reviewed in [164, 175]). Moreover, gonadotropin stimulation of the PI3K/PKB pathway was shown to prevent apoptosis and induce progesterone production in porcine CCs [44, 176].

PKC and MAPK pathways are also involved in the CC signaling pathways of oocyte maturation in many species [168, 177–179]. The addition of phorbol 12-myristate13-acetate (PMA), a PKC activator, to the culture media activates MAPK in CCs [177], induces gene expression cascades (including the EGF-like factors), and enhances oocyte competence in vitro. The inhibition of this pathway reverses these effects [79, 104, 177]. The PKC pathway PLC/PIP2/DAG/PKC is suggested to act in CCs upstream of the MAPK cascades which are necessary for gonadotropin-induced meiotic resumption before the GVBD and are involved in microtubule organization and meiotic spindle assembly thereafter [32, 177, 180]. EGF-like factors drive PKC induction of oocyte maturation (mainly through MAPK) in mice [181] and pigs [182, 183] (reviewed in [184]). MAPK induction of meiotic resumption in oocyte is believed to act through MOS/MEK/ERK1/2 in mammalian species [185].

Other oocyte-secreted factors, mainly the TGFβ family, are crucial in CC differentiation, support of oocyte maturation, and CC expansion [49, 50, 186, 187]. These TGFβ effects on CCs occur mainly but not exclusively through the Smad 2/3 pathway [59, 125, 188, 189].

Keeping in mind all the aforementioned signaling pathways, it clearly appears that CCs respond to gonadotropin, oocyte, and other intrafollicular factor (e.g., EGF-like factors) stimulation by expressing key genes and activating several signaling pathways. Despite their complexity, these signaling cascades look to act harmoniously in order to support suitable oocyte maturation and subsequent fertilization.

Contribution to the Ovulation Process

Ovulation is a complex mechanism that allows COC ejection to the fallopian tube following the rupture of the follicle and the ovarian epithelium. It is a crucial step in the reproductive function that ensures two key roles: release of the oocyte and luteinization of the remaining parts of the follicle. While the former is essential to fertilization, the latter is critical for pregnancy maintenance [5]. Following the LH surge, final and rapid changes occur including FF volume increase, CC mucification, and ECM water attraction. Following ECM expansion, the COC acquires viscoelastic properties that facilitate its release through follicle rupture [5, 74]. Impaired ECM structure or knocking out some of its structural genes such as TNFAIP6 and/or ADAMTS1 deeply affects ovulation rates and therefore fertility [190–192]. The ovulation process is triggered via many CC-expressed factors associated with an inflammatory-like response [73, 100]. These immune-related genes support the CC contribution in the ovulation and fertilization processes [193]. These findings also suggest that CCs and the ECM ensure the protection of the oocyte in an inflammatory and proapoptotic environment during ovulation [5, 193–195].

The other consequence of the LH surge is the rapid increase in progesterone. PGR induces transcription of ADAMTS1 and cathepsin L in CCs [196], which are key genes in the proteolytic events of follicle rupture. Prostaglandins (PGs) are also overexpressed in CCs and are involved in activating the proteolytic process that leads to follicular wall rupture by the activation of collagenases [100, 196]. Other cytokines and neuronal factors have been reported to be differentially expressed in human and mouse CCs and therefore

suggested as mediators of the ovulatory process [84, 106, 197]. In fact, recent gene expression analyses showed that CCs gained some immune and neuronal functions required during ovulation in many mammalian species including human [84, 99, 106], mouse [40, 198], rat [199], and bovine [104]. Overall, these findings support a significant role of CCs in ovulation.

Fertilization and Early Embryo Development

Following maturation, the expanded CCs provide a suitable coat for the oocyte that facilitates its ovulation and its transport inside the infundibulum [5]. Moreover, the ECM, and particularly HA, was shown to prevent CC apoptosis [200]. Because CCs are maintained in the vicinity of the oocyte during fertilization, the spermatozoa must pass between CCs and their ECM before binding to the ZP. Intriguingly, CCs and the ECM appear to be more permissive to spermatozoa with normal morphology, good motility, and an intact acrosome [201]. This selection may be mediated via ECM molecules such as HA [202, 203]. CCs were also reported to secrete sperm attractants that contribute to enhance fertilization rates [204]. This sperm chemotaxis may attract and concentrate the full-capacitated spermatozoa around the oocyte. This CC attractive action is reinforced by another chemotaxis exerted by the mature oocyte [204]. Progesterone was suggested as the suitable CC chemoattractant [66, 205]. These findings were supported by previous reports that showed impairment of ECM expansion or the removal of CCs led to fertilization problems in most mammalian species [124, 192, 206]. Following CC penetration, sperm binding to the ZP is achieved through the ZP3 protein. This ZP-sperm recognition induces acrosomal exocytosis and triggers the proteasome pathway [207–210]. Preventing harmful changes in the oocyte and ZP biochemical properties (e.g., zona hardening) was also considered as one of the main functions of CCs during maturation and until fertilization [7].

Successful early embryo development is the consequence of suitable achievement of all steps of maturation, ovulation, and fertilization. Consequently, CCs are important to the later achievement of the early embryo development. Interestingly, CC coculture with denuded bovine zygotes improved the cleavage, blastocyst, and hatching rates [211, 212]. It is important to mention that CCs are found in the embryo vicinity in the human oviduct at 80h following the LH surge. They maintain translational and steroidogenesis activities with first signs of luteinization both in human and bovine [213, 214] and are thought to have beneficial effects on fertilization, cleavage, implantation, and up to the first week of pregnancy [215–217]. Taken together, these findings argue for a supportive role of CCs during early embryo development.

Cumulus Cells as a Suitable Site of Expression of Oocyte Quality Biomarkers

To reach full competence, the oocyte must maintain optimal and mutual exchanges with CCs. These interactions are a prerequisite during the oocyte's journey to achieve developmental competence from maturation until pregnancy. While some studies used the immature CC morphology as an in vitro indicator of oocyte developmental potential [18, 218], others focused on its metabolic activities [138, 141]. However, and despite the important development in ART and the improvement in our understanding of the maturation events, accurate selection of good-quality oocytes is still challenging although there is evidence of improvement in pregnancy outcomes. Thus, oocyte quality is still assessed through successful fertilization and blastocyst yield. Since the morphological criteria used are subjective and lack the required precision to select highly competent oocytes, finding reliable tools that efficiently assess oocyte quality is required to predict its developmental potential. Therefore, studies of the expression of genes in CCs that are influenced by the oocyte [77] may intimately reflect its quality and strengthen the morphological criteria of its selection. In fact, successful embryonic development appears to be the result

Table 17.2 Main gene markers differentially expressed in CCs that reflect oocyte quality and subsequent developmental competence

	Biomarkers list	Species	References
Positive markers	DPP8, HIST1H4C, UBQLN1, CALM1, NRP1, and PSMD6	Human	[87]
	PGK1, RGS2, RGS3, CDC42 CYP19A1, SERPINE2, FDX1, and HSD3B1	Human	[233, 234]
	PTGS2, HAS2, and GREM1	Human	[99]
	PCK1, ADPRH, CABP4, SLAMF6, CAMTA1, CSPG2, and PRF1	Human	[88, 102, 229]
	HAS2 and GREM1	Human	[235]
	GREM1	Human	[228]
	PTX3	Human	[106]
	THBS1, EREG, UBE2N, and TNFAIP6	Bovine	[98]
Negative markers	CCND2, CXCR4, GPX3, HSPB1, DVL3, DHCR7, CTNND1, and TRIM28	Human	[219]
	TOM1	Human	[87]
	BDNF	Human	[228]
	CTSB, CTSS, CTSK, and CTSZ	Bovine	[103]
	TRIB2, ERRFI1	Bovine	[98]

of a precise and time- and site-specific gene expression program in CCs to support proper oocyte competence acquisition. Therefore, the identification of the gene markers that are expressed in CCs and prognostic of oocyte developmental potential will offer powerful tools to improve both oocyte selection efficiency and our understanding of the molecular pathways that underlie oocyte competence. Consequently, additional genomic markers could be added to the morphological criteria to accurately and noninvasively predict oocyte developmental competence. In this context, some gene candidates expressed in CCs were suggested as potential markers of oocyte competence mainly in bovine and human [87, 88, 98, 99, 219] (Table 17.2).

Investigation of the molecular pathways of competence through the identification of potential CC biomarkers of oocyte quality is a key step to demystify the complex mechanism of optimal oocyte final maturation. It should be noted that CC gene expression patterns have the advantage of being specific (for each oocyte) and noninvasive. This biomarkers-based approach is particularly crucial in human ART to prevent the incidence of multiple pregnancies and perinatal mortality as well as health problems for both the mother and the babies [220–222]. Elective single embryo transfer (eSET) has recently become a necessary approach to avoid such complications and improve the safety of the mother and the offspring [223–227].

Recent studies have identified several CC biomarkers that represent valuable, quantitative, and noninvasive prognostic tools for the assessment of oocyte developmental competence in human and livestock species [87, 101, 103, 104, 228–232]. The large-scale analysis of these differentially expressed genes associated with high-quality oocyte and successful pregnancy in different mammalian species and contexts should allow us to identify potential signaling pathways and biological processes driven by our gene predictors of pregnancy.

The analysis of the biomarkers list in Table 17.2 highlights a reduced and/or an absence of gene overlap across these studies. This may be due to the different experimental conditions such as incomplete target collection (many microarrays are incomplete), choices for PCR validation, superovulation schedules, as well as the timing of sample collection and processing. Therefore, a standardization effort (clinical trial) is required to accurately identify suitable biomarkers of high-quality oocyte and subsequent embryo development.

Conclusion

CC gene expression analysis is a strong approach that allows us to identify reliable biomarkers able to accurately and noninvasively predict oocyte developmental competence and reinforce the morphological criteria already used. According to several microarray and biomarkers research studies, good correlations were reported between molecular predictors of oocyte quality and the oocyte's developmental and/or implantation potential. We believe that the effectiveness of oocyte selection, in vitro media optimization, and suitable superovulation program depends on a profound comprehension of the molecular players and the time and space sequence of events from follicular recruitment to ovulation. Therefore, it is important to identify crucial meaningful points (time, oocyte stage, follicle stage, etc.) to launch a retrospective approach to link players together (using the cause-effect and/or inducer-target and/or dose–response relationships) and to try to progressively rebuild the molecular pathway of oocyte competence acquisition. The deep refinement of biomarkers research studies using well-designed experimental protocols should be the focus of future studies in order to yield insights into the molecular pathways of oocyte competence that still remains ambiguous. Since human tissues are not easily obtained and preclude exploratory experimentation, ovarian research programs should focus on animal models. In fact, several ethical and logistical restrictions ban repeated or prolonged examinations, invasive trial, frequent tissue collection, and dose optimization.

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Oocyte Polarity and Its Developmental Significance

18

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Abstract

Establishment of the animal-vegetal (AV) axis is one of the most important events of meiotic maturation in mammalian oocytes, as it extensively affects further embryonic development. Initially, in prophase of the first meiotic division (ProI), an oocyte is radially symmetric, with a nucleus localized in the cell centre. After resumption of meiosis, metaphase I (MetI) spindle is moved from the central position towards the cortex, marking an animal pole of the oocyte. Translocation of the meiotic spindle depends on actin cytoskeleton and leads to an extensive reorganization of the animal cortex, an event regulated by complex molecular pathways. Asymmetric localization of the oocyte chromatin is maintained in the metaphase II (Met II) stage. Migration of the spindle to the cortex ensures that both meiotic divisions occur in an asymmetric manner giving rise to small polar bodies and the big egg cell containing most of the maternal factors stored during oogenesis. Moreover, cortical reorganization caused by translocation of the oocyte chromatin prevents egg-sperm fusion in the vicinity of the animal pole and in consequence precocious mixing of maternal and paternal chromosomes that could disturb proper segregation

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of genetic material during the second meiotic division. Finally, recent research provides evidence that the AV axis formed in the oocyte may influence embryonic fate of the blastomeres, as cells containing either animal or vegetal components are differentially predisposed. We would like to present here the current stage of knowledge regarding molecular mechanism of AV axis formation in mammalian oocytes and developmental significance of this process.

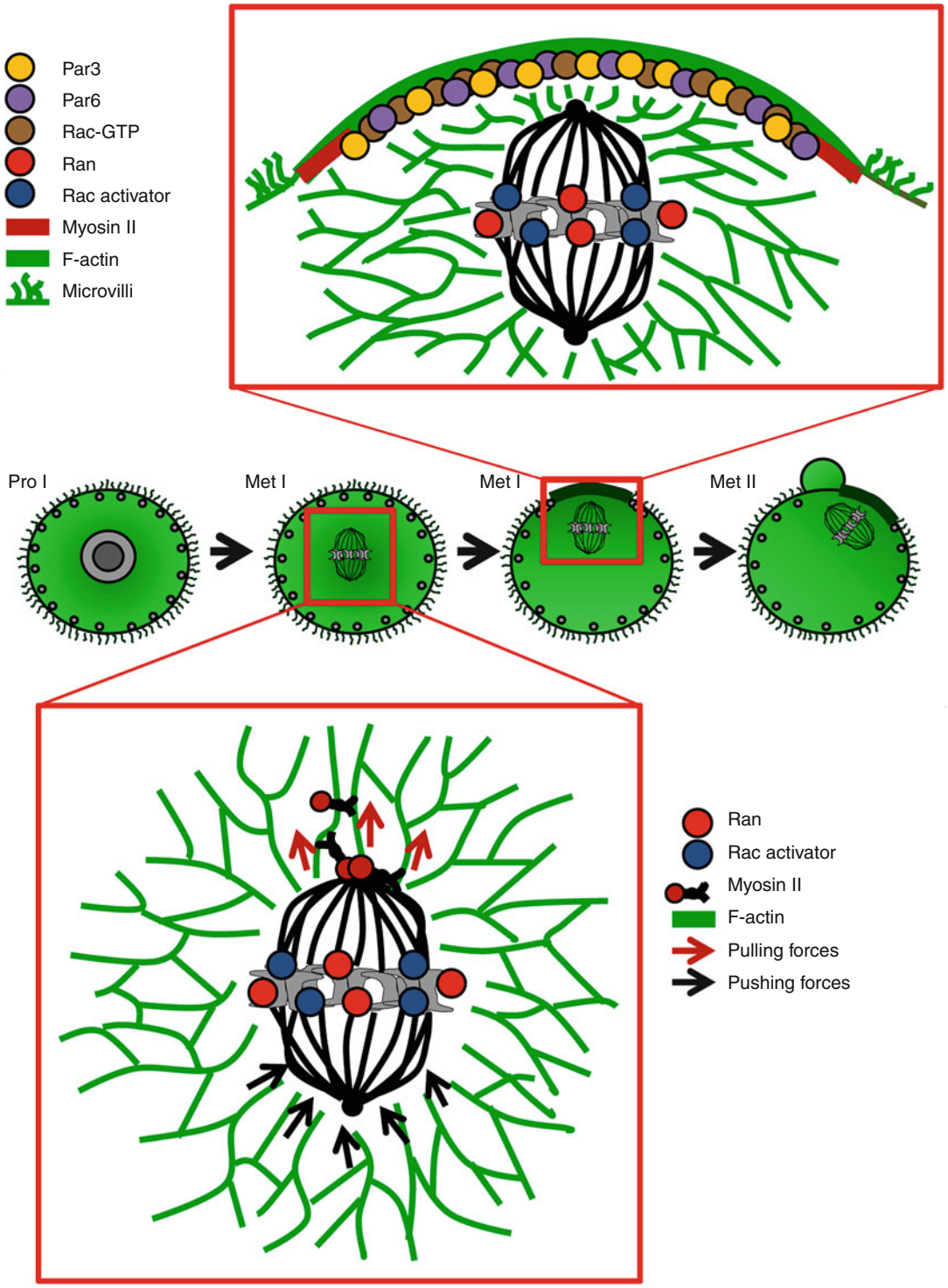
Keywords
Mammalian oocyte • Mouse • Polarity • Actin • Spindle • Microvilli Meiosis • Fertilization • Animal/vegetal axis • Developmental potential

Introduction

Establishment of cell polarity is one of the most important events occurring during meiotic maturation of mammalian oocytes. In prophase of the first meiotic division (ProI), oocyte is radially symmetric, with a nucleus (called germinal vesicle, GV) located in the center and surrounded by a ring of mitochondria and endoplasmic reticulum. Cortical granules containing enzymes, which will be subsequently engaged in the formation of the block to polyspermy after fertilization, are distributed uniformly under the oocyte cortex, and whole surface of the cell is covered with microvilli. As oocyte progresses through the first meiotic division, this configuration changes. Metaphase I (MetI) spindle is formed initially in the cell center, but then it migrates toward the cell cortex leading to the formation of the animal pole and, in consequence, the animal-vegetal (AV) axis of the egg. Area overlying the spindle becomes enriched in actin filaments and, at the same time, devoid of both cortical granules and microvilli. This spatial organization is maintained in metaphase of the second meiotic division (MetII), a stage at which oocytes become arrested awaiting for fertilization (Fig. 18.1).

Recent research has shed some light on molecular mechanism underlying the establishment of polarity in mammalian oocytes. It is now well known that migration of meiotic spindle is mediated by actin meshwork [1–4] and therefore requires function of actin regulators such as Cdc42 [5] and formin 2 [6, 7]. As the spindle migrates, the area above the spindle becomes enriched in Par proteins [5, 8–10] associated with polarity formation in other biological models, such as oocytes of *Caenorhabditis elegans*, neurons, or epithelial cells [11, 12]. Migration of the spindle to the cortex ensures that both meiotic divisions occur in an asymmetric manner, leading to the formation of small polar bodies and the big egg cell that will contain most of the maternal factors stored during oogenesis and crucial for proper development of the future embryo. It has been also shown that reorganization of actin cytoskeleton above the spindle, particularly the lack of microvilli, prevents sperm penetration in this region [13–15] and therefore precocious mixing of maternal and paternal chromosomes

Fig. 18.1 Formation of polarity in a mouse oocyte. Mouse *ProI* oocyte is radially symmetric with a nucleus located slightly off-center and cortical granules and microvilli distributed uniformly at its surface. After resumption of meiosis *MetI* spindle is formed and migrates toward the closest cortex in actin-dependent manner. This movement is mediated by two mechanisms: myosin-dependent pulling forces and myosin-independent pushing forces. Proteins transported together with the spindle, such as *Rac activator* or *Ran GTPase*, trigger reorganization of the cortical region overlying the spindle, leading to a replacement of microvilli with a thick layer of filamentous actin, accumulation of proteins involved in polarization (e.g., *Par3*, *Par6*, *Rac-GTP*, *myosin II*), and redistribution of cortical granules. Similar spatial configuration of the cortex is sustained at the *MetII* stage


Par3
Par6
Rac-GTP
Ran
Rac activator
Myosin II
F-actin
Microvilli
Pro I
Met I
Met I
Met II
Ran
Rac activator
Myosin II
F-actin
Pulling forces
Pushing forces

always migrates along its long axis toward the closest cortical region. It can be hypothesized that a shorter distance between the spindle and the cortex facilitates a more stable actin connection between these structures, which would increase the pulling myosin-dependent force exerted on the spindle from this direction [3]. Such pulling-associated movement may be supported by pushing forces acting from behind the spindle. Indeed, recent results suggest that the pushing force depends on formin 2, which tends to accumulate behind the migrating spindle rather than at its front [22] (Fig. 18.1).

Translocation of the MetI spindle induces changes in the local architecture of the cortex, namely, replacement of microvilli with a thick layer of actin filaments. Enrichment in cortical actin facilitates accumulation of a polarity protein Par3 in the area above the spindle [8]. Interestingly, Par6, a partner of Par3 in the polarity complex, localizes to the actin-rich domain in actin-independent way, as depolymerization of actin filaments with cytochalasin D does not prevent it [9]. At a molecular level, this cortical reorganization depends on Ran GTPase, and overexpression of a dominant-negative form of Ran prevents formation of the actin-rich domain above the spindle [23]. Ran is brought to the cortex on the migrating spindle and seems to be transported on chromosomes rather than the spindle microtubules, because depolymerization of the latter does not inhibit its effect on the cortical actin [23] (Fig. 18.1). Experiments with DNA-coated beads injected into an oocyte, either under the cortex or more centrally, showed that Ran acts in a distance-dependent manner: it has to be placed in the close vicinity of the cortex to exert its action [23]. There are also several reports suggesting involvement of Mos/MEK/MAP kinase pathway in reorganization of the cortex above the MetI spindle. In Mos-/-mice, spindle migration is hindered, and actin-rich domain does not form, even if chromatin is injected directly under the cortex [24–27]. Moreover, it has been shown that pharmacological inhibition of MEK also inhibits spindle migration [28, 29]. As myosin light-chain kinase, myosin 2 activator, is one of MAPK substrates, it seems plausible that Mos/MEK/MAPK pathway affects polarity formation through myosin 2. In support of this hypothesis, inhibition of myosin light-chain kinase or myosin 2 itself leads to defects similar to those observed in oocytes lacking Mos/MEK/MAPK activity (see previous paragraph) [3, 4, 21, 26].

Formation of the amicrovillar zone above the spindle coincides also with a removal of cortical granules from this region (Fig. 18.1). There are two mechanisms implicated in this process: Ca^{2+}-dependent exocytosis of cortical granules or their Ca^{2+}-independent redistribution. It has been recently proposed that formation of the cortical granule-free domain depends on redistribution of granules, not on their exocytosis [30]. Indeed, chelation of Ca^{2+} ions does not inhibit this process [30]. Moreover, an increase in cortical granule density at the periphery of the CG-free domain has been observed [31, 32]. However, CG exocytosis does occur during transition between MetI and MetII and probably contributes to enlargement of the CG-free domain observed at this time [30–33]. This removal of the CGs from the region above the spindle can facilitate formation of the cleavage furrow during meiotic divisions.

Polarity of an Oocyte Ensures Asymmetric Meiotic Divisions

From a developmental point of view, it is absolutely crucial that both meiotic divisions in an oocyte occur in an asymmetric way. Only then cytokinesis may result in a formation of two daughter cells of a drastically different size: a small polar body and a big egg cell. This difference in cell size prevents a loss of maternal material, such as proteins, mRNAs, organelles, that in case of symmetric division would be divided in two more equal portions. To ensure that meiotic divisions will be asymmetric, two conditions have to be met. Firstly, MetI spindle has to migrate to the cortex, and we described mechanism of this movement above. Secondly, MetII spindle, formed after completion of the first meiotic division, has to remain under the cortex until fertilization. Similarly to MetI stage, MetII spindle is also associated with an amicrovillar domain enriched

in cortical actin, myosin 2, and Par proteins [1, 8, 9, 21, 34], and recent research suggests that this domain participates in MetII spindle anchoring. One of the factors involved in this process is Rac GTPase, a protein distributed uniformly in whole oocyte cortex, but activated (GTP-bound) only in the cortex above the spindle. It has been proposed that this spatially restricted activation of Rac is caused by Rac activator or GTP/GDP exchange factor that is transported to the cortex on the migrating chromosomes [35] (Fig. 18.1). Inhibition of Rac leads to a displacement of MetII spindle from the cortex to the more central location [35]. It is likely that Rac acts through Arp2/3 complex, as it has been shown to be its indirect activator [36]. Indeed, Arp2/3 also plays an important role in the spindle anchoring as its inhibition leads to MetII spindle being moved away from the cortex in myosin 2-dependent way [37]. Detailed analysis of spindle and cytoplasm movements revealed that Arp2/3 activity prevented myosin-dependant cytoplasmic flow pushing the spindle away from the cortex [37].

Polarity of an Oocyte Prevents Misplaced Fertilization

Amicrovillar domain not only participates in asymmetric divisions of the oocyte but also prevents sperm-egg fusion in the immediate proximity of the spindle. Spermatozoa preferentially fuse with microvilli-enriched area, away from maternal chromosomes, minimizing chances of unwanted mingling of maternal and paternal DNA and incorrect segregation of the genetic material [13–15]. Scanning electron microscopy showed that sperm is enveloped by microvilli during sperm–egg fusion, and transmitted electron micrographs suggest that it first fuses with the microvilli rather than the planar membrane region between them [38, 39]. Depolymerization of actin filaments with cytochalasin blocks formation of the amicrovillar area [1] and leads to a uniform distribution of binding sites for fertilin alpha and beta (sperm proteins involved in sperm–egg interaction) around the oocyte surface [40]. Recent research has revealed that CD9, the only oocyte protein so far proved to be directly involved in the sperm fusion [41–43], is almost excluded from the region above the spindle and accumulates in the microvillar area [41, 44, 45]. Electron microscopy revealed that majority of CD9 localizes on the membranes of the microvilli as opposed to the membranes between them, explaining why sperm fuses preferentially with microvilli. Interestingly, it has been also shown that CD9 additionally facilitates fertilization by maintaining proper structure of microvilli. Oocytes from CD9 knockout mice have been reported to have short and thick microvilli, as opposed to thin and long microvilli of wild-type mice [44]. It seems probable that CD9 affects microvilli structure acting through EWI-2 and EWI-F, proteins that link CD9 to actin cytoskeleton [44].

Polarity of an Oocyte Provides Clues for Further Embryonic Development

In many animal species, information relating to the specification of the future body axis is asymmetrically localized in the oocyte, and in case of frog eggs, for example, it forms a gradient along the AV axis [46–48]. For a long time, mammals were thought to be an exception to this biological principle. This is because in mammalian embryos, development is regulative, which means that up to a certain point blastomeres can switch their fate in response to changing conditions. For instance, cleavage stage embryos can be aggregated, or individual blastomeres can be removed, but such manipulated embryos will still often develop into normal organisms [49–53]. Therefore, it has been believed that spatial information regarding embryonic development is not likely to be present as early as the oocyte or zygote stage. However, it has been unexpectedly observed that the AV axis of the oocyte correlates with the orientation of the bilateral symmetry axis of the mouse blastocyst [54]. In checking the physiological meaning of this correlation, experiments were carried out in which either animal or vegetal poles were removed from zygotes, and the effect of this operation on further development was examined. These experiments seemingly supported the view

that neither animal nor vegetal poles are essential for embryos to develop, as the resulting embryos were able to develop into viable pups (5 and 6 %, respectively) [55]. However, the low efficiency of embryos' survival, together with the subsequent finding that microsurgical doubling of the animal pole perturbs development and leads to numerous chromosomal abnormalities [56], suggests that there may be some maternal factors asymmetrically distributed within the embryo. Such factors would be associated with the oocyte and then zygote animal and vegetal poles and could bias (although not permanently determine) developmental fate of cells that would inherit them. In agreement with this view, further experiments revealed that the embryonic-abembryonic axis of the blastocyst is, in most cases, perpendicular to the plane of the first cleavage division of the zygote and in consequence also to the AV axis [54, 57]. Importantly, clones of cells derived from early blastomeres stay coherent up to the early blastocyst stage [58–65], suggesting that cytoplasm in different regions of the egg remains in its relative position within the "older" embryo.

A next important insight into the role of animal and vegetal poles of an egg has been provided by experiments in which the pattern of the first two embryonic divisions and the fate of the resulting blastomeres were followed in live mouse embryos. The first cleavage plane in most zygotes is meridional, which means it occurs along the AV axis [54, 56, 66, 67]. As a consequence, cytoplasm from animal and vegetal zygotic regions is equally distributed between both blastomeres in the 2-cell stage embryo. However, the divisions of the second cleavage can be orientated in two alternative ways: either meridionally (M, i.e., parallel to the AV axis), when daughter blastomeres inherit both animal and vegetal material, or equatorially (E, i.e., perpendicular to the AV axis), when animal and vegetal material is separated and inherited differentially by daughter cells [66, 68]. Therefore, after the second division, four types of embryos can be distinguished: ME, EM, MM, and EE (Fig. 18.2a), with ME and EM embryos accounting for roughly 80 % of embryo population (in 50:50 ratio) [66, 68]. Subsequent study revealed that distribution of zygotic material to the distinct blastomeres during the second cleavage division, as well as order in which these divisions occur, affects both fate of the resulting cells and their developmental potential [66]. Specifically, blastomeres of ME embryos have more predictable fates in comparison to other groups (Fig. 18.2a). In 85 % of ME embryos, embryonic part of the blastocyst is built by the progeny of the 2-cell blastomere that divided meridionally (Fig. 18.2b). In contrast, even though 79 % of EM embryos have embryonic and abembryonic regions built mainly by one of 2-cell clones, this can be either a clone originating from equatorial or meridional division (Fig. 18.2b). Hence, in this type of embryos, progeny of the blastomere that divided equatorially have equal chances to contribute to embryonic and abembryonic part of the blastocyst. The relation between division orientation and cell allocation appears random in MM and EE embryos, where specific regions of blastocyst are predominantly originating from one of the 2-cell clones in only 31 and 57 %, respectively [66].

More detailed time-lapse studies combined with 3-D analyses of behavior of all cells revealed that in ME embryos, progeny of the E-blastomere that inherited the vegetal material undertake significantly more symmetric divisions comparing to the progeny of other blastomeres [58]. This would explain why these cells were observed previously to contribute significantly more than progeny of other blastomeres to the mural trophectoderm of the blastocyst [69] (Fig. 18.2). In comparison, progeny of the E-blastomere containing animal material predominantly populates the boundary zone between inner cell mass (ICM) and trophectoderm [69]. Importantly, the pattern of segregation and inheritance of animal and vegetal material also correlates with developmental potential of the embryos. The majority of embryos in which at least two 4-cell stage blastomeres inherit both animal and vegetal material (i.e., embryos that have at least one meridional division) develop successfully to term (91 % of ME and EM embryos, 84 % of MM embryos). In comparison, only 35 % of embryos in which all 4-cell stage blastomeres have exclusively either animal or vegetal material (EE embryos) give rise to viable pups [66]. This result has been further confirmed by experiments in which developmental capability of embryos generated from 4-cell

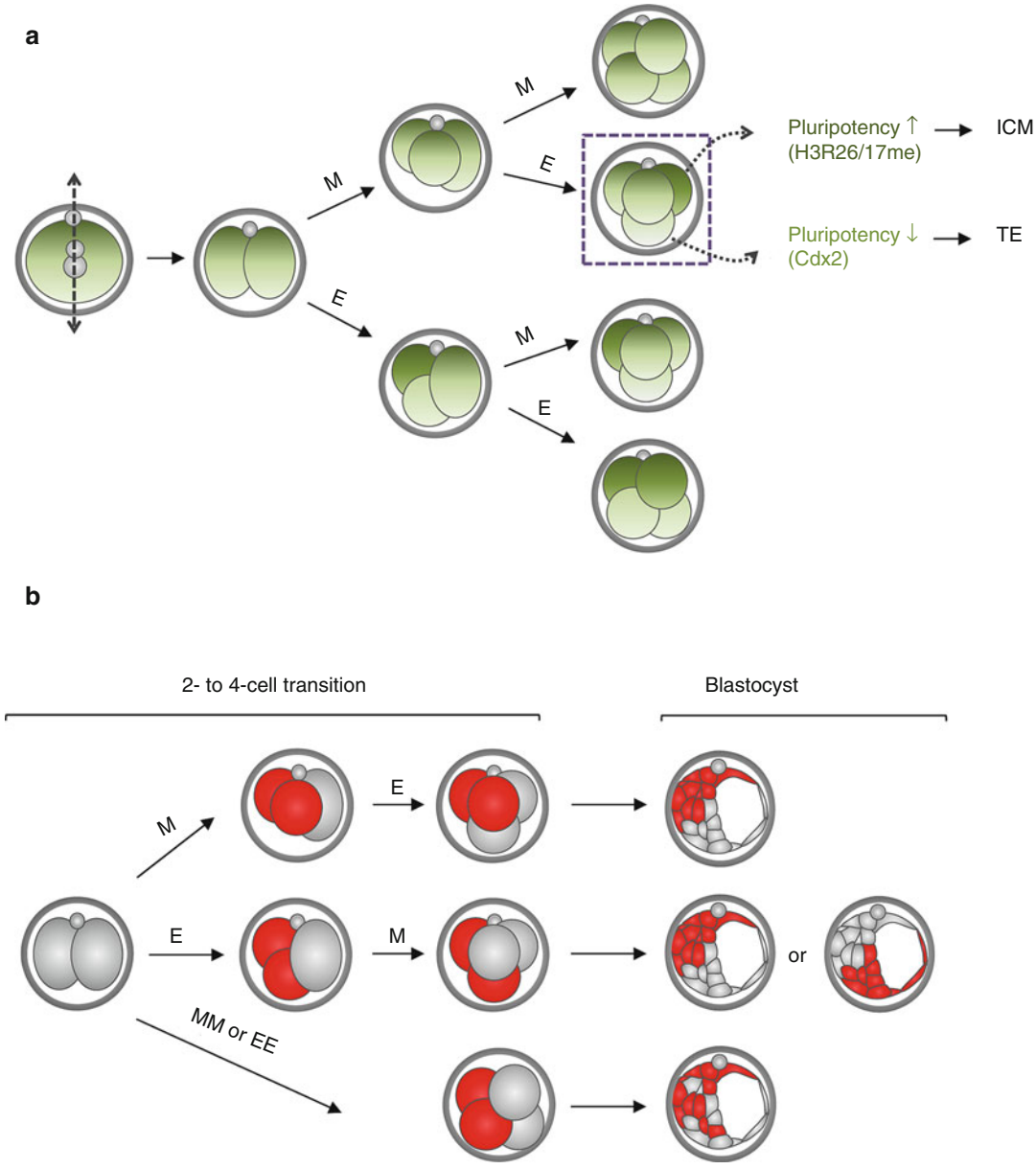


Fig. 18.2 Consequences of *AV* inheritance for early development. (**a**) Blastomeres of a 2-cell stage embryo can divide, with respect to the *AV* axis (as indicated by the position of the polar body), meridionally (*M*) or equatorially (*E*). This results in four categories of embryos at the 4-cell stage: *MM*, *ME*, *EM*, and *EE*, with tetrahedral *ME* and *EM*, constituting the most prevalent type (see text for details). Blastomeres of *ME* embryo, inheriting distinct *AV* regions, of the egg have defined fates. Pluripotent *A* blastomere (*dark green*) shows high levels of epigenetic modification associated with pluripotency (*H3R26/17me*; see text for details) and preferentially contributes to the *ICM*. *V* blastomere (*light green*), on the other hand, divides favorably symmetrically; its descendants express high levels of TE marker (*Cdx2*) and as a consequence contribute to the trophectoderm. (**b**) At the blastocyst stage, progeny of 2-cell blastomeres contributes to both pluripotent *ICM* and differentiated trophectoderm (*TE*); however, in tetrahedral *ME* and *EM* embryos, one contributes more cells to the embryonic part of the blastocyst (*ICM* and polar trophectoderm) and the other to the abembryonic part (mural trophectoderm and more superficial *ICM*). In remaining embryos (*MM* and *EE*), the allocation of blastomere progeny is random. Not only orientation but also order of the divisions matters, as when *M* division precedes the *E* division (*ME* embryos) the embryonic hemisphere is populated mainly by the first blastomere to divide (*red*). In EM embryos, the earlier to divide blastomere contributes either to the embryonic or to the abembryonic part (Adapted from [80])

stage blastomeres originating from either meridional or equatorial divisions of ME or EM embryos was examined. Embryos formed from 4 M-blastomeres developed to viable pups in 69–85 % of cases, whereas embryos consisting of 4 E-blastomeres had significantly lower developmental potential (only 30–46 % developed to term) [69]. More detailed analysis of the developmental potential of E-blastomeres of ME embryos revealed that cells resulting from equatorial divisions that inherited animal material can, when aggregated together into a chimera, develop to term in 18–27 % of cases. In contrast, chimeras obtained by aggregation of E-blastomeres containing vegetal material never gave rise to viable pups [69]. Even closer examination showed that chimeras from both types of E-blastomeres have hindered preimplantation development, with many embryos arresting at the morula stage and the rest forming blastocysts of reduced size. Moreover, when such blastocysts were transferred to pseudopregnant recipients, they often display a range of abnormalities at the egg cylinder stage (E6.5) [69].

Only over the last years these findings started to find molecular support and also explanation. First, it was found that individual 4-cell stage blastomeres differ in the extent of specific epigenetic modifications such as histone H3R26/17 methylation. Specifically, it has been discovered that in ME embryos, E-blastomere containing mainly vegetal cytoplasm shows significantly lower level of histone H3R26 and H3R17 methylation when compared to the rest of blastomeres [70] (Fig. 18.2a). The differences in H3R26 and H3R17 methylation were also observed in EM embryos, but here the correlation with specific types of blastomeres was less clear. Interestingly, when both divisions where meridional and thus all four 4-cell stage blastomeres inherited animal and vegetal components of the zygote, the differences between the cells in the extent of these two modifications were not apparent. These results suggest that inheritance of the vegetal components of the mouse zygote can lead in at least a subset of embryos to inhibition of certain epigenetic modifications. Experiments, in which Carm1 methyltransferase responsible for this specific H3 arginine methylation was overexpressed, revealed that this modification results in upregulation of expression levels of pluripotency markers Nanog and Sox2 and can predispose a progeny of the blastomere with high methylation levels to contribute to the ICM [70]. In support of these findings, recent experiments also reported that kinetics of nuclear import/export of another key pluripotency factor Oct4 differs among blastomeres of 4- and 8-cell stage embryos. Importantly, the difference in Oct4 nuclear kinetics is predictive of subsequent cell fate in the mouse embryo, so that cells with Oct4 staying long in nucleus take more asymmetric divisions and preferentially develop to ICM, whereas cells with more "mobile" nuclear Oct4 tend to divide symmetrically and differentiate into trophectoderm [71]. Although this study did not correlate the differences in Oct4 kinetics with the inheritance of animal or vegetal material, it is plausible that distinct nuclear kinetics of Oct4 originate from the AV asymmetries formed initially within the oocyte. These results are complemented by another study, revealing that 8-cell blastomeres originated from equatorial division in ME embryo are characterized by fivefold higher expression of a trophectoderm marker, Cdx2 [72] (Fig. 18.2a). Moreover, it was found that changing the level of expression of Cdx2 affects cell fate [72, 73]. This is most likely through the effect of Cdx2 on the extent of cell polarization [72, 74], which can affect division orientation and, consequently, cell position and fate [75]. Taken together these observations explains why progeny of cells inheriting mainly vegetal material have been found to undertake more symmetric divisions and preferentially contribute to the trophectoderm (see above) [58, 69]. Therefore, it seems that inheritance of specific regions of oocyte/zygote does have important consequences for the embryonic development and can bias cells toward differentiated or pluripotent fate.

The question remains what molecules asymmetrically distributed in oocytes decide about different properties of blastomeres resulting from meridional or equatorial divisions. Most of proteins that are currently known to be distributed asymmetrically in MetI and MetII oocytes,

such as filamentous actin or myosin, appear to lose their polarized localization after fertilization as embryo enters first mitotic interphase [21, 34]. It should be noted that hormone leptin, critical for multiple reproductive events, including implantation, and transcription factor STAT3 have been reported to localize preferentially in the animal pole of MetII mouse and human oocytes and later to be deferentially inherited by the blastomeres [76, 77]. Although this result has been received with skepticism [78], asymmetric distribution of leptin has been recently confirmed [79]. However, it has been also shown that localization of this hormone in oocytes and embryos may depend on hormonal stimulation (superovulation) and a strain of mice [79]. In addition, several other proteins have been reported to show asymmetric distribution in human oocytes and early embryos, including the growth factors TGFβ2 and VEGF and the apoptosis-associated proteins BCL-X and BAX [77]. Although these results are promising, thus far, there are no studies showing that any of the factors identified to have asymmetric expression in both oocyte and preimplantation embryo are functionally important for predisposing the blastomeres to a certain developmental fate.

Conclusion

Polarity formation in oocytes and its function have been thoroughly examined for the past 30 years, and our understanding of this aspect of mammalian embryonic development is definitely increasing. We know how the AV axis in oocyte is established, and that is an absolute requisite for asymmetric divisions, in consequence of retention of the majority of maternally derived material for the future embryo. We also know that lack of microvilli, and proteins associated with them such as CD9, protects the area above the MetII spindle from sperm penetration and potential precocious mingling of paternal and maternal chromosomes. On the other hand, it appears that a question of how oocyte polarity affects further embryonic development is more challenging to address. Although several studies now have clearly revealed that the AV axis of the oocyte and zygote can translate to the different developmental potential of the blastomeres, the molecular mechanism of this process remains to be discovered. An impressive improvement in sensitivity and reliability of molecular biology techniques that we have witnessed for the last few years provides hope that this developmental puzzle will be solved soon.

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The Role of the Plasma Membrane and Pericortical Cytoplasm in Early Mammalian Development

19

Jonathan Van Blerkom

Abstract

In a clinical context, each stage of mammalian oogenesis is usually considered in terms of nuclear and cytoplasmic processes that convey meiotic, fertilization and developmental competence to a preovulatory and ovulated oocyte, respectively. While the progression of changes in chromsomal configurations define specific stages of the meiotic cell cycle, which in the human can be decades long, cytoplasmic changes that lead to fertilization and developmental competence for the ovulated oocyte tend to be poorly understood. Yet, fertilization and early developmental failures are often attributed to largely undefined or putative defects in cytoplasmic development during oogenesis in general, and preovulatory maturation in particular. Failures of sperm penetration in meiotically mature oocytes, or the inability of a penetrated oocyte to support the resumption of meiosis or the normal evolution of pronuclei, are usually viewed as consequences of a global rather than focal cytoplasmic defect(s). It is likely that most clinical embryologists consider the human ooplasm to be spatially unstructured with respect to physiological, biochemical and regulatory functions.

Here, the notion that the mammalian oocyte may be functionally structured is discussed in the context of critical regulatory and physiological processes that current findings indicate may be differentially localized to the plasma membrane, subplasmalemmal and pericortical cytoplasm. Confirmation that spatial localization or compartmentalization of regulatory and developmental functions and activities exists in the in the mature

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human oocyte may have important clinical implications if a greater understanding of the fundamental cell biology of the human oocyte results in information that can identify new causes of infertility, or explain failures in treatments such as IVF.

Keywords
Oocyte competence • Oolemma • Subplasmalemmal cytoplasm Subcortical cytoplasm • Lipid raft microdomains • Mitochondria

Origins of Developmental Competence in Human Oocytes and Embryos

Introduction

Current laboratory methods commonly used to assess human oocyte and embryo competence in in vitro fertilization (IVF) programs are largely based upon static inspections of oocyte morphology prior to insemination, the timing of cell divisions, and the occurrence of stage-specific developmental landmarks during culture, with validation of sensitivity based on outcomes with transfer of fresh or thawed embryos. Although the predictive power of empirical schemes has long been controversial, developmental landmarks and performance characteristics have been shown to be generally beneficial when applied from the pronuclear through blastocyst stages [1, 2]. A further enhancement may be afforded morphokinetic documentation of performance by continuous (time-lapse) imaging of dynamic spatial and temporal characteristics of cleavage, compaction, morulation, blastocyst formation, and expansion [3]. More recently, outcome-based findings collected by different programs over many years led a consensus of experts to propose a standard scheme of competence assessment that characterizes developmental landmarks at specific times after fertilization, either by static or morphokinetic imaging [1]. This was the first realistic attempt in the human IVF field to create a simplified competence assessment scheme based upon specific and common oocyte and embryo characteristics that the collective results showed to be positive or negative for outcome. IVF programs were encouraged to adopt this model both for purposes of standardization by reducing operator- and program-related differences and biases and to allow empirical findings between programs to be compared on the same basis.

Metabolic parameters measured by Raman spectroscopy [4, 5], proton nuclear magnetic resonance [6], relative amino acid concentrations in spent culture medium [7], metabolite consumption [8, 9], and oxygen uptake [10] have been optimistically suggested to be potentially important, largely operator-independent, and objective criteria (i.e., independent of morphology or performance) of competence for oocyte and embryo selection. While the application of these microanalytical methods in clinical IVF programs often requires specialized equipment and expertise, a truly significant improvement in outcome (i.e., births) could justify the significant costs involved. This could be especially helpful where restrictions exist on the number of oocytes that are permissible for insemination or where the number of embryos that can be transferred is determined by agreement among IVF programs or by government mandates. However, as is often the case in this field, results from larger studies that are both prospective and randomized may fail to show truly significant improvements in outcome when compared to conventional morphological assessments or may require modifications of the study design to determine efficacy [11].

For metabolism in particular, a cell biological explanation of specific threshold bioenergetic values associated with competence has focused primarily on the contribution of mitochondria

to normal development in the maturing oocyte and preimplantation stage human embryo. Correlations between competence and mitochondria for the human [12] have largely focused on the following properties: bioenergetic status measured by ATP production [13, 14], mtDNA copy number [15], abnormalities in organelle structure [16] or cytoplasmic distribution [17], or apparent activity indicated by the relative potential difference across the inner mitochondrial membrane ($\Delta\Psi m$) detected in the living state with potentiometric fluorescent probes [17–19]. Specific threshold values for each parameter have been considered proximal causes of maturation and fertilization dysfunction for the oocyte and failure of the nascent embryo to progress beyond the early cleavage stages. However, with the exception of relative $\Delta\Psi m$ observations made on living cells, quantitative threshold values for ATP or mtDNA require lysis of intact oocytes or embryos. Because oocytes and embryos are obtained for procreative purposes in clinical IVF, mitochondrial and bioenergetic findings come primarily from instances of maturational, fertilization, or early embryonic failures, and for those assumed likely to be competent, their actual abilities cannot be determined if used for analysis. However, a developmentally significant relationship exists between mitochondrial bioenergetics and competence for individual blastomeres, and present evidence suggests that mitochondria in the subplasmalemmal cytoplasm of the maturing oocyte and early embryo may have important focal influences on critical developmental processes, including those localized to the plasma membrane (see below).

These notions imply levels of regulation at the cellular and cytoplasmic levels that, if perturbed, could explain certain developmental abnormalities and failures commonly observed in clinical IVF. This leads to the underlying theme of the science discussed in this chapter, namely, that developmentally significant activities localized to the plasma membrane and subplasmalemmal and subcortical cytoplasm are responsible for the establishment of developmental competence. In this context, this chapter examines (1) aspects of oocyte biology that could suggest new ways of considering how competence becomes compromised and (2) whether molecular and cellular influences on competence may be assessed for selective purposes in routine IVF practice.

In clinical practice, causes of fertilization failure after conventional IVF, abnormal development, or arrested cell division are often difficult to explain, especially when metabolic or morphokinetic assessment models are positive for outcome and no direct answers are forthcoming from laboratory findings. Thus, while success rates have improved markedly during the past three decades of clinical IVF and patients have a greater chance than previously of having a normal pregnancy, failure is still common, despite advances in how embryos are cultured and evaluated, including those shown to be chromosomally normal after preimplantation genetic diagnosis (PGD). The ability to understand failures in treatment cycles when preimplantation developmental landmarks are time and stage appropriate (up to the point development ceases) is perhaps one of the greatest challenges remaining in the clinical IVF field. To this end, here, emphasis is placed on functional and organizational aspect of the mature oocyte that can impact embryo developmental potential.

Known Influences on Human Oocyte and Embryo Developmental Competence

The relative contribution of the developmental biology of human gametes and early embryos to outcome is one significant element generally absent from discussions between clinician and patients, either prior to the start of an IVF cycle or afterward if failure occurs. This is not intended to be a criticism of either clinical or laboratory personnel, who are generally skilled and competent, but rather of how little we know of what is involved at the molecular and cellular levels in producing a competent female gamete that results in a developmentally viable embryo. With respect to fertilizability and outcome after transfer, mature oocytes and preimplantation stage embryos from the same cohort are frequently

developmentally heterogeneous. This repeated finding was recognized shortly after IVF became a primary treatment for infertility and largely ascribed to cellular and chromosomal defects in the oocyte [20–24]. Cytoplasmic defects were initially described as so-called dysmorphisms, i.e., cytoplasmic phenotypes found to be inconsistent with normal preimplantation embryogenesis, with some related to unusually high frequencies of chromosomal aneuploidies [21]. However, early cytogenetic studies of human oocytes repeatedly demonstrated that while aneuploidy was common, its occurrence did not preclude fertilization and lethal developmental consequences were often downstream of the preimplantation stages. Likewise, the effects on outcome of certain "lifestyle" factors, such as tobacco smoking [25] and obesity [26], are now universally recognized as very significant negative influences on the success of infertility treatment, including IVF, and owing to poor outcomes, are considered criteria for exclusion for IVF in a growing number of infertility programs.

Yet women who are significantly overweight, smoke tobacco, or are of advanced reproductive age can and do have normal outcomes. Undoubtedly, every IVF program has had patients who conceive without treatment or intervention after multiple failed cycles of ovarian stimulation, IUI, and IVF. This should reenforce a healthy skepticism that there may be no "absolutes" with respect to the predictive power of oocyte selection where an underlying biological cause of developmental incompetence has not been identified. At present, a seemingly unavoidable fact of the normal developmental biology of early human development is that competence for any specific MII oocyte or early embryo cannot be determined unambiguously, and at best, current methods of assessment realistically provide only a positive or negative bias for selection at a particular stage of preimplantation development. Likewise, no current assessment method has the proven power to predict or explain fertilization failure for MII oocytes that are normal at the chromosomal and cytoplasmic levels, but where a sperm has reached the oolemma but fails to attach or penetrate.

Although not a focus of this chapter, uterine receptivity is clearly a central element in the development success of an otherwise competent embryo after transfer. The molecular and cellular biology of uterine receptivity has been long studied in model systems and correlates with outcome after clinical IVF have been indentified [27]. Typically, assessments of uterine receptivity used in most IVF programs are based on ultrasonic evaluations and measurements of endometrial thickness with certain threshold values considered primary criteria for transfer or cryopreservation. In the absence of validated biomarkers whose detection can be accomplished noninvasively, receptivity assessments prior to transfer remain largely based on ultrasound findings. The detection of certain molecular and cellular biomarkers currently thought to be associated with normal receptivity [28, 29] would require a diagnostic endometrial biopsy prior to transfer. This may be an unacceptable intervention or counterproductive if the time required for analysis exceeded the optimal embryo "transfer window." At present, the relative contribution to implantation failure of apparently competent euploid embryos that may result from molecular or cellular defects in the embryo that originated in the oocyte, or placement in a suboptimal uterine environment, cannot be readily distinguished.

Here, we consider whether regulatory processes exist at level of the oolemma and subplasmalemmal and subcortical cytoplasm of the oocyte that could impact the normality of early human development. While much of the experimental findings discussed come from model systems such as the mouse and for the human, some notions are speculative; they are presented in the context of novel directions for research that may explain possible common causes of clinical IVF failure. It could be reasonably argued that the human oocyte or embryo is not an experimental system for developmental biology because in virtually all instances in which IVF is used, the singular intent of this treatment is to achieve pregnancy. For obvious reasons, the types of molecular genetic studies routinely performed in model mammalian systems, such as gene "knock-in" and "knockout" methodology,

used to investigate the consequences of the gain or loss of function associated with specific genes, gene networks, and signaling pathways have no possible application in the human. However, while it is reasonable to suspect that similar developmental pathways and activities identified and studied in model systems likely have similar functions and consequences for the human oocyte and early embryo, important and fundamental differences do exist. With respect to developmental problems that affect human oocytes and early embryos, the mouse may be a poor model because aneuploidy is very rare, and in natural estrous cycles, the number of pups born is largely equivalent to the number of oocytes ovulated. Other species such as the bovine or porcine have been advanced as suitable human models, but issues related to oocyte and embryo availability, and establishing IVM and IVF, may be impractical, problematic, or, depending on the clinical setting, impossible. In this regard, the human oocyte and preimplantation embryo may be the best system to study the origins of normal and compromised competence and the most practical one given the ready availability of source material and patient history.

While genetic manipulations are impossible for the human (e.g., transgenics), targeted gene "knockdowns" using morpholino oligonucleotides [30] may be the first step in identifying critical regulatory functions that contribute to the types of developmental abnormalities and demise observed during 5 or 6 days of human embryo culture. It is apparent from microarray analyses of the transcriptome and proteome that a large universe of potential gene targets exists and, to consider one to be more important than another with respect to developmental significance, may be difficult to validate if based solely on relative expression levels and, ultimately, to confirm as "the" etiology of a fertilization defect or compromised competence. The critical issue in designing targeted gene knockdown experiments using embryos donated to research is how to maximize the clinical relevance of possible findings by identifying which gene(s) to target. We know little about the regulation of gene expression in early human development, and considering the levels of regulation known to exist in eukaryotic cells, from microRNAs and RNA-binding proteins to differential transcription factor activity, findings from high-resolution methods such as Chip-seq and RNA-seq technologies will produce a catalog of potential targets, but where to place investigational emphasis? Which specific oocyte or embryo phenotypes may be mono- or multigenic or arise from a common regulatory defect? Indeed, it is currently impossible to rule out defects at the posttranslation level as a primary factor contributing to compromised competence. For example, early studies from this author's laboratory [31] demonstrated that a significant proportion of the changes detected by high-resolution proteomic analysis in the maturing oocyte and newly fertilized mouse egg were the result of posttranslational modifications (e.g., glycosylation and phosphorylation) rather than new gene expression.

The universe of potential targets may be reduced significantly by focusing on common defects observed in clinical IVF, such as penetration and fertilization failure, the arrest of cytokinesis, and the inability to compact or transition from a morula to a blastocyst or, for the blastocyst, to escape the confines of the zona pellucida. These defects may be especially suited for targeted gene knockdown methodology where proteomic or transcriptome analyses have identified candidate genes associated with these developmental abnormalities in the human. An early aim of such studies would be to confirm a role in the occurrence of an oocyte or embryo defect by duplicating a suspected phenotype (phenocopy) and correlating it with the level of gene expression detected when it arises spontaneously [32]. Other potential candidates in this regard are those involved in fertilization and the so-called maternal-effect genes (MEGs) that have regulatory functions during preimplantation embryogenesis, as discussed below. These may be particularly relevant as "first case studies" of competence because they are maternally derived developmental drivers of early embryogenesis and may be spatially compartmentalized in the ooplasm (see below). However, perhaps the first place to study "compartmentalized" functions is the oolemma

in instances where after conventional IVF with normal sperm and normal-appearing meiotically mature oocytes, sperm attachment or penetration fails. This is an unexpected and frequently inexplicable outcome that can necessitate the so-called rescue ICSI if detected early. Where a sufficient number of fertilized oocytes occur, the most common course is to simply discard unfertilized oocytes with no investigation as to cause, which is unfortunate.

The Oolemma and Competence: The Lipid Raft Theory

The oolemma is the interface between the cytoplasm and the external milieu, the site of sperm attachment and penetration, as well as site of transport and signaling activities. In this regard, it is surprising that so little is known about its organization in the human oocyte and early embryo and the extent to which functional defects may be responsible for fertilization and early embryonic failure. Modern membrane theory considers the plasma membrane as a microheterogeneous bilayer of lipid-ordered and lipid-disordered microdomains in which their state of organization can promote molecular diversity, compartmentalization, and polarization of functions (e.g., apical vs. basal portions of a cell). A central component of membrane theory involves lipid rafts, submicron-sized entities of cholesterol, sphingolipids, and protein that form microdomains which serve as concentrating platforms for a wide variety of membrane activities [33, 34].

A large body of experimental and analytical studies generally supports the notion that lipid rafts can be "functional units" of the eukaryotic plasma membrane, and that by virtue of dynamic changes in their state of aggregation, turnover, protein density, and composition, dynamically change membrane function(s) in response to different intrinsic and extrinsic conditions. Classes of proteins localized to lipid rafts in the exoplasmic leaflet of the lipid bilayer of the plasma membrane include a wide variety of receptors, transporters, G-proteins and GPI (glycophosphatidylinositol)-linked proteins, and other members of signal transduction cascades [35–38]. Lipid raft microdomains also serve as sites for catalytic activity, such as phosphorylation, and for anchoring molecules, including proteins involved in the regulation of ion channels, endocytosis, cell recognition, attachment, and communication (e.g., integrins and gangliosides). Lipid rafts are postulated to float freely in lipid-disordered regions of the plasma membrane and, by virtue of dynamic transitions in their state of aggregation, increase their net diameter from the submicron to micron range, which can regulate the relative density of associated molecules. In the same respect, disassembly or disaggregation of lipid rafts within ordered microdomains is postulated to reduce the density of associated molecules. By means of dynamic transitions in lipid raft density and organization, up- or down-regulation of membrane functions, as well as the gain or loss of function, can be accomplished globally or locally.

The molecular structure and organization of lipid rafts and the lipid-ordered microdomains they form in differentiated cells lead to the following questions for the human oocyte: (1) Does a similar membrane organization occur? (2) Does the oolemma undergo stage-related molecular remodeling of lipid raft microdomains? (3) Does remodeling establish defined regions of functional compartmentalized, and (4) Do defects in molecular organization occur and are they proximate causes of fertilization failure? While similar questions apply to the preimplantation embryo, here the focus is on the oocyte.

Lipid Rafts and Lipid-Ordered Microdomains in the Human Oocyte

Studies of lipid raft dynamics and membrane structure in the human oocyte face problems not encountered with tissues, cell cultures, or, for that matter, oocytes and embryos of model nonmammalian species. For biochemical analysis, lipid rafts are prepared by detergent extraction followed by ultracentrifugation through a sucrose gradient to obtain a detergent-resistant membrane fraction (DRF). Isolation of a DRFs enriched in lipid rafts is based upon their buoyant density

characteristics [38], and yields required for high-resolution structural, biochemical, and proteomic analysis are typically obtained from high-density cell cultures, intact tissues, and cellular aggregates such as the cumulus oophorus [39] sperm [40] and from species with either large oocytes or where massive numbers are released at one time [41]. Levels of detergent-resistant membrane required for lipid raft analysis make similar studies for the mouse problematic and for the human impossible.

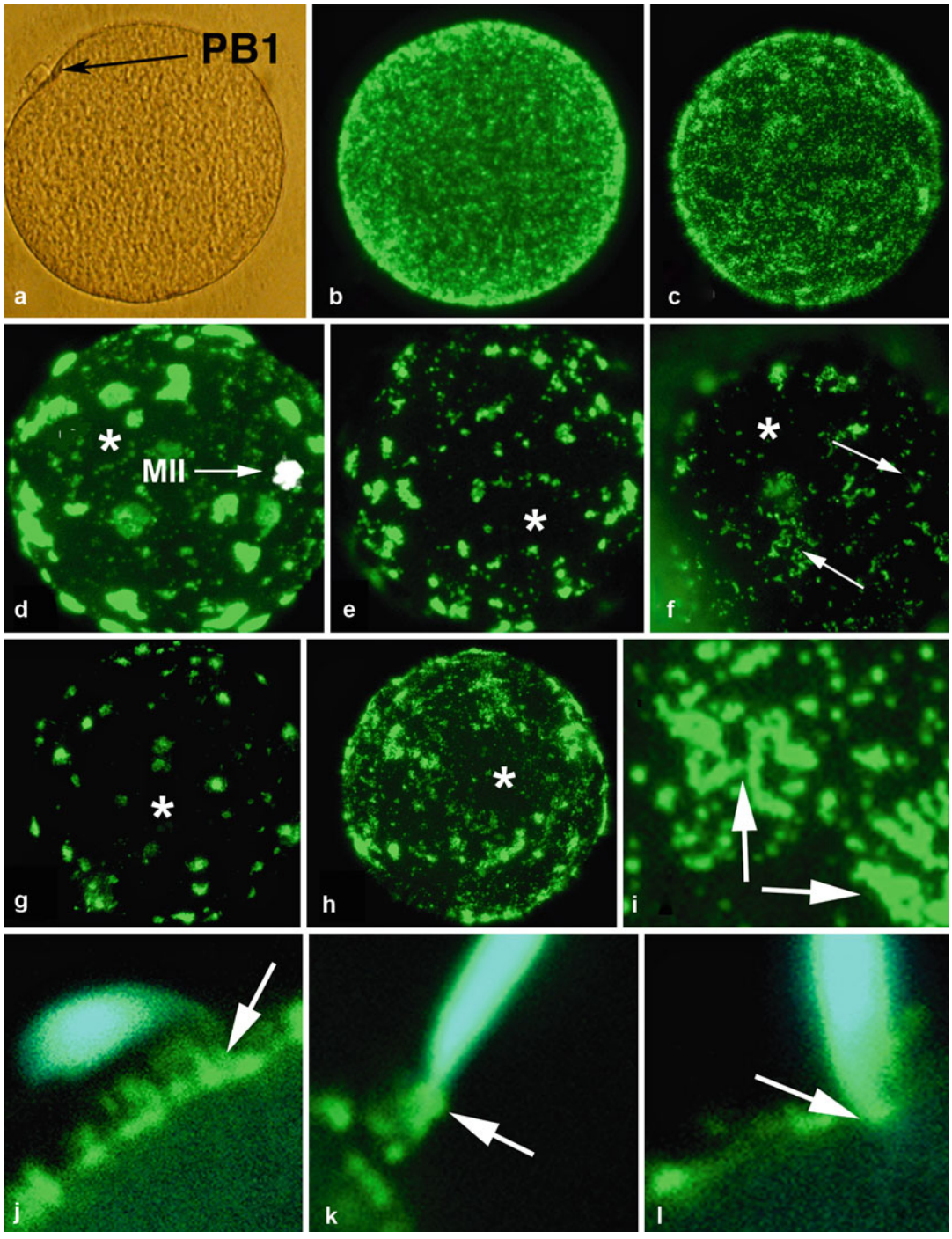
The B subunit of cholera toxin (CTB), by virtue of its high specificity and affinity binding to the relatively ubiquitous lipid raft ganglioside GM1 (monosialotetrahexosylganglioside), is one of the most common biomarkers used to identify lipid rafts [39, 42, 43]. When conjugated with a fluorophore (e.g., fluorescein or rhodamine isothiocyanate, Alexa 488 or Texas Red), constructs of this type report GM1-enriched microdomains in the exoplasmic leaflet of the plasma membrane. The direct fluorescent detection of GM1 lipid rafts permits studies of microdomain reorganization and membrane remodeling, as well as GM1 lipid raft turnover in living cells.

Whether GM1 microdomains occur in human oocytes and, if so, have a specific organization associated with fertilization competence was assessed by scanning laser confocal microscopy of meiotically mature human oocytes, obtained under the following conditions: (1) normal-appearing superfluous MII oocytes from ovum donation cycles; (2) immature at follicular aspiration but MII within 4 h of culture; (3) MII likely fertilizable, but intentionally not inseminated by ICSI owing to the presence of certain cytoplasmic features associated with adverse developmental consequences (Balaban [1, 21, 44]); and (4) unfertilized at 10 h after conventional IVF despite the presence of a sperm on the oolemma detected by differential interference contrast microscopy.

The confocal images in Fig. 19.1 are projections of fully complied section series showing in a single plane, all detectable CTB staining of the oolemma. Figure 19.1a shows the typical appearance of MII human oocytes used in the study of lipid raft microdomains. The typical pattern of CTB staining (Alexa Fluor 488 conjugate) of GM1 found to be routinely associated with normal penetration is one in which finely punctate fluorescence is well dispersed (Fig. 19.1b). A similar organization occurs in oocytes that completed of meiotic maturation in vitro (Fig. 19.1c) and in oocytes deselected for ICSI because of unfavorable cytoplasmic characteristics. In competent MII human oocytes, GM1 microdomains occur throughout the oolemma, whereas for unpenetrated oocytes, two predominant phenotypes are observed. The most common type, occurring in nearly 60 % of unpenetrated oocytes with sperm on the oolemma, showed intense fluorescence localized in dense, discrete clusters that appeared as "islands" surrounded by CTB-negative regions (Fig. 19.1d, e). For approximately 40 % of unpenetrated oocytes, punctate CTB fluorescence was relatively scant (arrows, Fig. 19.1f) with large regions of the oolemma devoid of a detectable fluorescent signal (asterisks, Fig. 19.1f). In MII oocyte(s) with a normal distribution of GM1 at retrieval (similar to Fig. 19.1b), restaining at 6- to 8-h intervals for up to 36 h during culture was associated with a progressive decline in the intensity and density of CTB fluorescence. This was also observed (in vitro aging) in oocytes that originally showed the predominant "island" phenotype associated with penetration failure (e.g., 12 h, Fig. 19.1g). These findings suggest that GM1 lipid rafts may be internalized (likely by endocytosis) and, if not replaced, effect a time-related loss of associated functions from the oolemma. Although relatively few oocytes from women of advanced maternal age (>40 years) have been available for study to date, Fig. 19.1h shows a common GM1 phenotype associated with fertilization failure after conventional IVF, where a sperm was observed in the perivitelline space but not attached to the oolemma. This phenotype is similar to the "island" type described above for younger women, with large areas devoid of CTB fluorescence (asterisk). While the islands generally appear to be of a lower density, fertilization failure may have the same biological consequences associated with of this type of organization as observed in younger women.

For oocytes that are likely fertilization competent at retrieval, CTB fluorescence is typically well dispersed with individual punctate elements clearly

detectable (similar to Fig. 19.1b, c). Figure 19.1i is a representative high-magnification image of what is likely a "fertilization-competent" organization of GM1 microdomains in the human oolemma. Based on current findings, this organization is considered indicative of the molecular topology of the GM1-enriched lipid raft microdomains the sperm "sees" (arrows) and can interact with when it reaches the



oolemma. According to the protocol presently in use, insemination for experimental purposes is not permitted. However, studies of zona-free mouse oocytes show that for each of the numerous sperm that bound to the oolemma within seconds after insemination in vitro, binding was always to discrete microdomains enriched in GM1 (arrow, Fig. 19.1j; [45]). However, for the few human oocytes where sperm were identified on the oolemma but had not penetrated ($n=28$), preliminary findings after CTB staining indicate that all docked at GM1-positive microdomains (arrows, Fig. 19.1j, k).

The possibility that a ganglioside such as GM1 may participate in sperm binding is a novel notion with respect to the process of fertilization in the mammal, but may not be too unusual given their known roles in cell–cell adhesion, recognition, and signaling, as noted above. Confirmation that GM1 microdomains are sites of sperm binding to the human oolemma would go a long way in suggesting possible causes of infertility and fertilization failure after IVF. For example, if sperm passage through the zona pellucida occurs at a site that is largely devoid of GM1 microdomains (e.g., asterisks, Fig. 19.1d–g), no attachment may occur, and because in this instance most of the tail will persist in an extended and motile state through the zona pellucida [46], the head will likely remain fixed in position with respect to the oolemma. This could explain fertilization failures observed in normal-appearing MII oocytes where GM1 staining was scant or undetectable in large areas of the oolemma, as shown above.

Contact with the oolemma at an intensely fluorescent GM1 island may be equally problematic if its density is too great to permit robust contact between gamete membranes. In the mouse, this degree of membrane association may be facilitated by a rapid lateral displacement of GM1 microdomains at the site of sperm contact and has been suggested to promote membrane fusion between sperm and oocyte [45]. If a similar focal microdomain reorganization is confirmed for the human, the inability of GM1 lipid rafts occurring at high density, such as the "island" phenotype, to dynamically partition into lipid-disordered regions and effect disaggregation or diminution of the corresponding microdomains in response to sperm contact may explain arrested fertilization where the sperm head remains firmly attached to the oolemma at an island.

Membrane Organization and Sperm Penetration

CD9, a known constituent of lipid rafts and member of the transmembrane 4 superfamily (so-called tetraspanin family) [47], has been shown to be a critical cell membrane protein that mediates fusion of male and female gamete membranes in the mammal [48, 49]. Figure 19.2a shows the typical distribution of CD9 immunofluorescence in MII human oocyte detected in a projection of a fully compiled section series taken by scanning laser confocal microscopy. Figure 19.2b is a 4-μm section from this series showing its complete circumferential distribution. Of particular note with respect to fertilization failure is that this distribution remains unchanged in instances of penetration failure where GM1 microdomains are scant or in the dense island configuration. These findings suggest that GM1 and CD9 may occur in unique lipid raft microdomains that may

Fig. 19.1 (**a**) Typical appearance of an *MII* human oocyte used in the study of lipid raft microdomains (PB1, first polar body). (**b**, **c**) Show the appearance of GM1 lipid raft microdomains reported by fluorophore (Alexa Fluor 488) conjugated cholera toxin subunit B that is consistent with normal fertilization. (**d**–**h**) GM1 phenotypes associated with fertilization failure after IVF. (**d**, **e**) Sperm showed strong attachment to the oolemma but there was no penetration. (**f**–**h**) Sperm were unattached or weakly attached to the oolemma. GM1 positive microdomains were scant (*arrows*, **f**) or undetectable in wide areas of the oolemma (*asterisk*, **e**–**h**). (**d**) DAPI fluorescent chromosomes on the metaphase II, MII, spindle are indicated by an arrow. (i) A high magnification view of GM1 microdomains from an area of the oolemma that in size, likely represents what the sperm head might 'see' for docking when it reaches the oolemma of a fertilization competent oocyte (e.g., **b**, **c**). (**j**–**i**) The initial docking of a mouse (**j**) and human (**k**, **l**) sperm to GM1 microdomains (*arrows*) on the exoplasmic leaflet of meiotically mature oocytes. All images were taken by scanning laser confocal microscopy (see text for details)

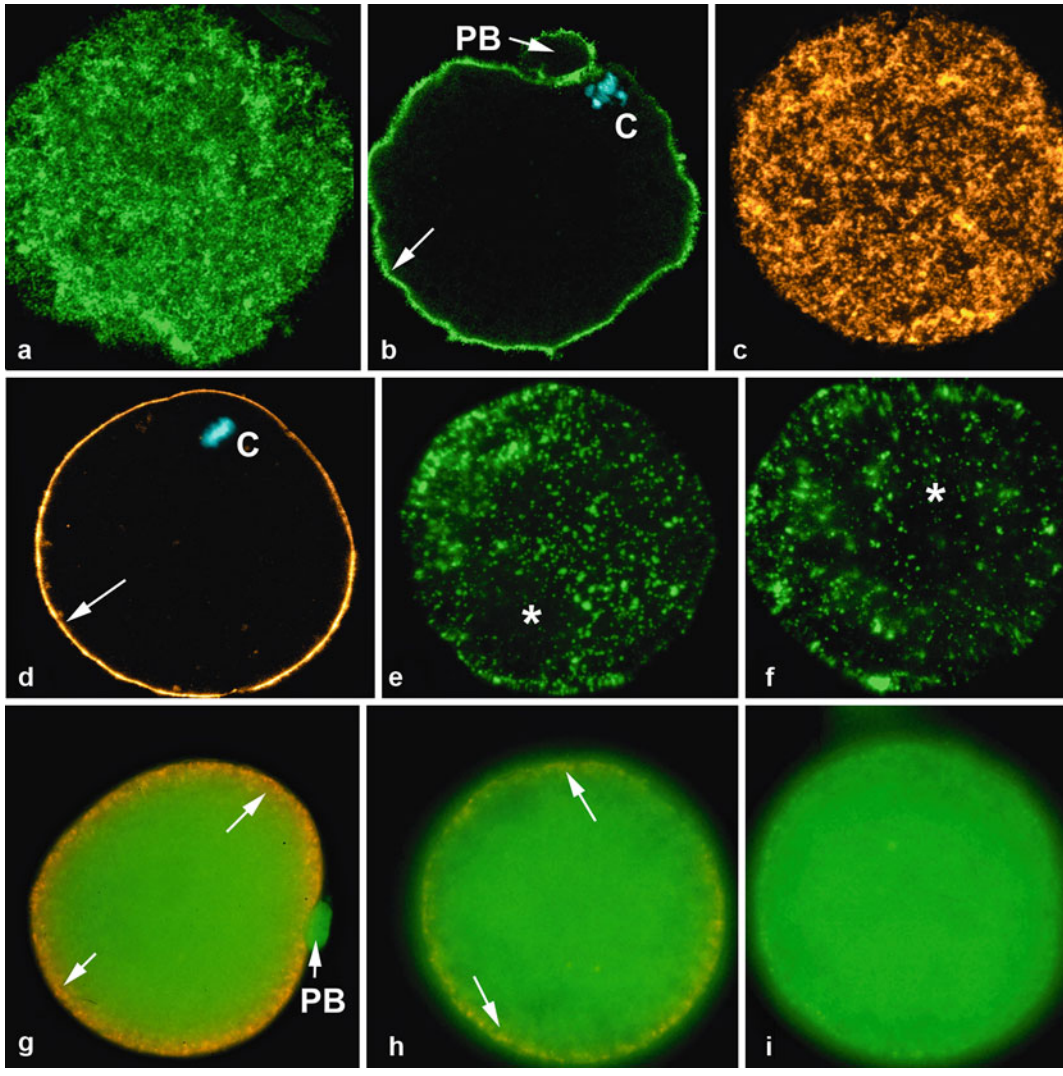


Fig. 19.2 Scanning laser confocal images of CD9 localization to the exoplasmic leaflet of the oolemma (*arrows*, **b**, **c**) in normal human (**a**, **b**) and mouse (**c**, **d**) *MII* oocytes, shown as fully compiled projections of section series (**a**, **c**) or in a midline cross section (**b**, **d**). The *MII* spindle is stained with DAPI to show chromosomes (*c*). (**e**, **f**) Projections of fully complied section series of normal-appearing *MII* human oocytes showing diminished CD9 fluorescence and regions with no detectable signal (*asterisks*) where sperm attachment but not penetration occurred. (**g**–**i**) Human *MII* oocytes stained with the potentiometric fluorescent dye JC1 to identify high-potential ($\Delta\Psi m$) mitochondria. In normal and presumably fertilizable oocytes, high-potential mitochondria occur in a distinct subplasmalemmal domain (*arrows*, **g**). Oocytes with reduced (**h**) or no fertilization competence show scant (*arrows*, **h**) or no high-potential forms (**i**) (see text for details)

be subject to different modes of regulation. For the human, it would appear that a critical protein involved in sperm penetration exists in the plasma membrane of unpenetrated human oocytes, which is consistent with the notion that certain common instances of fertilization failure may be related to the organization and distribution of GM1-enriched microdomains. In support of this possibility are findings from Jegou et al. [50], who proposed that CD9 promotes the formation of "fusion competent sites" on the oolemma by mediating the close opposition of gamete membranes necessary for

sperm penetration in the mouse. The extremes of GM1 microdomain distribution observed in human penetration failures in the presence of a normal CD9 phenotype may preclude this activity in GM1-scant or GM1-negative regions or where these microdomains occur at abnormally high density. Sperm attachment and penetration failures observed in human IVF also suggest that the presence of CD9 alone may be insufficient to achieve the level of close membrane association that has been suggested to be required for penetration in the mouse.

An unexpected finding in our survey of normal-appearing but unpenetrated human oocytes was the presence of CD9-negative regions of varying size and distribution, as shown in Fig. 19.2e, f. These MII oocytes showed grossly normal cytoplasmic morphology after insemination and a normal distribution and organization of GM1 microdomains. For the human, regions of the oolemma largely devoid of CD9 lipid raft microdomains could identify a new etiology of fertilization failure where sperm attachment to GM1 microdomains is normal, but there is no corresponding domain to facilitate close membrane opposition and penetration.

The above studies represent intriguing insights into potentially new etiologies of unanticipated fertilization incompetence and possible diagnostic means for their detection in clinical applications in IVF. Although ICSI is commonly used for insemination and would be expected to overcome defects in the oolemma that may contribute to failure after insemination in vitro, it is not necessarily the first line approach in most IVF programs and would obviously not remedy fertilization failure in vivo if similar oolemmal microdomain organizations are responsible. It is also unclear whether GM1/CD9 microdomain defects in the oocyte that are likely overcome by ICSI have adverse downstream development consequences that could contribute to signaling defects and poor embryo performance or arrested development during cleavage. For example, CD9 and GM1 are detected in the plasma membrane of human blastomeres during cleavage (Van Blerkom, unpublished), and while their function(s) during early embryogenesis remains to be determined, based on known activities, a role-promoting intercellular contact and communication are distinct possibilities. Likewise, how microdomain distribution may be differentially regulated could offer novel insights related to the acquisition of developmental competence in general and information that could be especially helpful in identifying idiopathic causes of reduced fertility. The findings discussed here show potential relevance in regard to poor IVF outcomes related to idiopathic infertility and, potentially, why fertility declines with maternal age. The detection and localization of other lipid raft proteins whose membrane functions have been characterized in other cell types can be an important step in ongoing efforts to map the molecular organization of the human oolemma as it relates to the acquisition of developmental competence.

The regulation of the molecular organization of the oolemma during early development is another area that warrants investigation with respect to developmental competence. The organization of lipid raft microdomains in exoplasmic leaflet of certain somatic cells has been shown to be directly influenced by two interacting elements in the subplasmalemmal cytoplasm: cortical actin microfilaments and the phospholipid-binding protein annexin 2. Annexin 2 is a calcium-dependent protein that resides in lipid rafts in the cytoplasmic leaflet of the plasma membrane [51], and its state of oligomerization regulates lipid raft density in the exoplasmic leaflet of certain differentiated cells [52], and changes in the state of oligomerization directly influence the activity of lipid raft microdomains as concentrating platforms in the exoplasmic leaflet. Annexin 2 has been shown to occur in the inner leaflet of the mature mouse oocyte [45], and if a similar location is confirmed for the human oocyte, its calcium dependence and regulatory interactions with subplasmalemmal actin microfilaments may suggest means by which local changes in subplasmalemmal conditions could directly influence molecular organization in the exoplasmic leaflet and fertilization competence.

In this regard, preliminary findings from human oocytes with a normal GM1 distribution

prior to cryopreservation (programmed freezing) show that after thawing, the subplasmalemmal actin microfilament network is disrupted, GM1 distribution shifts to the "island" phenotype, and oolemmal annexin 2 immunostaining occurs as distinct foci of intense fluorescence; a similar state of annexin 2 organization occurs in fresh oocytes with the so-called island phenotype (Van Blerkom, unpublished). While additional study is necessary to validate these findings, they tentatively suggest a possible mechanism by which fertilization competence by conventional IVF is largely lost after thawing of oocytes that were likely fertilization competent prior to cryopreservation.

Functional and biochemical characterizations of the human oocyte and early embryo plasma membrane are at an early stage of investigation, but notwithstanding current analytical limitations, they offer the promise of identifying molecular conditions that influence developmental normality for both oocyte and early embryo. The extent to which the specific conditions or defects noted above directly influence fertility in general, and outcome in infertility treatment in particular, remains to be determined. However, the important questions that need to be answered are whether the type of analytical findings described above can (1) be directly related to problems of developmental competence commonly encountered in clinical IVF and (2) produce diagnostic methods that may explain instances of fertilization or preimplantation failure, which, for the latter in particular, are not associated with chromosomal abnormalities. Tagging GM1 with CTB may be one method to identify causes of fertilization or early cleavage arrest that has potential applications in clinical IVF. Preliminary studies in the mouse indicate that staining with a fluorescent-CTB construct does not inhibit fertilization in vitro and that turnover of GM1 leads to a progressive loss of the original fluorescence signal, which is barely detectable after ~24 h (Van Blerkom, unpublished). However, before clinical application could be contemplated for oocyte selection (e.g., ICSI or conventional IVF), it would have to be shown to be developmentally benign from fertilization to postnatal development in model species. That said, application as a diagnostic biomarker in fertilization failures may well provide a developmental biological basis for failure that could have important implications in planning subsequent treatment for affected patients and could indicate a possible etiology of a patient's inability to conceive naturally.

The Subplasmalemmal Cytoplasm

The subplasmalemmal cytoplasm occupies about a 5- to 10-μm zone beneath the oolemma. Interest in this region has traditionally focused on the organization and function of actin microfilaments and cortical granules and how ambient free calcium levels change in response to the fertilizing spermatozoon. More recently, whether mitochondria located in the subplasmalemmal cytoplasm have regulatory roles in early development has become a topic of investigation for mammalian oocytes and early embryos, including the human [12, 18, 53].

The subplasmalemmal cytoplasm of the MII mouse and human oocyte is populated by circumferential domain of underdeveloped mitochondria that, while structurally identical to their more numerous counterparts in other parts of the ooplasm, are distinguishable by a higher-potential difference across the inner membrane ($\Delta\Psi m$), as reported by mitochondria-specific potentiometric fluorescence stains, such as JC1 [18, 54, 55]. In differentiated cells with fully developed and metabolically active mitochondria, the relative magnitude of $\Delta\Psi m$ is usually high (termed hyperpolarized) and can be related to several critical mitochondrial activities, including levels of ATP generation, protein import and functional modification, the regulation of organelle volume homeostasis, and calcium sequestration and release signaling through mitochondrial calcium-induced calcium release [18, 56, 57]. In somatic cells, the magnitude of $\Delta\Psi m$ (−mV) fluctuates in response to intracellular conditions, such as mitochondrial density [58], and extracellular influences such as the extent and type of intercellular contact [59]. Indeed, regions of differential $\Delta\Psi m$ indicating differences in specific

levels of activity can be spatially localized in the same fully developed mitochondrion in cultured cells [54]. Similar rapid transitions in ΔΨm are undetectable with current live-cell imaging methods for mammalian oocyte mitochondria because of their small size (~1 μm) and underdeveloped state (few, short cristae that do not traverse a dense matrix; [60, 61]).

In MII mouse and human oocytes, the subplasmalemmal cytoplasm has been estimated to contain ~2–3 % of the total mitochondrial complement and is distinguished from the majority of mitochondria both by a relatively high potential and their spatial stability in the subplasmalemmal cytoplasm during oocyte maturation and early postfertilization development [57, 62, 63]. This apparent stability is in contrast to their lower potential that can change position with cytoplasmic movements or is translocated along microtubular arrays to perinuclear locations [64, 65]. The apparent stability of this domain and proximity to the plasma membrane led us to suggest that by virtue of compartmentalization, they may have specialized regulatory functions or influences during early development on local bioenergetic, ionic, or redox states [12, 53, 66]. In this context, defects in number, distribution, or magnitude of ΔΨm may have developmental consequences. Van Blerkom et al. [18] reported that subplasmalemmal ΔΨm in unpenetrated human oocytes was often relatively low, and Jones et al. [67] showed that a shift from high to low potential in this domain was associated with cryopreservation and that after thawing, the persistence of a low-potential state could render the oolemmal refractory to penetration. Van Blerkom and Davis [68] showed that penetration (but not sperm attachment) was reversibly inhibited in the mouse by experimentally shifting ΔΨm in the subplasmalemmal cytoplasm from a high to low to high. However, we have not observed a spontaneous shift of this type in any unfertilized human oocyte during subsequent culture.

The typical distribution of high-potential mitochondria reported by JC1 defines a circumferential domain just beneath the oolemma (arrows, Fig. 19.2g). The fluorescence emission characteristics of JC1 are dependent upon the potential difference across the inner mitochondrial membrane (i.e., −mV), with low-potential mitochondria fluorescing green (indicating the presence of the molecule as a monomer), while those with a higher ΔΨm fluoresce orange red (indicating the presence of multimers). Potential-driven multimerization results in a shift in fluorescence emissions from shorter (green, monomeric) to longer wavelengths (multimeric, orange red) due to the formation of multimers, termed J-aggregates [18, 54]. It is worth mentioning that similar to other potentiometric fluorescent stains, the potential difference across the inner mitochondrial membrane reported by JC1 does not indicate a specific mV value but rather a relative range that can be described as high or low. The presence of green-fluorescing mitochondria in JC1-stained oocytes or early embryos has often been misinterpreted to indicate metabolic inactivity. This conclusion seems to be odd considering that virtually all mitochondria in the mouse or human oocyte would have to be considered inactive, requiring that the bioenergetic requirements of the oocyte (i.e., ATP) be served by a minute fraction of the total mitochondrial population located in proximity to the oolemma. Intuitively, this is problematic from a bioenergetic perspective because the fully grown oocyte is the largest cell in the body and nascent ATP generated by mitochondria is largely utilized to drive biological processes located close to the producing organelles. While ATP is comparatively stable in aqueous solutions, its intracellular half-life is measured in microseconds. It would seem likely that in the oocyte, ATP is used where produced mitochondria and the comparatively enormous cytoplasmic volume of the oocyte are not favorable for diffusion from subplasmalemmal to more interior locations. Indeed, inhibitor studies of mouse oocytes that effect different portions of the oxidative metabolic pathway or which collapse ΔΨm show a precipitous drop in ATP levels after inhibitor exposure [69].

Oocyte mitochondrial activity and ΔΨm should have the same general relationship observed for somatic cells, but in the case of the oocyte, ATP levels supported by structurally immature and low-activity organelle are presumably compensated for

by the size of the mitochondrial complement, which is orders of magnitude greater than in a typical cell. Therefore, while ATP generation/mitochondrion may be relatively small when compared to levels when fully developed (e.g., in the trophectoderm), in aggregate, it is obviously sufficient to maintain a net cytoplasmic content consistent with oocyte function. For normal developmental competence, several investigators suggest a net ATP content for the human oocyte around 1.8–2 pM may be an optimal level [13, 14].

If JC1 fluorescence in the oocyte and early preimplantation embryo do correspond to differences in activity levels, the occurrence of high-potential forms in the subplasmalemmal cytoplasm would seem to have higher functional capacities or activity. The origin of a higher ΔΨm detected in oocytes denuded of cumulus and coronal cells remains to be determined, but it may be due to a proximity to oxygen diffusing across the oolemma. If oxygen levels are higher at this domain and used for oxidative phosphorylation, an oxygen gradient may be established within the cytoplasm such that increasingly levels of hypoxia (to virtually anoxic) may occur with distance from the oolemma. Although presently speculative, this could limit the ability of most mitochondria in the oocyte to transition to a higher potential.

Nitric oxide (NO) may be another factor that naturally regulates ΔΨm in the subplasmalemmal cytoplasm. In the intact oocyte, nitric oxide (NO) produced by the cells of the cumulus oophorus and corona radiata appears to have a suppressive effect on the level of ΔΨm in subplasmalemmal mitochondria of the mouse oocyte, most probably, by competing with oxygen at the level of cytochrome c oxidase located on the inner mitochondrial membrane [70]. The putative suppressive effect appeared to diminish at MII in instances where the cumulus oophorus was fully expanded. Suppression of high ΔΨm and upregulated levels of metabolism in the subplasmalemmal domain prior to the terminal stages of oocyte maturation could be compensated for by fully developed mitochondria in transzonal processes located in proximity to the oolemma [53]. These mitochondria may be a source of ATP needed to support activities in the subplasmalemmal cytoplasm before an increase in ΔΨm of the resident mitochondria. Prior to the resumption of meiosis (GV stage), gap junction-mediated communication between the oocyte and somatic cell compartment by means of transzonal processes provides a direct conduit for ATP passage into the oocyte. However, after their detachment and the disruption of gap junctions that initiates the resumption of meiosis, the termini of the processes remain in close proximity to the oolemma [61], and NO released from the residual processes may still have a suppressive influence on subplasmalemmal mitochondria until ovulation.

For the human, mitochondria in the subplasmalemmal cytoplasm appear to be under the same regulatory influences observed in the mouse [70]. A systematic investigation has not been undertaken to determine if the size, degree of expansion, or state of organization of the somatic cell compartment of the human cumulus-oocyte complex is related to the occurrence and distribution of high ΔΨm in newly retrieved MII oocytes. However, there are findings that support this notion. MII human oocytes are observed in which high-potential forms are scant and discontinuously distributed (arrows, Fig. 19.2h) or all of low potential. Figure 19.2i is an example of an MII oocyte where no detectable high-potential forms were observed, and in this instance, all eight unfertilized MII oocytes from the same patient (with a long history of infertility) showed this phenotype after conventional insemination. Although sperm were present within the matrix of the zona pellucida, what was noted as unusual at follicular aspiration was that the cumulus oophorus for each oocyte was relatively small, dark, and highly compact and that 16 h later, when denudation was done to determine fertilization (so-called 2PN check), removal of both cumulus and coronal cells was unusually difficult and, as a result, incomplete.

How different subplasmalemmal ΔΨm phenotypes arise is unclear, and a few “interesting” IVF cases, such as the one described above, may be suggestive but are by no means definitive. Where feasible, a systematic study of extrinsic factors that may regulate mitochondrial potential at MII

could offer new insights into whether mitochondrial activity in this domain influences the molecular organization and developmental competence of the plasma membrane. In this regard, preliminary findings suggest that the GM1 island phenotype (e.g., Fig. 19.1d, e) may be associated with a scant population of high-potential forms. Further investigation should demonstrate whether a membrane microdomain organization consistent with fertilization, which we suggest is associated with a dispersed and punctate GM1 distribution, is an energy-requiring process with ATP provided from the subplasmalemmal domain. The finding that high-potential forms tend to be scarce or absent in the MII oocytes of women of advanced maternal age (>40 years) may also be related to a relatively scant population of GM1 lipid raft microdomains, as noted above (e.g., Fig. 19.1f). Confirmation that oolemmal fertilization competence requires a circumferential domain of high-potential mitochondria could have important implications in instances of unexpected fertilization failure, particularly for women of advanced maternal age with long histories of infertility and repeated failure after conventional IVF.

The subplasmalemmal location of high-potential mitochondria observed for the mouse and human MII oocyte is also seen at the pronuclear stage and in blastomeres during early cleavage [18]. It is not until blastocyst stage that high-polarized mitochondria are the predominant form detected throughout the cytoplasm but only in the trophectoderm; mitochondria in the inner cell mass (ICM) are largely characterized as low potential with JC1 (green fluorescent [18, 71]). Here again, cell geometry, ambient oxygen availability, and rates of gaseous diffusion across the plasma membrane may play important roles in providing intracellular conditions permissive for high potential to occur in mitochondria that are now fully (i.e., structurally) developed with respect to shape, size, cristae number, and organization [60, 61]. In comparison to the ICM, trophectodermal cells are highly flattened and with a greater surface area for diffusion that is increased significantly by a highly microvillus exoplasmic surface [61]. These features are typical of a high-energy-consuming transporting epithelium, and the development of conditions supportive of a high $\Delta\Psi m$ in the trophectoderm is consistent with the function of this cell layer as a transporting epithelium [72]. For example, trophectodermal mitochondria are in close proximity to the plasma membrane, while oxygen must pass through the trophectoderm and dissolve in the blastocoelic fluid to reach the ICM. While the precise contribution to mitochondrial potential of spatial proximity to the plasma membrane remains to be determined, in a low-ambient-oxygen environment, as likely occurs in the fallopian tube and uterus, an oxygen threshold for high potential appears to be met in the trophectoderm but not in the ICM. For the ICM, this changes rapidly when these cells are placed under conditions that support their growth as stem cells [57]. In this regard, an oxygen concentration threshold permissive to shift $\Delta\Psi m$ from low to high in the subplasmalemmal cytoplasm may be a relatively narrow one. If differences in cell dimension, shape, and location at the blastocyst stage effect $\Delta\Psi m$ and corresponding mitochondrial functions and activity levels that are potential driven or dependent, they could contribute to an explanation of the comparatively lower levels of ATP generation by ICM cells [73].

The extent to which ambient oxygen levels may be regulatory with respect to mitochondrial function in the oocyte in general and subplasmalemmal cytoplasm in particular will require additional study. However, confirmation that such a regulatory relationship exists and may be dependent upon follicle-specific intrafollicular oxygen tension [74], or cumulus/coronal cell organization and function, could have important implications in the design of culture systems for in vitro oocyte growth and maturation or for embryonic development, if stage-specific adjustments of oxygen concentration prove to be beneficial.

In a developmental context, a domain of mitochondria in the subplasmalemmal cytoplasm that may be responsive to extrinsic conditions could be viewed as an adaptation by uniquely large and spherical cells that permits local regulation of critical stage-specific developmental processes. The contribution of subplasmalemmal mitochondria to

net cytoplasmic ATP content is likely minimal, which is not unexpected considering they represent a minute portion of the total mitochondrial complement. However, the ability to focally upregulate ATP production in the subplasmalemmal cytoplasm, without altering ΔΨm globally, may have important developmental implications for membrane structure and function, as well as for local cytoplasmic physiologic conditions that could influence critical signaling transduction pathways. For example, spatially compartmentalized, high-potential mitochondria may effect ambient free calcium, ATP, ROS levels, or cytoplasmic redox state that influence the function of molecules, including those involved in signal transduction, whose activity is regulated by these cytoplasmic factors [12, 53, 68, 75].

In support of this possibility are experimental findings that show that a transient reduction in ΔΨm in the subplasmalemmal cytoplasm of the MII mouse oocyte from high to low is inconsistent with sperm penetration (but not attachment to the oolemma) in zona-free oocytes [68]; however, penetration occurs after high potential is restored to its normal state. As noted above, mitochondria in the subplasmalemmal cytoplasm of human oocytes that remain unpenetrated after conventional insemination for IVF frequently show no high-potential forms, or J-aggregate-positive forms that are scant or localized in small clusters discontinuously distributed in the subplasmalemmal cytoplasm. In these instances, a bioenergetic deficit or alterations in free calcium levels localized to the subplasmalemmal cytoplasm [69] may prevent (1) the occurrence of molecular activities in the oolemma that are permissive for the penetration of a sperm docked at a GM1 microdomain or possibly (2) attachment, if the molecular organization of the oolemma consistent with fertilization competence is dependent upon energetic and ionic conditions in the subplasmalemmal cytoplasm. Confirmation that defects of this type occur and are related to fertilizability could go a long way in explaining the origins of conception failure in subgroups of infertile patients.

Loss of subplasmalemmal high-potential mitochondria to minor spontaneous fragmentation in early human blastomeres is often associated with an arrest of cell division or a grossly unequal cell divisions [62], yet ATP levels in affected embryos are within those measured for normally developing embryos at the same stage. Here too, loss of high-polarized forms localized in the subplasmalemmal cytoplasm may have cell-specific effects that are not directly associated with net bioenergetic status. In these instances, present evidence indicates that loss of most or a significant portion of the subplasmalemmal domain to small fragments is not corrected by replacement from the more abundant lower potential organelles, further indicating that this domain may be unique with respect to stability and function. This interpretation is supported by experiments in which subplasmalemmal mitochondria that partitioned into small fragments were labeled with JC1, shown to high potential (JC1 red fluorescent), and then transferred to an intact blastomere(s) in the same or different embryo [63]. After deposition on the cell surface, most fragments spontaneously fused with the plasma membrane; their mitochondria retained high potential and remained during subsequent cell division(s), in the same subplasmalemmal location where they first entered the cytoplasm.

The above findings should not be interpreted to mean that the bioenergetic state of the early embryo is not important. Indeed, the ATP content of individual blastomeres does have a direct effect on developmental competence. Stage-specific ATP thresholds consistent with normal cell function were demonstrated for normally progressing human embryos up to the late cleavage stages and for embryos in which one or more blastomeres arrested cytokinesis [65]. For normally progressing embryos, the mitochondrial and ATP contents were relatively uniform between cells, but very different values were found in blastomeres that underwent asymmetric divisions or ceased to divide. In these instances, blastomere-specific differences of developmental significance were associated with disproportionate mitochondrial segregation between cells at the first and second cleavage divisions. Unequal mitochondrial inheritance in blastomeres was traced to the symmetry of mitochondrial distribution at the pronuclear

stage, with different degrees of asymmetry creating corresponding differences in mitochondrial complement at the first and subsequent cell divisions [65].

ATP levels consistent with normal cell division, arrested cell division, or, in the case of very low levels, cell lysis support the notion of stage-specific bioenergetic thresholds during human preimplantation development. Particularly relevant to the outcome was the finding that disproportionate numerical mitochondrial segregation at the earliest stages of embryogenesis was not uncommon in human IVF. The critical clinical issue is whether different patterns of mitochondrial inheritance can be recognized noninvasively without the use of fluorescent probes. In this regard, advantage could be taken of mitochondrial autofluorescence at infrared frequencies, which has been shown to have no adverse developmental effects during the preimplantation stages for primate embryos [76].

The Cortical/Subcortical Cytoplasm: Maternal-Effect Genes

In the MII mouse oocyte and early cleavage-stage embryo, the subcortical cytoplasm, the region immediately below the subplasmalemmal zone described above, may be the site of regulatory activities that are compartmentalized in a stable complex of proteins and cytostructural elements [77]. Maternal-effect genes are transcribed during oogenesis, and their downstream function after fertilization is recognized by mutations in the maternal genotype that produce phenotypes that impair the normal progression of preimplantation embryogenesis. Li et al. [78] described three basic functions in which maternal-effect genes are likely involved during early development: (1) molecular remodeling of the paternal genome (e.g., additional of maternal histones), (2) degradation of certain maternal polyadenylated transcripts and proteins that are either no longer needed or replaced by embryonic forms, and (3) completion of zygotic genome activation. In addition to these essential activities, they may have roles in blastomere-specific activities involved in the establishment of the inner cell mass and trophectoderm [79]. Maternal-effect genes have been long postulated to exist in mammals, but it was not until 2000 that their occurrence was confirmed [80]. Developmental defects and abnormalities observed in mouse and attributed to null mutations in certain maternal-effect genes do resemble some of the commonly occurring abnormal phenotypes seen during the in vitro culture of human embryos, such as cleavage arrest or numerical/organizational deficiencies in the trophectoderm or ICM [81, 82]. Human homologues of maternal-effect genes in the mouse have been identified, [81] and defects associated with their normal expression are likely causes of early embryo developmental failure that could certain types of idiopathic infertility [83].

Storage of Maternal-Effect Gene Products

The notion that maternal-effect genes have important functions during early mammalian development is attractive from a regulatory perspective when viewed in the context of ensuring that critical events and processes required during the preimplantation stages occur without the need for new transcription. Recent findings indicating that MEGs may be stored during oogenesis in the subcortical cytoplasm in specialized cytoplasmic elements lend credence to this possibility. What makes these elements particularly interesting from a developmental biological perspective is that they disassemble by compaction and are largely undetectable by the late morula to early blastocyst stage [84], which would be consistent with a putative storage form of oocyte-derived factors (e.g., proteins) that support preimplantation development prior to full activation of the embryonic genome. In addition to maternal-effect genes [85], they are also storage sites of monomeric tubulin and ribosomal components [86–88]. With regard to how these lattices form, Kim et al. [89] indicated the involvement of the maternal-effect gene MATER in lattice assembly, suggesting functions during both oogenesis and early embryogenesis.

While species-specific differences in the fine structure of the cytoplasmic lattices are observed between rodents, their distinct submicroscopic architecture in rodents is not observed in other mammals, including the human. Nevertheless, it has been suggested that a unique fibrous network with similar functions is a characteristic of other mammalian oocytes and early embryos [84] and that changes in organization during the preimplantation period are regulatory with respect to critical stage-specific developmental transitions. For the mouse, a central role in development for cytoplasmic lattices is largely based on the lethal developmental consequences of experimental disruption of these elements or mutations that prevent their formation. In the mouse, maternal-effect genes whose knockout phenotypes are developmentally lethal during the early preimplantation or fetal stages (e.g., FILIA, Dnmt1, FLOPED, MATER, TLe6) have been shown by immunofluorescence and immuno-electron microscopy to have a specific localization in the subcortical cytoplasm of the mature oocyte [90]. Spatial compartmentalization within the ooplasm could be developmentally relevant if MEG functions in the subcortical cytoplasm are linked to, or dependent upon, local changes in subplasmalemmal cytoplasmic physiology related to mitochondrial potential, bioenergetic and redox state, or calcium homeostasis that occur at fertilization or during cleavage. In this regard, Tong et al. [90] showed by immuno-electron microscopy that mitochondria were a specific site of MATER localization in the mouse oocyte. Whether this finding has developmental relevance for mitochondrial function or activity is unknown.

A new area of investigation into the origins of competence in the early human embryo could become apparent if regulatory molecules that are spatially compartmentalized in the oocyte become functional in response to dynamic changes in specialized storage platforms after fertilization. The most serious challenge to this notion has come from a recent report by Morency et al. [91], who showed that the subcortical localization of two maternal-effect genes in the mouse, PADI6 and MATER, was dependent upon the method of sample fixation and processing. Based on variations of fixation and processing for immunofluorescence, the authors concluded that the distribution of these proteins was not spatially localized within the ooplasm. The potential importance of these findings needs to be considered in investigations seeking to relate spatial compartmentalization, including MEGs, with functional compartmentalization that could have localized development effects or based on distribution during cleavage, cell fate determination [92]. This caveat should not imply that certain gene products do not have specific addresses within the ooplasm or that a maternally derived structural complex that may serve as a storage site of regulatory proteins, including those classified as maternal effect, is an artifact. What it does mean is that such assignments need to be rigorously established.

Whether the malexpression of regulatory genes that may be of maternal origin plays a critical role in early human embryogenesis is an exciting possibility with respect to possible explanations of the variable development performance of preimplantation embryos in the same cohort. If confirmed, follow-up studies could ask whether intrafollicular conditions associated with controlled ovarian hyperstimulation could influence the function of inherited/stored molecules, perhaps by altering bidirectional communication between the oocyte and cumulus cells [93, 94] and, as a consequence, local physiological conditions (e.g., redox potential, bioenergetic or ionic state, kinase activity) in the subplasmalemmal and subcortical cytoplasm. This would represent new areas of investigation with respect to developmental competence and would be a departure from phenotype-driven evaluations of oocyte and embryo competence.

Future Directions

Certain preimplantation embryo performance characteristics are common in IVF and repeatable for the same patient during multiple attempts. Why

they reoccur despite changes protocols of ovarian stimulation or culture is unknown. While some phenotypes can be positively or negatively related to outcome, the molecular or cellular conditions that may be responsible are at a very early stage of investigation and characterization. Current transcriptional and translational microarray technology provides information on the relative expression levels of a large number of genes, but the findings are presently in the "cataloging and correlating" phase. It remains to be seen whether this methodology will ultimately have sufficient predictive power and reliability to justify routine use in IVF programs as blastomere biopsies would be required. Even if a set of transcripts or proteins could be generally associated with competence for the embryo, similar diagnostic procedures for the oocyte would likely require the extraction of cytoplasm during an ICSI procedure with selection made some time late on embryos when gene expression results became available. Whether analysis of the secretome could be of benefit in this regard remains to be validated but could be of use for section if developmental roles for candidate secreted proteins are understood and their occurrence in culture medium shown to be a normal and consistent feature of early human embryogenesis. In the same respect, gene expression levels from microarray analyses of cumulus cells that may be predictive of developmental competence for the corresponding oocyte are a promising approach [95] that needs further validation before it can be considered sufficiently sensitive and reliable to justify the cost and effort for use in routine IVF programs.

Live-cell imaging with fluorescent probes can reveal the fine details of plasma membrane and cytoplasmic organization that may be related to developmental competence. To date, this method has been shown to be an effective and informative tool in trying to diagnose instances of fertilization and early developmental failure after the fact. However, whether it can be used proactively for selective purposes is either questionable (e.g., mitochondrial stains) or unknown (e.g., CTB). For example, fluorescent-tagged CTB appears to be able to distinguish between normal and abnormal microdomain configurations that, if applicable clinically, could identify oocytes for conventional IVF or ICSI. However, for such an application, its use and detection would have to be demonstrated to have no adverse downstream consequences for fertilization or embryogenesis, and the probe itself would have to be shown to be developmentally benign. A similar demonstration would have to be undertaken for JC1 or any other mitochondrial (or other organelle-specific) reporter if the organization and distribution of high-potential mitochondria in the subplasmalemmal region, or cytoplasm in general, can indeed provide developmentally significant information for oocyte or embryo selection. While current mitochondrial probes may be problematic in this regard because of potential functional toxicity after ultraviolet illumination, JC1-stained GV stage mouse oocytes and cleavage-stage embryos examined briefly (~5 s) by conventional epifluorescence illumination at reduced intensity (using neutral density filters in the optical path) mature to MII or continue cell division, respectively (Van Blerkom, unpublished), indicating that continued study in this regard may be productive. In the same sense, mouse oocytes stained with CTB are fertilizable several hours after staining, presumably owing to the spontaneous turnover of GM1 [45]. In addition, precedents exist in reproductive biology for selection with fluorescent stains. For example, insemination with X- or Y-bearing sperm stained with intercalating fluorescent DNA probes such as DAPI and separated by fluorescent-activated cell sorting does not appear to have any adverse developmental consequences, as shown by normal offspring in the bovine where this method is used commercially. Whether procedures can be developed that take advantage of the unique reporting properties of existing probes such as those noted above, or new probes can be developed that enable the organization or activity of cellular components to be assessed without adverse consequences, presents the real possibility for competence selection based upon established cell biological criteria of normal oogenesis and early embryogenesis. Whether they can ultimately be applicable for routine clinical IVF practice warrants further investigation.

Summary

For nearly 35 years, IVF programs have correlated oocyte morphology with fertilization, embryo performance in culture, and clinical outcomes. With the exception of numerical chromosomal disorders that arise during preovulatory meiotic maturation, an understanding of how normal developmental competence is established or becomes compromised at the molecular and cellular levels in euploid embryos has advanced little. The intent of this chapter was to consider new findings and avenues of investigation that could better define and identify how developmental competence in the human oocyte is acquired. Whether these findings could lead to the use of "competence biomarkers" capable of distinguishing between competent and developmentally compromised human oocytes and embryos is the central question that future studies will determine. To this end and with respect to function, the organization of the oolemma, subplasmalemmal, and cortical cytoplasm are considered in terms of functional compartmentalization of regulatory activities and molecules and the question of whether their developmental expression may be mutually dependent and temporally and spatially coordinated for successful fertilization and embryogenesis. While some of the notions presented are speculative, mammalian oocytes and early embryos are eukaryotic cells, albeit with certain unique and stage-specific properties, but would be expected to use molecular pathways and have dynamic membrane and cytoplasmic organizations similar to those of somatic cells. As noted above, there is no a priori reason to suspect that defects detected in vitro are not also primary causes of fertilization and early developmental failure in vivo. It is therefore relevant to investigate when and how these defects may arise, especially if more frequent after ovarian hyperstimulation, and whether the types of developmental defects discussed in this chapter for the oocyte can be associated with follicle-specific intrafollicular conditions and biochemistry [95, 96, 97].

It is worth repeating that while some of the notions discussed are tentative, they can offer new avenues for human oocyte and early research that may establish a developmental biological foundation for fertilization and early developmental defects that, while long documented empirically in clinical IVF, are not well understood in terms of etiology. This may be especially important if the state of the oolemma can be linked to abnormal conditions in the subplasmalemmal cytoplasm or reflective of defects in subcortical activities that have negative consequences for development after fertilization. The most significant challenges ahead will be to establish whether such linkages exist and to determine whether fluorescent reporters used in live-cell imaging can have a place in clinical IVF.

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The Choreography of Fertilization

20

Giovanni Coticchio and Fausta Brambillasca

Abstract

At fertilization, a continuum is established between the final phases of oogenesis and the formation of a new individual. In mammals, the two processes are overlapped. The fertilizing spermatozoon represents the paternal contribution to zygote constitution and at the same time the trigger for the completion of meiosis. Oocytes can mimic fertilization, being able to recapitulate autonomously many of the events of early embryonic development. However, without the sperm contribution development to term cannot occur. The sperm, in fact, carries not only the paternal chromosomes, but also cytoskeletal elements and biochemical cues that are essential to complement and regulate the oocyte cellular legacy. Therefore, oocyte-sperm fusion creates a unique cellular machinery whose regulation in time and space influences the long term destiny of the ensuing embryo.

Keywords

Oocytes • Activation • Fertilization • Pronuclei • Development

Introduction

Fertilization is the process by which the male and female gametes recognize each other, interact, and fuse to give rise to a new cell from which a fully formed individual will ultimately develop. It, therefore, represents the bridge between generations, accomplishing the functions of reproduction and transmission of genetic variation.

From a mammalian "oocentric" standpoint, fertilization is also a time of transition, during which oogenesis is finally achieved with the completion of meiosis, while the journey of the newly formed embryo has already started through the fusion of the oocyte with the spermatozoon. The mature oocyte is at the same time the stage and the main actor of the performance of fertilization. It can in fact undergo activation, i.e., recapitulate many of the events of fertilization – including cortical granule release, completion of meiosis, and formation of the female pronucleus – in the absence of paternal contribution. Nevertheless, this choreography would be largely imperfect without the participation of the spermatozoon. This chapter describes the major events occurring

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during fertilization in human and other mammalian species. In particular, sperm-oocyte interaction, gamete fusion, activation events, pronuclear formation and development are illustrated with the intent to give emphasis to both physiological and applicative implications – i.e., in vitro fertilization (IVF) for fertility treatment – of the fertilization process.

Initial Contact: Involvement of Cumulus Cells in Fertilization

Cumulus cells have an important role in fertilization in vivo. It has been shown that the organization of the oocyte, cumulus cells, and extracellular matrix is essential for successful fertilization in the oviduct [1, 2]. In particular, infertility of female mice defective for pentraxin synthesis, a structural constituent of the cumulus mass extracellular matrix, is caused by severe cumulus mass abnormality and inability to support oocyte fertilization in vivo, but not in vitro [3]. The cumulus mass functions physically to entrap spermatozoa and guide them to the oocyte [4, 5], having a chemotactic effect probably mediated by chemokine signaling [6, 7]. Once reached the cumulus cell-oocyte complex (COC), spermatozoa search a passage through the extracellular matrix. The disassembly of the extracellular matrix, observed in vitro in the presence of nonphysiological excess of spermatozoa, may be due to sperm motility and sperm hyaluronidase activity [8] or may be regulated in an autocrine or paracrine manner by cumulus cells. In vivo, only a few spermatozoa may be found around the oocyte at any one time, a condition that probably allows persistence of an intact cumulus mass for a relative long period of time after fertilization.

Sperm–Oocyte Interaction and Fusion

Once the spermatozoon reaches the oocyte surface, the two gametes recognize, bind, and fuse with each other. In the 1970s, studies in invertebrate models suggested the concept of the existence of species-specific sperm receptor(s) on the oocyte vestment, the zona pellucida (ZP) [9]. In mammals, sperm competence to recognize the oocyte is acquired as a consequence of a posttesticular maturation process. This transformation is started in the epididymis where sperm mature, acquiring the ability to swim progressively, advance through the female reproductive tract, undergo capacitation and acrosome reaction, and finally fertilize the oocyte [10].

During capacitation, spermatozoa change their movement to a hyperactivated motility [11, 12]. At the same time, the membrane surface is extensively remodeled. Of particular importance is the activation of the signal transduction pathway of sperm protein kinase A and protein kinase C [13–15], making spermatozoa competent to bind the ZP and respond to this cell recognition event with the initiation of acrosome reaction. In mouse, the ZP is formed from three glycoproteins, ZP1, ZP2, and ZP3 [16]. Even if the molecular mechanism that directs sperm-ZP binding remains controversial, one of the most accepted hypotheses is that spermatozoa interact in a species-specific manner with O-linked carbohydrate ligands of ZP3 [17], provided that this zona protein is structurally associated with ZP2 [18]. In the last several years, a number of candidate ZP3 receptors have been identified. At present, the prevailing opinion is founded on the idea that a sperm multimeric complex is responsible for the interaction with ZP3 and that the assembly of this complex is mediated by chaperons that are themselves activated during capacitation [19–21].

Following sperm-ZP binding, the acrosome reaction is induced. The inner acrosomal membrane is exposed, and enzymes, such as a serine protease and acrosin, are secreted permitting the digestion of the ZP [22]. The hyperactivated sperm cell can then drill through the zona pellucida with sequential local zona digestion and rebinding.

The next steps are adhesion and fusion of the gametes' plasma membranes. After ZP penetration, the spermatozoon accesses the perivitelline space and begins to fuse with the oocyte surface starting from the equatorial segment, located between the inner acrosomal membrane and the plasma membrane overlying the nucleus in the posterior half of the sperm head [23]. Microvilli

present on the oocyte surface envelop the sperm head, preceding sperm–oocyte fusion. On the basis that sperm rarely fuse with mouse oocytes in regions of the oolemma lacking microvilli [24], many studies have been undertaken to find binding and fusogenic molecules present on oocyte microvilli and the sperm equatorial segment [25, 26].

Cell adhesion molecules have been proposed to be involved in sperm–oocyte adhesion, as they mediate somatic cells adhesion. In particular, a role has been suggested for integrins after the identification of an integrin ligand-like disintegrin domain in fertilin α (alpha), fertilin β (beta), and cyritestin sperm ligands [27, 28].

Fertilin α (alpha) (ADAM 1), fertilin β (beta) (ADAM 2), and cyritestin (ADAM 3) are members of the ADAM family (a disintegrin and metalloproteinase domain-containing protein). Fertilin β (beta) was originally identified in the guinea pig as an antigen for an antibody (PH-30) that blocked fertilization [29]. Fertilin α (alpha) was found to form a heterodimer with fertilin β [30]. In turn, cyritestin was originally identified in mouse [31] and monkey [32]. Subsequently, all these molecules have been identified in various other rodents and primates, but functional fertilin α and cyritestin genes have not been identified in human [33]. ADAM proteins contain a metalloproteinase domain, a disintegrin-like domain, a cysteine-rich domain, an EGF-like repeat, and a transmembrane segment with a short cytoplasmic tail. Knockout mice for the genes of three ADAM proteins showed some sperm deficiencies in oolemma-binding activity, but not fusion [34–37].

Integrins are a heterodimeric protein family of a combination formed from 18 α and 8 β subunits [38]. They have been recognized as oolemmal receptors for sperm–oocyte interaction and, in particular, as candidates to recognize sperm ADAMs.

Some integrins are expressed on the surface of mouse oocytes. $\alpha_6\beta_1$-integrin was the first identified candidate for sperm–oocyte binding and fusion [39]. This integrin is also implicated as a receptor for fertilin β and cyritestin [39–41], although studies on oocytes from α_6-knockout mice show that α_6-expression is not required for fertilization [42].

Another family of proteins, tetraspanins, characterized by four transmembrane regions and two extracellular loops, are involved in cell adhesion and other physiological processes. These proteins associate in the plane of the lipid bilayer of the oolemma with other membrane proteins, including integrins, immunoglobulins, proteoglycans, complement regulatory proteins, and growth factor receptors, forming multimolecular complexes, referred to as tetraspanin web [43]. In particular in mouse, CD9, a member of this family, is involved in gamete membrane interaction. In mouse, CD9 is localized over the entire oocyte surface, except the area lacking microvilli where sperm rarely fuse with the oocyte [44]. The same distribution pattern characterizes α_6-integrin. CD9 knockout female mice ovulate normally, and oocytes reach the metaphase II stage but are rarely fertilized. Sperm can bind to oocytes but gametes are generally unable to fuse either in vitro or in vivo [43–45]. In addition, oocytes from CD9 knockout mice can be fertilized by ICSI and give rise to embryos that develop to term, while CD9 knockout females infrequently conceive spontaneously [45]. These observations suggest that CD9 may act in a cooperative fashion with other molecules to facilitate sperm–oocyte fusion. ZP-free oocytes treated with anti-CD9 antibodies showed reduced levels of binding of sperm ligands fertilin α, fertilin β, and cyritestin [40, 41, 46, 47]. These and other data of binding assays suggest a role for CD9 in strengthening the adhesion mediated by ADAMs.

Members of the CRISP family (cysteine-rich secretory protein) are localized on sperm and are thought to be involved in fusion. CRISP1/DE was first identified in rats. It is synthesized by the epididymal epithelium and assembled on rat sperm during epididymal transit. In capacitated rat sperm, CRISP1/DE protein is localized in the equatorial segment [48] and is implicated in gamete membrane interaction. Purified CRISP1/DE protein binds to the entire oocyte surface, except the area lacking microvilli [49]. In addition, anti-CRISP1/DE antibodies prevent sperm fusion with zona-free oocytes [50].

In conclusion, several studies have been carried out to identify molecules involved in sperm–oocyte interaction. Collectively, they suggest the presence of multimeric complexes necessary for interaction on both sperm and oocyte membranes. CD9 appears to be a key component of these complexes in the oocyte. In the case of sperm, multimeric complexes seem to be particularly important, as indicated by the phenotype of fertilin β and cyritestin knockout mice. Furthermore, the fact that antibodies against such molecules cause a reduction in gamete adhesion/fusion is supportive of these hypotheses.

Block to Polyspermic Fertilization

Mammalian oocytes display different strategies to prevent polyspermic fertilization (polyspermy) and avoid the formation of nonviable polyploid embryos.

These blocks occur at the level of the plasma membrane and the zona pellucida. In nonmammals (such as sea urchins and frogs), a rapid (30–60 s) and transient depolarization of the plasma membrane potential is responsible for the membrane block (referred to as "fast block") [51]. On the contrary, in some mammalian species, the membrane block is established approximately 1–2 h after insemination [52–55] and is not generated by a change in oolemma electrical polarization [51]. Rather, inhibition of sperm–oocyte fusion may be dependent on a reduction in sperm adhesion to the oocyte plasma membrane or a detachment after brief or weak adhesion [53, 56–58]. There is evidence that changes in the organization of membrane lipids occur after fertilization [59] and also that fluidity of membrane proteins changes [60], so that molecular differences are present between fertilized and unfertilized oocytes. The use of an intracellular Ca^{2+} chelator has shown that if postpenetration Ca^{2+} signaling (see below) is gradually attenuated, oocytes are increasingly fertilized by more sperm [61], suggesting that the oolemma does not become receptive to sperm according to an all-or-none reaction, but rather that the membrane block is a graded response.

The main system assuring prevention of polyspermy in mammals involves the exocytosis of cortical granules (CGs) from the cortex of the oocyte. CGs fuse with the overlying oolemma and release their contents of enzymes into the perivitelline space (an event referred to as cortical reaction). During the cortical reaction, glycoprotein ZP2 is cleaved by a protease, while β-hexosaminidase B digests the oligosaccharide receptor on glycoprotein ZP3, so that sperm cannot bind to the zona for the absence of a receptor [61]. Furthermore, ZP tyrosine residues are cross-linked preventing proteolytic cleavage. In such a way, sperm penetration is hampered.

The Key Role of Calcium Oscillations in Fertilization

At the intracellular level, Ca^{2+} ions mediate fundamental processes in various cell types. Reports in the sea urchin *Arbacia lixula* consistent with an involvement of Ca^{2+} in fertilization date back as early as the 1930s [62], but the concept that this element was a universal signal for triggering oocyte activation emerged decisively only in the 1970s [63]. Initially in fish [64] and sea urchin [65], a release of Ca^{2+} able to promote activation after sperm-egg fusion was observed as a single and transient increase in cytosolic-free ions, crossing the ooplasm and lasting for several minutes. In mammals, Ca^{2+} changes at fertilization unfold in a different fashion, taking the form of a series of low-frequency, high-amplitude oscillations lasting for hours after sperm penetration [66]. Understanding how and why these oscillations are generated is crucial to appreciate the contribution of the spermatozoon to fertilization and pre- and postimplantation development.

Several lines of evidence suggest that Ca^{2+} oscillations are generated by release of stores of this ion stockpiled in the smooth endoplasmic reticulum. In particular, at the membrane level of this organelle, the inositol trisphosphate (InsP3) receptor appears to be centrally involved in such a mechanism, by binding its ligand and acting as a release channel for Ca^{2+} [67]. In fact, blockage of the binding ability of this receptor with a specific antibody [68] or downregulation of its expression [69] prevents the generation of Ca^{2+} oscillations in

hamster and mouse oocytes, respectively, exposed to sperm. The same effect can be obtained in mouse by inhibiting the receptor expression through injection of siRNA [70]. The critical involvement of the InsP3 receptor implies an increased phosphoinositide metabolism and InsP3 generation. This has been shown to occur in sea urchin [71] and frog eggs [72]. In mammals, technical constraints have prevented direct measurement of InsP3, but the fact that fertilization causes downregulation of the InsP3 receptor is consistent with a mechanism in which sperm penetration generates a rise in InsP3 [69]. Although several hypotheses have been formulated on how a Ca^{2+} response is orchestrated by the oocyte at fertilization, it seems now clear that after gamete fusion the initial trigger for Ca^{2+} oscillations is provided by a factor delivered and released by the spermatozoon. The original clue was the finding that the injection of soluble sperm extracts was able to induce Ca^{2+} oscillations in hamster oocytes [73]. Moreover, a factor derived from mouse sperm was shown to cause oscillations in free Ca^{2+} by inducing the InsP3 pathway [74]. The nature of this factor(s) remained elusive for years, but it is currently accepted that it corresponds to a novel form of phospholipase C, described as PLCζ, described for the first time less than a decade ago [75]. Sperm extracts immunodepleted of this activity are unable to induce Ca^{2+} oscillations in oocytes [76]. A physiological role for PLCζ (zeta) is suggested also by the fact that the amount required to stimulate Ca^{2+} oscillations in microinjected mouse oocytes is comparable to the quantity presumably contained in a single sperm [77].

The discovery of PLCζ (zeta) has been a milestone in the understanding of the involvement of Ca^{2+} in fertilization, but other aspects remain only partially explained. The question of how PLCζ can generate an oscillatory phenomenon is critical in this respect. Repetitive release of Ca^{2+} from the smooth endoplasmic reticulum might be associated to the activity of the InsP3 receptor. It has been proposed that, in the course of a single oscillatory event occurring in mammalian oocytes, the Ca^{2+} releasing activity of the receptor is initially stimulated by a stable increase in InsP3 and inhibited in a successive phase by a negative feedback mechanism elicited by the same Ca^{2+}. This model can explain why a single injection of InsP3 can induce Ca^{2+} oscillations in unfertilized mouse oocytes [78]. However, a particular characteristic of PLCζ suggests that, during fertilization, Ca^{2+} dynamics may also depend on another mechanism. Contrary to other PLCs, PLCζ is in fact promoted by very low concentrations of Ca^{2+} [79]. After gamete fusion, resting concentrations of Ca^{2+} in oocytes are probably sufficient to stimulate an increase in PLCζ activity and, as a consequence, cause a rise in InsP3 concentration. The following release of Ca^{2+} is then anticipated to enhance further this mechanism. These two models based on InsP3 and PLCζ are not mutually exclusive and together could explain the observed oscillatory changes in Ca^{2+}. In fact, the positive feedback mechanism based on the sensitivity of PLCζ to Ca^{2+} could account for the initial Ca^{2+} rise, while the negative feedback action of Ca^{2+} on the InsP3 receptor could be responsible for resetting the ion concentration to resting levels.

The diverse events that concur to the fertilization process are triggered by different intensities of the oscillatory stimulus. For example, a single transient may be sufficient to induce meiotic resumption, while repeated oscillations are required to cause a decrease in kinase activities that regulate the cell cycle [80, 81]. This implies that, irrespective of the apparatus that governs Ca^{2+} dynamics, a downstream biochemical interface is needed for translating the digital language of Ca^{2+} oscillation patterns in precise instructions, expressed in an analogue form, to the terminal effectors. Although calmodulin-dependent kinase II (CaMKII) and protein kinase C (PKC) have been proposed as possible decoders [82, 83], the way by which Ca^{2+} oscillations are finely interpreted by the fertilization machinery remains unexplained. It is interesting to note that in mouse oocytes, while a single release or short sequences of Ca^{2+} oscillation are insufficient to trigger full activation, more prolonged series of Ca^{2+} transients are much more efficient in eliciting responses associated to fertilization [84]. The question then arises on the significance of mechanisms that generate and decrypt Ca^{2+} oscillations in mammals, unlike other organisms. Studies in rodent parthenogenetic embryos and fetuses offer a possible

answer to this matter. In mouse blastocysts developed from activated eggs, the relative abundance of cells of the inner cell mass and trophectoderm appears to be influenced by the duration of exposure to Sr^{2+}, an agent which causes Ca^{2+} oscillations [85]. Furthermore, the pattern of Ca^{2+} oscillations by which activation is experimentally obtained in rabbit oocytes has been shown to influence the growth and morphology of the ensuing parthenogenetic fetuses [86, 87]. Therefore, although the most obvious reason for the occurrence of Ca^{2+} oscillations at fertilization lies in the necessity to coordinate the activation events, the dynamics of Ca^{2+} may play a role well beyond fertilization, extending over much later stages of development.

Metaphase II Arrest and Meiotic Resumption

Achievement of meiotic maturation requires a modified cell cycle mechanism, by which two consecutive chromosome segregation events occur without an intervening phase of DNA replication. Although detailed description of regulation of meiosis is given elsewhere in this book (see Chap. 13), the present paragraph offers some basic elements on the same subject in the context of the fertilization process.

In mammals, meiosis is initiated already in the fetal life but is arrested at the prophase stage shortly afterward. The process is reinitiated only at the time of ovulation, with the consequence that human oocytes can remain in meiotic prophase arrest for up to four decades. Luteinizing hormone (LH) surge at ovulation produces a signaling cascade that ultimately promotes meiotic resumption and progression to metaphase II. This effect is mediated by a characteristic dynamics of a key cell cycle regulator, the kinase activity M-phase-promoting factor (MPF), whose constituents are Cdc2 (referred to also as cyclin-dependent protein kinase 1/CDK1) and cyclin B1. At the GV stage, MPF activity is low. Its increase prompted by the ovulatory stimulus causes meiotic resumption (signified by GV breakdown) and entry into meiosis I (MI). At the transition between MI and meiosis II (MII), cyclin B1 undergoes only limited degradation, which coincides with a partial and brief reduction in MPF activity. This determines that entry into interphase and chromosome duplication are prevented, so that haploid oocytes can be ultimately generated. Exit from MI and extrusion of the first polar body is therefore followed by direct entry into metaphase II, after further synthesis of cyclin B1 and reestablishment of high MPF activity. At the metaphase II stage, the oocyte can pause for several hours waiting for the sperm [88, 89]. Metaphase II arrest is made possible by maintenance of high MPF levels. MPF activity tends to decay rapidly, a reason why in somatic cell it can be detected only transiently (30–40 min) during the M phase [90]. In fact, a soon as metaphase II is attained, cyclin B1 is usually subjected to ubiquitination by the anaphase-promoting complex/cyclosome (APC/C) and proteasome degradation. Instead, in oocytes that have extruded the first polar body, MPF is stabilized for a much longer period of time to prevent exit from metaphase II. The nature of the regulatory element responsible for MPF stabilization, referred to as cytostatic factor (CSF), has remained elusive for decades, but evidence gained in the last few years has shed new light on its molecular identity. Originally in the early 1970s [91], CSF was functionally described as an activity meeting some key criteria including (a) appearance and steady increase peaking at metaphase II during maturation, (b) ability to induce M-phase arrest in blastomeres upon injection, and (c) inactivation following augmented levels of intracellular Ca^{2+} that occur at fertilization. Studies conducted mainly in *Xenopus* over the subsequent 20 years established that CSF could coincide biochemically with a pathway including Mos, MAPK/extracellular signal-regulated kinase (MEK), mitogen-activated protein kinase (MAPK), and ribosomal S6 kinase (Rsk) [88]. However, how the Mos pathway could ultimately inhibit the activity of APC/C and thereby prevent ubiquitination and degradation of cyclin B1 was unclear for another decade. More recent evidence has suggested that endogenous meiotic inhibitor 2 (Emi2) is the missing link between the Mos-MAPK pathway and APC/C [92–94].

Emi2 is targeted by both Cdc2-cyclin B1 and the Mos-MAPK pathway. Emi2 phosphorylation by Cdc2-cyclin B causes the dissociation of the Emi2-APC/C complex [95]. The free form of APC/C is active and could induce exit from metaphase II. This effect is antagonized by the Mos-MAPK pathway, which promotes Emi2 dephosphorylation, reconstitution of the Emi2-APC/C complex, and APC/C inhibition [96]. In this regulatory network, it is important to note how a rise in intracellular Ca^{2+} can influence Emi2 activity and, by this means, promote meiotic resumption. In fact, upon fertilization, activation of CamKII by Ca^{2+} transients causes a regulatory cascade involving a series of Emi2 modifications including phosphorylation, formation of a complex with Plx-1, ubiquitination, and ultimately degradation [97]. In this manner, APC/C can be converted in the active form and drive exit from metaphase II. Future studies will clarify whether this model is also applicable to mammalian species.

Centrosome Origin and Formation in the Fertilized Egg

Following completion of oocyte meiosis with the extrusion of the second polar body (PBII), the haploid maternal and paternal chromosome complements become organized in their respective pronuclear structures. At the beginning of their life cycles, pronuclei are usually distant from each other. Therefore, on a cell scale, they need to sustain a potentially long journey before they can make contact in the innermost part of the oocyte and share their chromosomal contents upon breakdown of pronuclear envelopes. This translocation is carried out and directed by an aster of microtubules originating from a single centrosome that acts as an organizing center for microtubules.

Differences exist among mammals, but generally the centrosome of the fertilized egg includes a centriole of paternal origin and pericentriolar material (PCM) of maternal derivation [98]. Exception to this rule is the mouse in which the spermatozoon does not contribute a centriole and the centrosome is entirely maternally derived [99]. The dual origin of the centrosome observed in the fertilized egg of most species reflects the history of this organelle in oocytes and spermatozoa during gametogenesis. Early during oogenesis, the two centrioles are lost while centrosomal proteins are retained [100]. Acentriolar centrosomes are characteristically found at the poles of the metaphase I (MI) and metaphase II (MII) spindles during oocyte meiosis [101, 102]. These centrosomes include proteins, e.g., γ-tubulin and nuclear mitotic apparatus (NuMA) protein, found in the more canonical centriolar centrosomes of the mitotic apparatus. Centrosomal proteins are also dispersed in the ooplasm. They are not easily detectable, but may be observed as aggregates of increasingly larger size in response to pH and intracellular Ca^{2+} changes or stimuli that cause oocyte activation [103]. A different picture characterizes mature sperm, in which most pericentriolar material has been shed while the two centrioles, termed proximal and distal, have been retained [104]. The proximal centriole, adjacent to the basal plate of the sperm head, is distinguished by nine triplets of microtubules [105] and some pericentriolar proteins, including γ-tubulin [106]. This is the sperm centriole that will exclusively contribute to the constitution of the centrosome of the fertilized egg, upon fusion of the two gametes [98]. The distal centriole, positioned perpendicularly to the proximal centriole and coaxially to the sperm flagellum, is regarded as a degenerated structure because it does not show an intact nine-triplet organization, typical of normal centrioles [105]. It also includes appendages, whose function is still little understood, that are normally found connected to the mother centriole of mitotic centrosomes [107].

Once the fertilizing spermatozoon has been incorporated into the oocyte, maternal and paternal constituents cooperate for the assembly of the zygotic centrosome. Complementary contribution from both parents after selective reduction/modification of centrosomal components during gametogenesis probably responds to the need to avoid the presence of two complete centrosomes at the time of gamete fusion, an event that would lead to tetrapolar spindles during the first mitosis

and disruption of chromosome segregation in the two daughter blastomeres. Following gamete fusion, an early step in centrosome formation is the proteasome-mediated detachment of the sperm proximal centriole from the distal centriole [108]. Proteasome activity at the centrosomal level appears critical because its inhibition has been shown to be associated with abnormal aster formation. Once released, the paternal proximal centriole represents the nucleating force of the rising centrosome. Although some γ-tubulin of paternal origin is associated to the sperm centriole, in the fully formed centrosome, most of this protein is recruited from a diffused ooplasmic reservoir [109]. The amount of maternal γ-tubulin that progressively organizes around the paternal centriole is crucial for centrosomal function, because it determines the size and function of the microtubular aster that drives pronuclear relocation [101, 110]. The aster is formed from microtubuli anchored with their minus ends to the centrosome, while their plus ends progressively grow and radiate to distal ooplasmic locations to make contact with pronuclei. In this fashion, the cytoskeletal network that allows the essential process of pronuclear repositioning is generated. However, centrosomal and aster function embraces other aspects of fertilization. The nucleating capacity of the centrosome and the plasticity of the aster, together with associated motor proteins, represent a logistic infrastructure by which organelles (e.g., mitochondria) and macromolecules are displaced, reassorted, and regulated. An example of that is the centrosomal protein PMC1 and associated factors, such as centrin and pericentrin, which are recruited from distal locations, conveyed along microtubules through a dynein-mediated mechanism, and concentrated in the pericentriolar compartment where they participate in the recruitment of γ-tubulin [111].

Pronuclear Formation, Migration, and Breakdown

Pronuclear formation and development is the crucial process during fertilization by which the parental chromosome complements merge and give rise to the zygotic genome. Pronuclei are formed shortly after gamete fusion. They grow, migrate, and juxtapose over several hours and undergo breakdown immediately before the first cell cleavage. The entire process has been observed in detail in human, initially by conventional microscopy [112] and more recently by time-lapse microscopy [113]. Perturbations in time or space of the unfolding of these highly regulated steps are often diagnostic of developmental failure [114].

Before pronuclear formation, the maternal and paternal chromosome sets follow different paths. In the mature oocyte, the meiotic process is arrested at the metaphase stage of the second division. Activation induced by the spermatozoon prompts resumption of meiosis, separation of bivalents, and elimination of one array of chromatids through the extrusion of the PBII. This sets the stage for the formation of the female pronucleus. Chromatids, while still localized near the point of emission of the PBII, establish contact individually or in groups with membranes probably derived from the Golgi apparatus and become progressively incorporated into vesicles. Immediately after their assembly, these vesicles coalesce to form the female pronucleus. The preliminaries of male pronuclear formation are different. The DNA of the fertilizing sperm is in fact associated with protamines, proteins rich in lysine and arginine required for tight chromatin packaging in the sperm head. Protamines must be removed and replaced by histones before the male pronucleus may be formed [115]. Studies performed with leftover material derived from IVF treatments have described in detail the dynamics of male pronuclear formation in human [112]. Immediately after incorporation into the oocyte cytoplasm, the sperm head undergoes a phase of decondensation occurring progressively according to an anterior-posterior axis and signaled by decrease in opacity and loss of definition of its outline. These two changes in the sperm head are followed by swelling of the chromatin mass whose projected area increases sevenfold in about 60 min. During these steps, it is presumed that disulphide bonds linking sperm proteins are reduced, protamines are shed, and chromatin is decondensed in a more relaxed conformation. Afterward, the mass of the decondensed chromatin

regresses to form a rounded shape, decreasing to a size that however remains larger than one of the original sperm head. This phase of re-condensation is caused by packaging of the sperm DNA with maternal histones and occurs in a time frame of 40–60 min. The re-condensed sperm head then undergoes a second wave of expansion accompanied by appearance of a distinct outline. In the following 30–60 min, numerous small masses of sperm chromatin appear as nucleolar precursors. In such a way, the male pronucleus is formed.

Studies in the rhesus monkey suggest that the application of intracytoplasmic sperm injection (ICSI) to achieve fertilization may affect the process of sperm head decondensation [116, 117]. In fact, when a spermatozoon is microinjected into an oocyte, in some cases the condensation of the apical (acrosomal) part of the sperm head is delayed, perhaps as an effect of partial persistence of the acrosomal cap, which vice versa is completely shed during standard IVF before the spermatozoon penetrates through the zona pellucida. A delay in the disassembly of the acrosomal cap and the underlying perinuclear theca might have significant implications for the remodeling of paternal chromatin. In particular, it might cause a lag in processing of apical chromatin during pronuclear formation, an area where the X chromosome chromatin is preferentially located [118, 119]. Together, these factors might interact and ultimately play a role in the generation of a small increase in sex chromosome aneuploidies detected in children born from ICSI treatments [120]. In microinjected rhesus oocytes, in which delayed chromatin decondensation is observed, formation of the zygotic centrosome and the microtubular aster is unaffected either structurally or temporally [116], a sign that during pronuclear formation the male chromatin does not regulate the egg cytoskeleton. This is consistent with the observation that aster formation is noticed also in oocytes in which the sperm head fails to decondense completely [121].

In human, in general male and female pronuclei are formed almost synchronously. In standard IVF, early observation of pronuclear formation is prevented by presence of cumulus cells surrounding the egg, but after ICSI – case in which cumulus cells are removed – pronuclei become visible as early as 3 h postinsemination (p.i.). More commonly, they may be first observed between 4 and 7 h p.i., while formation delayed to beyond 10 h p.i. is highly likely to be associated with developmental failure. Time-lapse microscopy has had a fundamental role in determining pronuclear dynamics [113]. Usually, the two pronuclei are formed at different locations in the fertilized egg. The female pronucleus emerges invariably next to the site where the PBII is extruded, while formation of the male pronucleus can occur anywhere, depending on the site where gamete fusion has occurred or the spermatozoon has been deposited after microinjection. In mouse, sperm penetration is inhibited in the area surrounding the point of emission of the PBII. Therefore, the male pronucleus is always formed at a significant distance from the female pronucleus. In human, pronuclear formation is accompanied by a still poorly understood cytoplasmic phenomenon described as circular waves of subcortical contractions, occurring 2–10 times with a periodicity of 20–50 min [113]. Another cytoplasmic event, referred to as cytoplasmic flare and concomitant with pronuclear formation, is the propagation across the cell of a single wave of contraction [113]. Considering that this wave usually originates from the site of appearance of the male pronucleus, it is tempting to speculate that it represents a manifestation of the radiation of the sperm aster. Once pronuclei are formed, they are displaced by cytoskeletal forces to establish mutual contact (Fig. 20.1). In particular, the female pronucleus is drawn toward the male counterpart. This phase, occurring in most cases between 6 and 9 h p.i., is crucial. In fact, fertilized eggs that fail to assist pronuclear juxtaposition (Fig. 20.2) are destined to develop into embryos affected by massive blastomere fragmentation and early developmental arrest [122, 123]. Pronuclei may juxtapose while they are still positioned eccentrically and only subsequently be transported to a central or paracentral position. Alternatively, they are moved independently, from separate peripheral positions to the center, where they finally make contact. Peripheral displacement of pronuclei after their initial central localization is also predictive of developmental failure (Fig. 20.3). During the

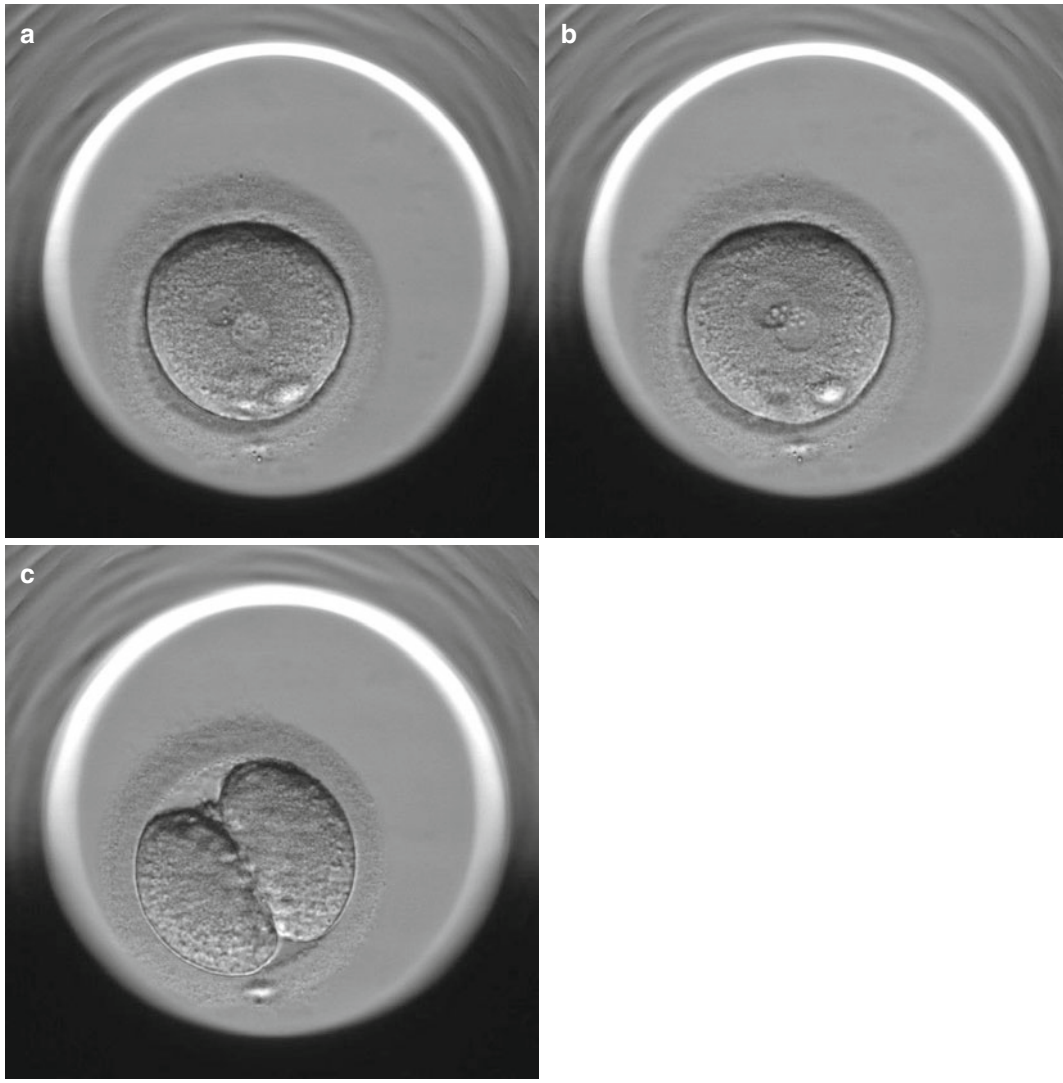

Fig. 20.1 Human fertilized egg showing juxtaposed pronuclei in which chromatin is organized in four to seven nucleoli aligned along the pronuclear contact area (**a**). This organization, which is normally maintained during the later stage during fertilization (**b**), is suggestive of higher implantation ability of the ensuing embryo (**c**)

phases of transportation and juxtaposition, pronuclei increase in size. The diameter of the male pronucleus changes from approximately 16 μ to over 24 μ(mu)m, while the female pronucleus reaches a size of about 22 μm at the end of the growth phase.

Chromatin also rearranges during pronuclear development. In both pronuclei, initially it becomes visible as small masses that subsequently aggregate in nucleoli. Nucleoli are continuously motile while undergoing partial coalescence and alignment along the edge of the nuclear envelope where pronuclei juxtapose. It has been suggested that chromatin organization at such an early stage of development may be predictive of embryo viability. In particular, at the time of fertilization check during a human IVF procedure (16–18 h p.i.), it appears that embryos with higher chances of establishing a pregnancy are characterized by nucleolar alignment along the juxtaposition area

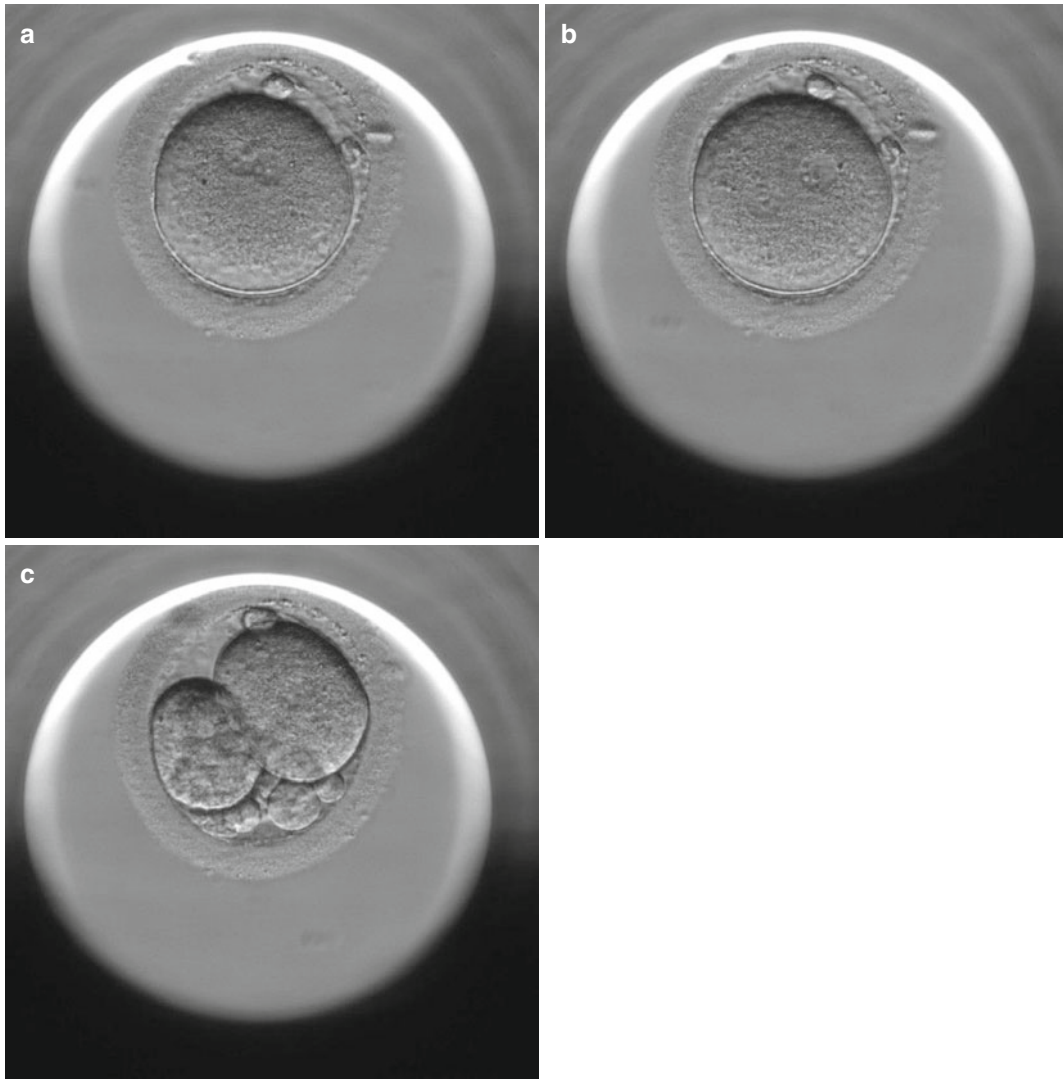

Fig. 20.2 Human fertilized egg in which the process of pronuclear juxtaposition failed. Pronuclei initially juxtaposed (**a**), but separated afterward (**b**). The first cleavage generated two blastomeres of different sizes (**c**), a phenomenon associated with poor developmental competence

of the two pronuclei and a number of nucleoli comprised between four and seven in both pronuclei (Fig. 20.1) [114]. Several studies seem to confirm the developmental significance of nucleolar organization [122, 124], although the underlying biology remains elusive.

Pronuclear and nucleolar development are extensive and active processes requiring extraordinary mechanical and energetic support from the egg. This justifies the massive organelle rearrangement that accompanies pronuclear migration from the periphery to the center. In particular, the entire population of mitochondria is reasserted, driven by movements that are believed to be under the control of dynein molecules shifting on tracks of microtubules [125]. These organelles, which provide the major ATP energy source for mechanical and biochemical processes, may be found aggregated in clusters of various sizes throughout the cytoplasm in mature unfertilized

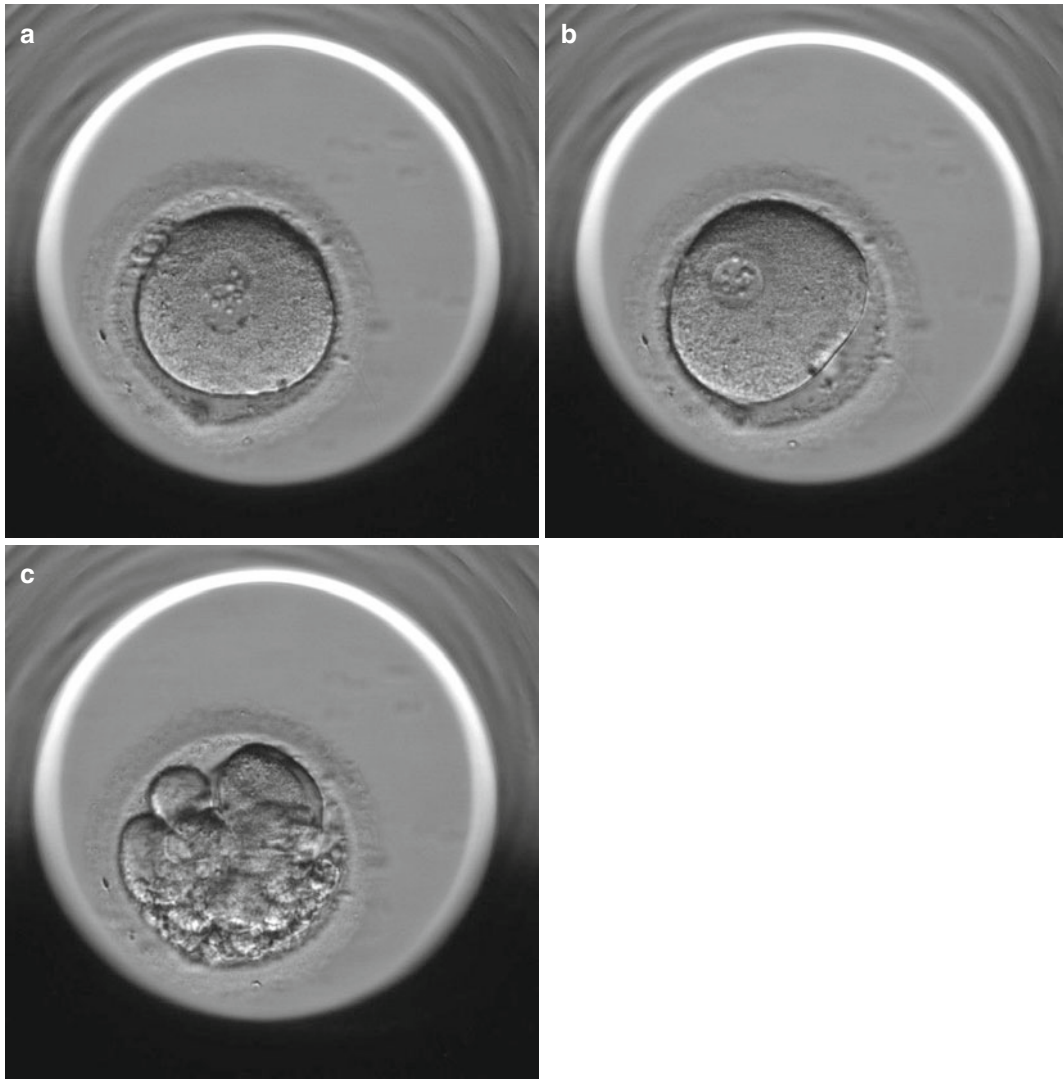

Fig. 20.3 Human fertilized egg. Pronuclei were initially localized in a central position (**a**), but were subsequently displaced to the cortex (**b**), The resulting two-cell embryo was affected by very severe fragmentation (**c**)

oocytes of the rhesus monkey [126]. With the onset of fertilization, they are progressively relocated toward more central positions forming two distinct masses of accumulation along either side of the surface of pronuclear juxtaposition. This arrangement seems to have a developmental significance because in its absence extensive blastomere fragmentation has been observed after the 8-cell stage of the ensuing embryo. Mitochondrial accumulation around the pronuclei has been observed also in the human fertilized egg [127]. Other organelles, such as tubuli and cisternae of the endoplasmic reticulum, are rearranged in a similar fashion, with the effect that starting from 8 to 10 h p.i., the cortical domain, left relatively deprived of subcellular structures, appears as a thinner cytoplasmic halo under conventional microscopic observation [124]. Likewise pronuclear position and nucleolar arrangement, formation of a cytoplasmic halo has been proposed to be predictive of higher implantation potential. As fertilization progresses, the egg also undergoes a significant overall contraction corresponding to a decrease

in diameter of 5–10 μm [113]. This massive phenomenon is not well understood but might be generated by the same mechanisms that control centripetal organelle displacement.

Pronuclear juxtaposition/relocation in central position, formation of the cortical halo, and contraction of the cortex are followed by an apparently uneventful phase lasting several hours in which movement of nucleoli is the only obvious manifestation of cellular activity under light microscopy observation. In reality, as early as 8–10 h p.i., chromosomal DNA starts to duplicate. The S phase of the fertilized eggs extends over 4–7 h and is usually completed by 14–17 h p.i. [128]. At the end of this phase, the 2*n*/4C DNA content is established in preparation for the first cleavage. The timing of DNA synthesis has practical implications with respect to the practice of zygote cryopreservation in human IVF. Irrespective of whether controlled rate freezing of vitrification is applied, during cryopreservation the fertilized egg is subjected to massive physical and biochemical stress that could affect DNA integrity in a phase of synthesis in which this macromolecule is particularly vulnerable. Therefore, cryopreservation should be postponed at around 20 h p.i., at a time when DNA duplication has been completed.

Once DNA duplication has been achieved, the fertilized egg is equipped to deliver two equivalent sets of chromosomes to each of the blastomere of the 2-cell embryo. This requires simultaneous breakdown of pronuclei in order for chromosomes to be released in the cytoplasmic compartment and pooled together in a single set. Pronuclear breakdown usually occurs at around 23–25 h p.i., but its time range is included between 19 and 35 h p.i. [129]. Two to three hours before the breakdown of pronuclei, the cortical cytoplasm undergoes a short event of contraction that accompanies the disappearance of the cortical halo. This might signify a redistribution of mitochondria, endoplasmic reticulum, and other organelles. Once in direct contact with the cytoplasmic environment, chromosomes are progressively recruited onto the mitotic spindle that meanwhile is assembled in a central position.

The Final Act of Fertilization

Fertilization is concluded by the first mitotic division. Geometrically directed by the central position of the mitotic spindle, the cleavage furrow divides the zygote cytoplasm into two blastomeres of equal size. Observations in human suggest that timing, geometry, and regularity of the first cleavage are indicative of the destiny of the ensuing embryo. In human IVF, early timing of the first cleavage appears to be positively associated with implantation ability. In 1997 for the first time, in a retrospective study, a higher pregnancy rate was reported in transfers whose embryos underwent the first cleavage by 25 h postinsemination [130]. The existence of a positive association between early cleavage and implantation ability was subsequently confirmed by a number of studies [131–133]. The recent introduction in routine clinical embryology of time-lapse microscopy (TLM) has opened entirely new opportunities to interpret the significance of embryo developmental morphokinetics, allowing monitoring at short intervals (2–20 min) rather than very few observations at fixed time points. It has been reported that, together with other parameters, the duration of the first cytokinesis is predictive of embryo developmental ability [134]. In particular, embryos that develop to blastocyst perform the cleavage process within a precise time interval (14.3 ± 6.0 min). In addition, embryos able to reach the blastocyst stage start and finish the first cytokinesis in a smooth, controlled fashion. On the contrary, in embryos destined to cleavage arrest, the first cytokinesis occurs over a long period of time, and the cleavage furrow is accompanied by extensive membrane instability. Meseguer et al. [129] have also adopted TLM to predict embryo implantation ability, concluding that in embryos with higher implantation ability, the first cleavage occurs between 24.3 and 27.9 h p.i. and daughter blastomeres are comparable in size. Therefore, it appears that the first cleavage, i.e., the final act of fertilization, is already to some extent informative of embryo developmental ability.

Conclusions

Fertilization joins the final phases of the oocyte life with the first steps of the journey of a newly formed individual. The oocyte is therefore central to the process of reproduction. However, the role of the male gamete is crucial, not only to restore biparental diploidy. In fact, the paternal-derived PLCζ and centriole provide biochemical and cytoskeletal cues that orchestrate distinct fertilization events, such as meiotic resumption and pronuclear formation and migration. In such a way, the legacies of the female and male gametes lay the foundations for successful development.

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Determinants of Oocyte Quality: Impact on In Vitro Fertilization Failures

21

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Abstract

Fertilization failures diminish the number of zygotes available for fertility treatment. When considering the potential etiologies of fertilization failures, oocyte determinants merit careful attention. Multiple oocyte-borne defects may hinder successful fertilization. The list of cellular and molecular components that render an oocyte competent for fertilization is growing, among which are the meiotic spindle and chromosomes, organelles, a calcium response machinery, structural and accessory proteins, a redox state, bioenergetic stores, and signaling or regulatory proteins. Specific instances are presented, along with the timing of when during development the oocyte progressively acquires these elements, which together endow the oocyte with full fertilization potential. Key changes in the fertilization potential of the oocyte occur within the confines of the developing follicle, and under the influence of its components. The developmental transitions that are relevant include: oocyte growth, pre-maturation, maturation, post-maturation; together, these transitions lead up to a window of maximal fertilizability that is followed by post-ovulatory aging. Along with basic studies, a careful evaluation of failed to fertilize oocytes has

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augmented our understanding of oocyte determinants of fertilization success, such as ones involved in cytoskeletal remodeling, sperm aster formation, and pronuclear assembly and dynamics. Identifying the exact cause(s) of fertilization failure can not only facilitate diagnostic efforts but also tailor potential therapies. A mastery of in vivo and in vitro factors influencing the acquisition of fertilization potential is essential to the optimal retrieval and handling of oocytes.

Keywords
Oocyte quality • Fertilization failure • Oocyte development • IVF • ICSI Zygotes • Infertility • ART • Gametes

Fertilization Failures

Despite the improvement of in vitro fertilization techniques in the last years, fertilization failure or suboptimal fertilization rates are a recurrent phenomenon that has been historically mainly explained in terms of oocyte's chromosomal abnormalities. In humans and most mammalian species, fertilization takes place at metaphase of the second meiosis (MII), a stage at which oocytes are arrested after maturation. Oocyte quality is critically important for successful fertilization, and a variety of different criteria have been used to assess its quality. However, in many cases, the reasons for fertilization failure remain unclear and cannot be determined based on presently available morphological and molecular/biochemical data [1]. For this reason, it is important to evaluate specific factors that are known to play a role in oocyte quality and affect successful fertilization. Recently, several studies focusing on mammalian oocytes analyzed by confocal microscopy techniques under fluorescence showed different types of fertilization failures after conventional in vitro fertilization (IVF) and intracytoplasmic sperm injection (ICSI) [2–5]. In these reports, sperm chromatin remodeling during decondensation, oocyte activation, meiotic resumption, and pronuclear migration have been described in humans. As first defined by Asch et al., Simerly et al., and Van Blerkom et al. [6–8], fertilization failure in mammals may have different etiologies. Firstly, the injected oocyte can fail to initiate the biochemical processes necessary for oocyte activation (activation failure) [9]. Alternatively, this process may be initiated but may not occur normally, leading to an incomplete or abortive activation. Further, the spermatozoon may remain poorly accessible to oocyte factors required for chromatin decondensation and formation of the female pronucleus [10–12]. Finally, either the structural problem at the level of the zona pellucida causing the block to polyspermy after IVF or the injection within the oocyte metaphase plate after ICSI can occur, albeit at a low frequency.

In an effort to establish the causes of fertilization failure in humans, the use of epifluorescence and confocal microscopy permits a sophisticated and precise analysis of stages at which human fertilization fails. In brief, immunofluorescence analysis may require removal of the zona pellucida (depending on the targeted antigen) by a short incubation in Tyrode's acid solution. Denuded oocytes are then fixed and permeabilized in a microtubule-stabilizing buffer. Anti-α-acetylated tubulin monoclonal antibodies can be used to identify the sperm tail, and anti-β-tubulin-Cy3 monoclonal antibodies for analysis of the meiotic spindle. Chromatin can be identified by counterstaining with Hoechst 33258 or TOTO-3. Before DNA staining, the TUNEL technique can be applied to the samples in order to explore the degree of DNA fragmentation (sperm DNA, oocyte chromosomes, or both can be affected). The processed material is mounted between

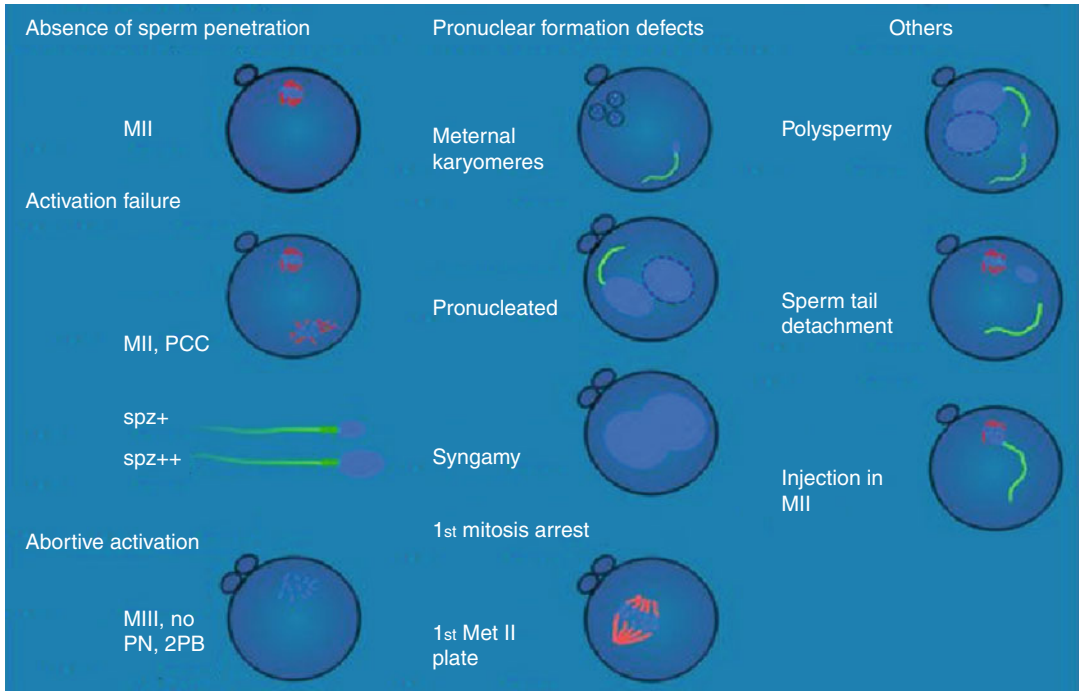


Fig. 21.1 Representation of stages at which human fertilization fails after IVF and ICSI. Six different categories were identified after immunofluorescent analysis of oocytes that did not show pronuclei 20–40 h after insemination or injection. Within some of the categories, several levels were detected and schematically represented as seen under the microscope. *MII* or *MIII* metaphase II or III oocytes, *PCC* premature chromosome condensation, *2PB* second polar body, *PN* pronuclei, + indicates the degree of sperm decondensation while in the oocyte (Obtained with permission from Rawe et al. [5])

a slide and a coverslip and examined using epifluorescence or confocal microscopy [3].

Flaherty et al. [13] and Liu et al. [14] have shown that total failed fertilization is a rare event after ICSI (3 %) and is more frequent in cycles in which only one or two oocytes are injected. The risk of failed fertilization reduces from 37 %, when one oocyte is injected, to only 0.8 % when five or more oocytes are injected. The rationale of the cellular analysis of fertilization failures is to try to elucidate cellular/molecular aspects of the failed interaction of both gametes to understand the etiology of each particular case. With a clear diagnosis in hands, clinicians can take an appropriate conduct in future cycles and even include therapeutic tools to improve the prognosis.

Using immunofluorescence, we studied a total of 871 "non-fertilized" human oocytes after IVF and ICSI, in which no pronuclei were visualized, and, in some cases, the extrusion of the second polar body had not occurred after 20–40 h post-insemination or sperm injection. The work is part of previously published results [4, 5, 15–17] and can be summarized in different categories as represented in Fig. 21.1. Among the total failed fertilized oocytes, the absence of sperm penetration was the main reason after conventional IVF (57.2 %). Microtubule and DNA analysis revealed that 32.1 % of the IVF-failed oocytes initiated the fertilization process but arrested at specific stages. On the other hand, 10.7 % of the "non-fertilized" IVF oocytes showed more than one sperm within the ooplasm. These results agree with those previously described [18, 19].

The main reason of fertilization failure after ICSI was oocyte activation failure (39.5 %). The sperm contains a temperature-sensitive cytosolic

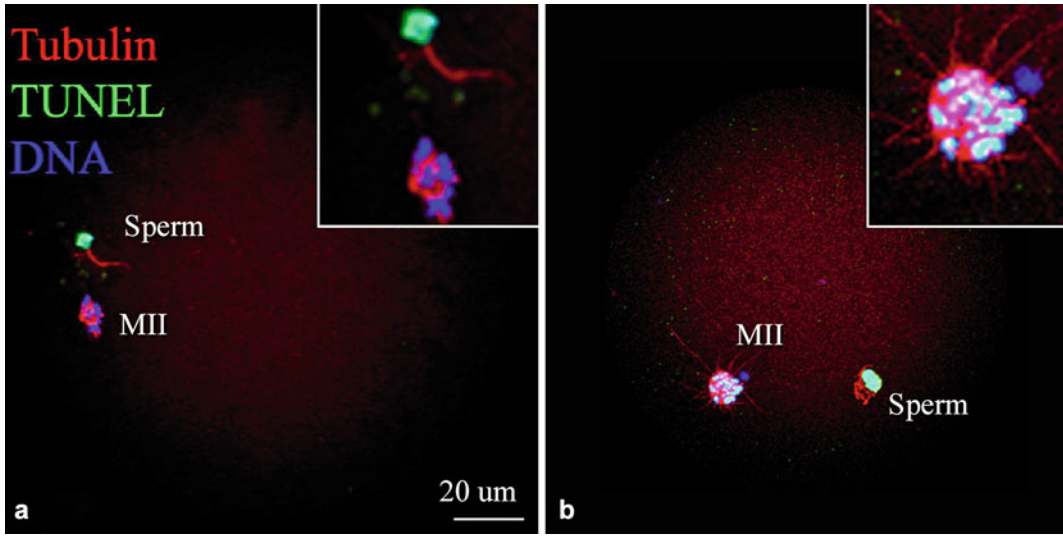


Fig. 21.2 Confocal microscopy analysis of fertilization failures after ICSI. In both cases, oocytes are arrested at metaphase II (*MII*) stage, and a lack of sperm chromatin decondensation with fragmented DNA (*green*) is visualized. Tubulin is visualized (*red*) as microtubules of the spindle (itself associated with chromosomes in *blue*) and unpolymerized subunits (background like aspect) throughout the cytoplasm. Non-fragmented and fragmented oocyte chromosomes (**a** and **b**, respectively) are shown as magnified images in *upper right corners*. The TUNEL technique was used to identify DNA fragmentation (*green*)

factor called "oscillin," which triggers activation when injected into the oocyte [20–22]. Today, the sperm-borne trigger candidate common to all animal oocytes at activation is phospholipase C (PLC), zeta 1 [23–25]. The absence of this factor or the lack of activity in "weak" sperm as those used in ICSI procedures could partly explain the activation failure observed after ICSI [26–28]. One of the most striking types of sperm defect that cause lack of oocyte activation is the "round-headed" sperm or globozoospermia. As the focus of the present chapter is on oocyte's related causes, the reader can refer to Dam et al. [29] for this specific sperm abnormality.

After sperm injection, there was a significantly higher rate of DNA fragmentation in sperm that was partially decondensed compared to the sperm that was not [19]. This observation may either suggest a correlation between the rate of DNA fragmentation and the underlying severe male factor present in ICSI semen samples or could represent the incomplete remodeling of the sperm chromatin by oocyte factors. The latter alternative could be linked to an inappropriate capacity of oocytes to transform the compact sperm chromatin into a fully developed male pronucleus. Unpublished observations showed up to 50 % of ICSI failures carrying sperm DNA fragmentation visualized by TUNEL (Fig. 21.2). Interestingly, among the studied materials, 34 % of the oocytes also showed some signs of DNA fragmentation at the MII plate.

Premature chromosome condensation (PCC) was observed in 8 % of the oocytes studied after IVF and ICSI. Schmiady et al. [30] originally described this process, and the essential prerequisites are no oocyte activation and the presence of condensing factors (e.g., high levels of M-phase promoting factor, MPF) in the ooplasm, in turn preventing the transformation of sperm nuclei into pronuclei. Metaphase II arrest in mammalian oocytes is supposed to be maintained by persisting high concentrations of MPF stabilized by a cytostatic factor (CSF) [31]. MPF inactivation is one of the principal events in oocyte activation that prevents the male pronucleus from entering metaphase prematurely.

The term "abortive activation" was used to describe an event occasionally observed after IVF and ICSI (less than 2 %), which is characterized

by the formation of a third metaphase plate (MIII) and the inability to progress to interphase. It has been shown that MIII formation is clearly dependent of the rhythm of Ca^{2+} oscillations that drive oocyte activation. Perturbation in frequency and/or amplitude of Ca^{2+} repetitive spikes leads to abortive activations [32].

Defects in pronuclear formation and/or migration have been found at a similar incidence after IVF and ICSI (32.1 and 28.3 %, respectively). Schatten [33] have shown that this situation can arise from an inability in the assembly of the microtubules around the paternal centrosome (see section on "Ooplasmic Factors During Fertilization"). An arrest at metaphase plate of the first mitosis of the embryo (first mitosis arrest in 21.1) is more frequently observed after ICSI than IVF (11 % vs. 6 %, respectively). One possible explanation is that during the sperm injection (ICSI), the correct pattern of calcium oscillations is perturbed in the ICSI oocyte, causing an arrest in the first mitosis more frequently than with IVF failures. In human oocytes, the early calcium reaction to the spermatozoon is characterized by an initial group of three to six rapidly occurring calcium spikes which is then followed by lower frequency spikes for the rest of the calcium period [34]. The difficulties for ICSI oocytes to go through cell cycle checkpoints could be the consequence of a high disruption of Ca^{2+} oscillation pattern. In mouse, ICSI-generated zygotes cleaved at a slower rate had lower cell numbers and lower hatching rates. ICSI and IVF induced similar initial $[Ca^{2+}]i$ responses, although ICSI zygotes exhibited shorter durations of $[Ca^{2+}]i$ oscillations and showed diminished degradation of IP3R-1. ICSI-induced $[Ca^{2+}]i$ responses are not equivalent to those initiated by IVF, and they may thus have developmental consequences [35].

Given the complex nature of the cellular interactions at fertilization, failure may occur at one or more steps; defects are often tied together and difficult to distinguish. The causes may also stem from the oocyte and/or the sperm, thereby necessitating the need to identify the culprit in instances of fertilization failure. We focus the rest of this chapter onto oocyte factors that may help explain clinical instances of fertilization failure.

Oocyte Development: A Time to Become Fertilizable

The oocyte undergoes a remarkably long and complex journey prior to reaching MII in a state that renders it fully competent for fertilization. A myriad processes prepare the oocyte for fertilization; thus, a deficiency in any one or more event(s) will render the oocyte refractory to fertilization and possibly result in fertilization failure in the laboratory. All of the preparatory events occur during the protracted timeline of oogenesis within the unique and highly differentiated milieu created by the follicle. Thus, to reach an understanding of fertilization failure, we must consider the developing follicle and its influences on oocyte quality (i.e., developmental competence or the ability to become fertilized and support early embryonic development). With such knowledge, we may be able to manipulate follicles and strive for the retrieval of oocytes that can all be fertilized. The follicle consists of several compartments, including the cumulus-oocyte complex, follicular fluid, and granulosa cells. A large body of evidence has demonstrated the determining influences of the differentiation state of the follicle on the quality of its enclosed oocyte [36–38]. Follicle development is a characteristically dynamic process; in a cyclic fashion, follicles grow and differentiate as a group or cohort while a single follicle will be selected and continue development as the dominant or preovulatory one. Consequently, all follicles within a recruited cohort (other than the dominant one) are not naturally selected for ovulation; it is thus not surprising that during routine IVF, oocytes are retrieved from some follicles that have not completed the developmental program necessary to support an oocyte to full competence. Retrieved oocytes may be thus intrinsically deficient in preparatory steps that are essential for fertilization, thereby potentially accounting for many instances of oocyte-borne fertilization failure.

Figure 21.3 summarizes the cellular and molecular attributes that render an oocyte prepared to support fertilization, and Fig. 21.4 depicts the developmental transitions that ensure the making of a fertilizable oocyte during follicu-

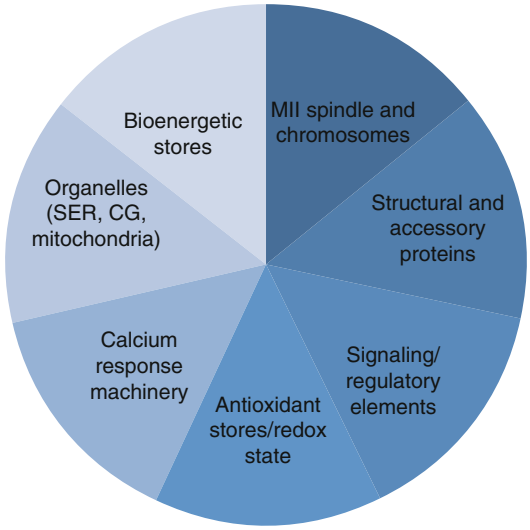


Fig. 21.3 Cellular and molecular components that render an oocyte fully competent for fertilization. As presently understood, some of the main elements that provide the oocyte with fertilization potential are depicted as pieces of a pie; there are however functional overlaps between some of the categories as for instance with the SER, signaling proteins, calcium response machinery, and bioenergetic stores. Each category is discussed throughout the chapter, including the timing of their acquisition (see Fig. 21.4). The importance of the oolemma, zona pellucida, and cumulus cells is not represented here. *MII* metaphase II, *SER* smooth endoplasmic reticulum, *CG* cortical granule

logenesis and oogenesis. The accompanying text that elaborates on both figures follows.

Oocyte Growth

In human and large domestic species, the bulk of oocyte growth occurs prior to antral follicular development (in primary and secondary preantral follicles), although a little growth continues in the antral follicle [39–42]. The oocyte grows while arrested in prophase of meiosis I, and its growth phase is characterized by the storage of cytoplasmic components, including organelles and molecules necessary for fertilization [43–45].

Several organelles accumulate and undergo modifications, including their positioning toward the periphery of the oocyte at a site most suited to their functions. Of relevance to fertilization competence are mitochondria, smooth endoplasmic reticulum (SER), and cortical granules. Given the entirely maternal inheritance of mitochondria at fertilization, the full-grown oocyte becomes endowed with a very large number of them (after multiple rounds of replication), making it the most abundant organelle in the oocyte [46]. Cortical granules, upon their regulated secretion, are responsible for blocking polyspermy during fertilization [47]. The contribution of an extensive SER network to fertilization is presented below (under "Oocyte Maturation"). Oocyte growth is a time when the zona pellucida is synthesized as the extracellular matrix that is crucial for gamete interactions during routine IVF [48–50]. It is also during oogenesis that the layer of cumulus cells becomes highly specialized, rendering its associated oocyte fertilizable [51]. The growing oocyte also experiences a remarkable accumulation of ribosomes, RNA transcripts, and proteins, some of which are required for the continued differentiation of the oocyte itself and others for fertilization [45].

Through the growth phase, the oocyte becomes meiotically competent or able to resume meiosis in a stepwise fashion. Depending on the species, meiotic competence may not be attained until antral folliculogenesis, a period during which the oocyte will continue acquiring developmental competence. Many of the modifications taking place during oocyte growth are obligatory for later differentiation events when the oocyte becomes endowed with further specializations for fertilization.

Oocyte Prematuration

Even when nearly fully grown, the oocyte experiences fine-tuning events, including ones required to support fertilization. These final steps occur immediately prior to and during oocyte maturation, which refers to the progression from prophase I to MII in response to the LH surge. The developmental phase prior to meiotic resumption is referred to oocyte capacitation or prematuration [44, 52–54]. Oocyte prematuration entails molecular events during which transcripts and proteins are stockpiled in the ooplasm, in anticipation for their use during fertilization and later development. Even though oocyte prematuration is now established as a prerequisite to the development of fully competent oocytes, its exact nature remains poorly defined. Notably, the list of molecular modifications is likely

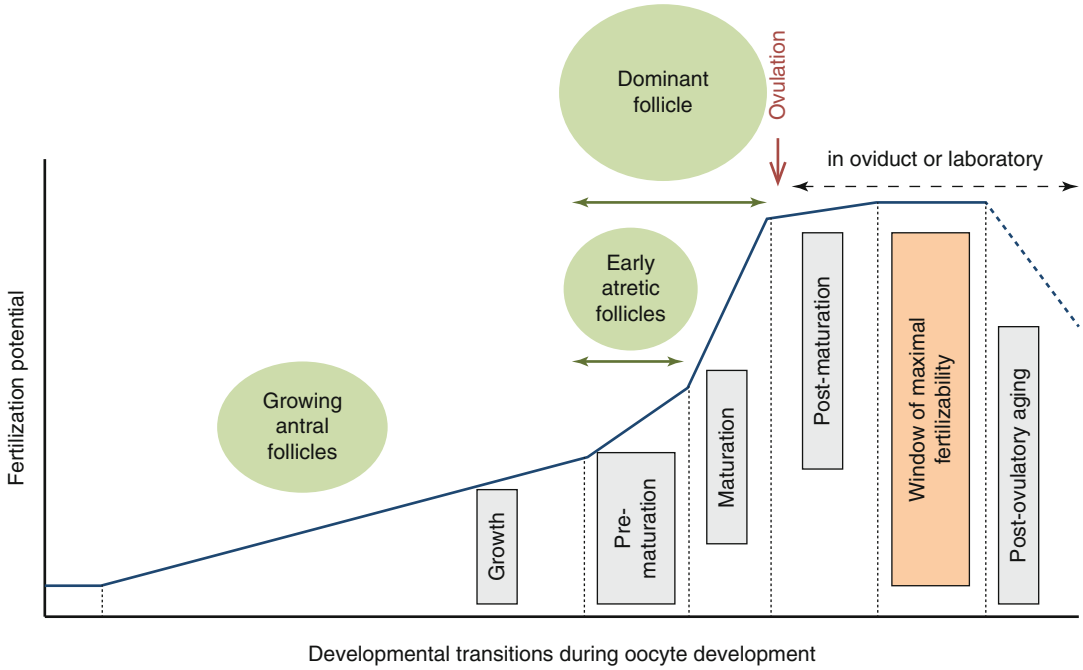


Fig. 21.4 Developmental continuum in the life of an oocyte when its fertilization potential changes. Only relative changes in the ability of an oocyte to sustain fertilization are depicted on the Y-axis. Shown above the *graphed line* are the times when each developmental transition occurs within follicles of a certain type (growing, early atretic, or dominant), the oviduct, or in culture following oocyte retrieval. The steepness of each segment on the line attempts to approximate the extent of fertilizability that is gained by the oocyte during the respective developmental period. Furthermore, each transition depends on the previous one being completed, culminating in the formation of an oocyte of maximal potential. Supporting details are provided in the section entitled "Oocyte Development: A Time to Become Fertilizable"

more exhaustive than currently envisaged. Modifications and rearrangements of organelles (including cortical granules that are necessary for the block to polyspermy at fertilization) also take place during prematuration [40, 44].

Oocyte prematuration and maturation occur in the final stages of antral folliculogenesis, and both processes are influenced by a follicular microenvironment that has become highly differentiated. Only a dominant follicle that is one selected as the preovulatory one will provide the necessary signals, somatic support cells, and follicular fluid to enable the oocyte to complete its prematuration and maturation. More specifically, prematuration takes place during the final differentiation phase of antral folliculogenesis, while the dominant follicle is in a stationary growth phase and poised to receive the preovulatory LH surge [37]. Interestingly, an oocyte in the preovulatory follicle is not the only one capable of supporting fertilization and early development; indeed, oocytes in follicles showing early signs of atresia exhibit a developmental competence that is not only comparable to an oocyte in a preovulatory follicle but also superior to oocytes in all other follicles [55–58]. Mechanistically, the unique microenvironment of early atretic follicle would mirror the one of preovulatory follicles, thereby supporting the final prematuration steps of the enclosed oocyte. This phenomenon is of relevance to oocytes retrieved during routine ART, since aspirated follicles are likely in various stages of atresia, some of which may be early atretic. The heterogeneity in follicle sources may thus underlie the inability of a proportion of retrieved oocytes to undergo fertilization, more precisely due to the failure to complete some of the essential prematuration events. Prematuration merits further attention; a thorough

understanding of its processes and regulation should shed light on not only causes but also treatment of fertilization failure. Notably, ovarian stimulation and oocyte retrieval methodologies during ART should be aimed at permitting the complete differentiation of the oocyte (by enabling a plateau phase following follicular growth).

Oocyte Maturation

The final preparatory steps that the oocyte undergoes prior to fertilization occur in response to the ovulatory signal, during oocyte maturation, and within the confines of the preovulatory follicle in vivo. Oocyte maturation itself can proceed thanks to a series of developmental processes that took place during growth and prematuration; it includes both nuclear and cytoplasmic events, all of which impart on the oocyte its full developmental competence. During nuclear maturation, the oocyte progresses from prophase I arrest through meiosis I before arresting again at MII. Cytoplasmic maturation entails morphological and biochemical changes that are under the critical influence of the antral follicular milieu [36, 54]. Cytoplasmic maturation is not yet completely defined, but despite some of its elusiveness, cytoplasmic maturation is unequivocally imperative for fertilization. Further attesting to the complexity of making an oocyte of full competence is the need for nuclear and cytoplasmic maturation events to be coordinated [59]. Indeed, a recurring theme is the accumulation of cytoplasmic components while the oocyte is progressing through maturation, such that by the time MII is reached, the bulk of cytoplasmic events are completed. Nuclear and cytoplasmic events must thus occur along an optimal timeline, the specific sequence of which we do not yet fully grasp. Indeed, nuclear and cytoplasmic events are not necessarily always interdependent; for instance, a cytoplasmic modification may not be strictly dependent on a nuclear stage. This has been shown for the ability to sustain calcium oscillatory patterns [60] and the reorganization of ER clusters in the oocyte cortex [61]. Noteworthy is the need for at least germinal vesicle breakdown, particularly when it comes to cytoplasmic specializations that are necessary to render the oocyte fertilizable [59]. Cytoplasmic maturation is a multifaceted process, but a few specific changes merit mention when considering the maternal underpinnings of fertilization. We will largely focus on the accumulation of the antioxidant glutathione and the acquisition of a calcium-dependent response.

Every cell must maintain a redox environment that permits normal cell function, and the oocyte does not escape such rule. Pertinent to fertilization is the reliance of sperm processing on maternal stores of glutathione; indeed, sperm decondensation entails the exchange of protamines for histones, a process that begins with the reduction of disulfide bonds of protamines, in turn causing their release from sperm chromatin [62, 63]. The ooplasm must thus provide a strong reducing environment, which is achieved, thanks to a large concentration of the small tripeptide glutathione (GSH). The levels of GSH vary during oocyte development, notably during oocyte maturation with highest GSH found in hamster MII when compared to earlier meiotic stages [64]. Functional evidence includes the inability of rodent and bovine oocytes depleted or deficient in GSH to become fertilized, with a specific failure in sperm decondensation [62, 65, 66]. Most recently, a mouse model with the expression of glutamate cysteine ligase or *Gclm* (the rate-limiting enzyme in GSH synthesis) lacking only in oocytes resulted in compromised fertilization, with a decreased incidence of paternal pronucleus formation; concomitantly, maternal GSH was low in the ooplasm of ovulated oocytes [67]. Interestingly, GSH may ensure the normal function of the maternal meiotic spindle, and in turn the formation of a single female pronucleus at fertilization [68], although conflicting results exist [66]. While species-specific requirements and/or variations in the extent of GSH depletion may explain different responses, the sensitivity of fertilization to oxidative stress also merits attention.

Another fertilization event that is deeply rooted in oogenesis is the process of oocyte activation. While triggered by a sperm factor [23, 24], activation can only proceed in an oocyte that contains all of the necessary elements for the myriad calcium-dependent responses [69, 70]. Upon entry into the oocyte, the sperm factor (now identified as phospholipase C zeta or PLC-ζ)

triggers the formation and subsequent release of 1, 4, 5-inositol triphosphate (IP3) from the maternal oolemma. The second messenger IP3 then binds to its receptor (type I IP3R), itself a calcium channel at the oocyte's smooth endoplasmic reticulum (SER). Lastly, once released from SER stores into the ooplasm, calcium ions act as another second messenger molecules that in turn regulate cell cycle molecules (themselves stockpiled during oocyte development), exocytosis, maternal RNA recruitment, and protein translation machinery [70], or histone assembly onto sperm chromatin [71], all of which are events crucial to fertilization. An immature oocyte arrested in prophase I does not yet possess such potential in contrast to the later stages of maturation [60, 72]. To elicit a satisfactory response, development must endow the oocyte with an extensive SER network [73] that is enriched in IP3-gated calcium channels (IP3-receptors), itself biochemically modified [74–77]; sequesters a high concentration of calcium ions [60, 72]; and is placed in the proper locale (in the oocyte cortex, near the site of sperm-oocyte fusion) within the large volume of the cell [61, 78–81]. Evidently, it is only by the end of meiotic maturation that the oocyte possesses all of these cellular and molecular features that will enable the sperm PLC-ζ to trigger the release of internal calcium stores into the ooplasm [69, 79, 82, 83]. Furthermore, fertilization is characterized by not just a single release of calcium from maternal SER stores, but rather, an oscillatory activity must exist so as to allow a species-specific pattern of increases and decreases in cytoplasmic calcium. This oscillatory activity may be rooted in oogenesis, with the search for the controllers of calcium influx and their origins still ongoing [84]. Further adding to the complexity of calcium control is the demonstrated contribution of oocyte mitochondria to calcium signaling at fertilization [85]. As aforementioned, a large proportion of fertilization failures are attributed to defects in oocyte activation; evaluation and treatment of such failures must thus consider inherent deficiencies in the calcium-signaling machinery of the oocyte.

A sufficient maternal store of ATP is expectedly required for fertilization, since it is an energy-consuming process; for example, energy is needed to sustain calcium oscillations (for the reuptake of calcium into SER stores) and to allow pronuclear formation (for the assembly of histones onto paternal chromatin). There is thus an increase in ATP consumption at fertilization. Interestingly, ATP levels are tightly controlled via a coupling mechanism between mitochondrial ATP supply and demand; as a result, the oocyte may lessen the potential risks associated with increased ROS generation, in turn maximizing fertilization competence [85, 86]. Maternal mitochondria may indeed play a determining role in the ability of mature oocytes to be fertilized. Some clinical evidence supports associations between fertilization outcomes and number of mitochondrial DNA genomes [87, 88], or mitochondrial gene expression [89]. In the pig animal model, the control of mitochondrial DNA replication during oocyte development may also influence fertilization [90, 91]. Although awaiting further evidence, another cytoplasmic event that promotes successful fertilization may be the establishment of high-polarized mitochondria in the maturing oocyte [92, 93]. The normal biogenesis of mitochondria thus merits attention during oocyte development, notably with respect to its impact on fertilization competence [94].

Fertilization is a tightly regulated and rapid process that relies on maternal control mechanisms established during oocyte maturation. Signal transduction components accumulate and become positioned properly within the oocyte, including a large number of protein tyrosine kinases [95]. Downstream signaling molecules are also central to the activating effect of calcium oscillations at fertilization [70, 96]. Any abnormalities in transcription, translation, control of activity, and positioning of signal transducers during oocyte development will eventually cause fertilization failures.

There are other maternal influences that shall not be overlooked, including the accumulation of proteins necessary at fertilization, such as histones (for sperm chromatin remodeling), lamins (for pronuclear formation [97]), and γ-tubulin (for sperm aster assembly [33, 98, 99]). For

example, it is during oocyte maturation that the oocyte acquires the functional activity required to replace protamines with maternal histones [71, 100, 101]. More precisely, the mRNA transcripts of oocyte factors necessary for histone assembly accumulate during the growth phase of oogenesis, but their translation only occurs upon meiotic maturation. Not discussed here but presented elsewhere [1] is the machinery developed in the oocyte for sperm-oocyte binding and fusion. While already present in sufficient numbers, cortical granules do not yet assume their final and necessary cortical distributions (for exocytosis to block polyspermy) until completion of meiotic maturation [102, 103]. Another type of cytoplasmic immaturity, albeit not yet defined at the molecular level, has been documented as underlying a certain type of fertilization failure, notably an arrest prior to sperm chromatin remodeling. For instance, a cytoplasmic state that would prevent inactivation of the M-phase cell cycle regulator (maturation-promoting factor or MPF) would then favor the premature chromosome condensation (PCC) of sperm [104–106]. Other instances when the cytoplasmic endowment of the oocyte influences fertilization events are presented in a separate section below (e.g., cytoskeletal dynamics, nuclear formation).

A complete nuclear arrest of the oocyte may also explain a fertilization failure. All retrieved oocytes may be arrested at prophase I in the germinal vesicle (GV) stage, or metaphase I (MI), or a mixture of both GV and M-I [107]. Not having reached MII in spite of ovarian stimulation, these oocytes are expectedly unable to be fertilized. Although maturation arrests occur during a developmental period that precedes fertilization, they remain relevant to consider during any cycles of fertilization failure. Indeed, what may be grossly described as a failure in fertilization may simply stem from the retrieval of immature oocytes rather than a specific impairment at fertilization. Based on animal studies, there are several molecular candidates (i.e., factors that control meiotic progression) that may be perturbed and cause a complete maturation arrest [107, 108]. Diagnostic efforts have pointed toward spindle organization defects in some instances [109–111], or chromosomal aberrations [109, 112–114]. Studies to date consist of case series or reports that attempt to define the defect and document management and/or treatment strategies. Albeit instrumental to augmenting our collective knowledge of maturation arrest issues, none of these studies have yet pinpointed a single culprit or mutation that would explain a failure to mature to the index fertilizable MII stage. Adequate cell cycle control in the oocyte is evidently pivotal for fertilization to ensue.

Beyond Metaphase II

Along the same line, oocytes may fail to arrest at MII and progress to a cell cycle stage that is incompatible with fertilization. For instance, any oocyte that undergoes spontaneous activation has exited M phase, and such premature resumption of meiosis II has been described in a recurring case of complete fertilization failure [115]. This case also illustrates the need to undertake a routine analysis of failed to fertilize oocytes, particularly in situations of uniform and recurrent failures even following ICSI. The knowledge obtained can then be used for management, as for example, counseling the patients toward oocyte donation whenever a clear oocyte-borne defect is at play.

The retrieval of a cohort of oocytes at a uniform time post-hCG may not allow all of the oocytes to reach a similar, and complete, maturity state. This may occur due to variations in the differentiation states of punctured follicles (due to current ovarian stimulation protocols), thereby potentially accounting for varying maturity states and fertilization potentials across a cohort. Even once the oocyte has reached MII and it has presumably completed the developmental program to support fertilization, some penultimate differentiation events still take place prior to fertilization. After all, it is not immediately following its release from the ovary that the ovulated oocyte interacts with sperm and becomes fertilized, but rather after a delay period in the oviduct. Once in the oviduct, further cellular/molecular alterations may take place in the oocyte as for instance a final maturation of the zona pellucida, a process

that actually began during folliculogenesis [116]. The oocyte may also continue to acquire full activation competence during the protracted period of MII arrest, as demonstrated in the mouse [117]. Routine laboratory practices may allow additional cytoplasmic alterations during MII arrest that is during a short delay prior to sperm encounter. Indeed, fertilization rates appear to benefit from a preincubation of MII oocytes between the times of retrieval and either insemination (IVF) or sperm injection (ICSI) [118–122].

While permitting a delay prior to insemination or sperm injection may enhance cytoplasmic maturity and in turn fertilization, too long of a lag may prove detrimental [122]. Detrimental changes occur during post-ovulatory aging, and given the risks and benefits, a balance must thus be struck. Studies in a mouse model support significant modifications in cellular elements necessary for fertilization [123]. Indeed, calcium oscillations become altered (with aberrant calcium release and reuptake across the ER) [124, 125], and the regulation of intracellular ATP regulation at fertilization differed [126] in in vivo-aged oocytes. Alterations in calcium stores and oscillations were confirmed during the in vitro aging of mouse oocytes, together with compromised activation [127, 128]. Further attesting to the disrupted activation of mouse oocytes aged in vitro are dysfunctions in several later activation events: the completion of meiosis II, exocytosis of cortical granule exocytosis, changes in zona pellucida, and recruitment of maternal mRNAs [129]. These aging-associated alterations may thus compromise the ability of some oocytes to be fertilized (notably ones that are more vulnerable depending on their initial quality). To constitute a clinically relevant problem, consideration should be of course given to the length of aging under study. Some of the deteriorations may not occur until more than a day such as for cytoskeletal perturbations [130, 131]; thus, these are not directly pertinent to routine fertilization practices in the ART laboratory. However, several of the aforementioned studies did test post-ovulation times that demonstrate the ephemeral nature of fertilization competence, with deleterious changes detected as early as 4–6 h in the mouse [124–126, 128, 132, 133]. Confirmatory results await in the human.

Also noteworthy is the fact that a mature oocyte with full developmental competence may still fail to fertilize. Besides the changes associated with in vitro aging, there is a temporal window within which activities that were acquired during oocyte development can act at fertilization. For instance, the remodeling of sperm chromatin can only take place after several hours following activation [101]. In the same vein, activation itself may induce further modifications that are necessary for completion of the multiple events of fertilization; an instance exists in sea urchin oocytes with the rearrangements of cytoplasmic membrane domains at fertilization, thereby permitting assembly of the male pronuclear envelope [134]. Preparatory events thus continue in the oocyte, even during fertilization.

Ooplasmic Factors During Fertilization

Cytoskeletal and Chromosomal Abnormalities of the Oocyte

The oocyte cytoskeleton is critically important for successful fertilization and for all stages of subsequent development. Therefore, it is important to evaluate specific factors that are known to play a role in oocyte quality and affect successful fertilization. The most prominent structure of the MII oocyte is the MII spindle containing the maternal chromosomes that are aligned at the metaphase plate and connected to centrosomes at the opposite spindle poles by kinetochore microtubules (kMTs). Along with nucleation of kMTs from spindle pole centrosomes are pole-to-pole microtubules that are important for chromosome separation [17]. The integrity of spindle fibers as well as centrosome is an important criterion for oocyte quality as it reflects the general condition of the individual oocyte including oocyte aging that may occur during the process of IVF [135].

As the oocyte lose centrioles during gametogenesis, the MII spindle is organized by acentriolar centrosomes consisting of numerous maternal centrosomal proteins, including γ-tubulin, centrin, and nuclear mitotic apparatus (NuMA). In

humans, the MII spindle is localized perpendicular to the cell surface, and it is a barrel-shaped to pointed structure. Although it appears static in immunofluorescence and TEM images, the MII spindle is a highly dynamic structure that maintains its shape by a complex set of regulatory kinases and other regulatory proteins [135]. The main functions of the MII spindle are to precisely separate chromosomes and extrude one set of sister chromatids into the polar body; consequently, diploidy is restored after fertilization when the sperm contributes the paternal set of chromosomes. Thus, any failure in MII spindle functions can result in cellular and developmental abnormalities that may lead to abortion, disease, or birth defects [135]. Misaligned chromosomes may result from dysfunctional microtubules and spindle proteins. For instance, recent studies in human oocytes have clearly shown the importance of oocyte-derived NuMA in the correct assembly of the MII spindle and functions of the male pronucleus [99, 136, 137]. The studies by Alvarez Sedó et al. [136] carefully monitored the presence of NuMA during human oocyte maturation; furthermore, abnormal MII spindles were formed, and fertilization failure took place after chemical inhibition of NuMA.

The MII spindle is therefore a crucial structure that requires precise regulation by important ooplasmic factors (centrosomal proteins, molecular motors, kinases, and phosphatases) and sperm during fertilization. An intact MII spindle is a key criterion when assessing oocyte quality [2].

Sperm Aster Formation During Fertilization

One of the essential cytoplasmic structures that human sperm contribute to the oocyte during fertilization is the centrosome. Still not completely understood at the structural and functional levels after more than 100 years since its discovery, the centrosome serves as the dominant microtubule-organizing center in the majority of animal cells [138]. Consisting of an orthogonal pair of centrioles and surrounded by an amorphous cloud of pericentriolar material, the centrosome nucleates microtubules from a central structure known as the γ-tubulin ring complex [139]. In most mammalian species, including humans, the centrosome degenerates in the oocyte and is retained in the sperm during the maturation process [33].

Following the insemination of oocytes, the sperm centrosome, which lies attached to the sperm nucleus in the oocyte cytoplasm, nucleates microtubules to form a structure known as the sperm aster. Defined as a radial array of microtubules anchored at the centrosome, the sperm aster grows and expands to reach both the surface of the female pronucleus as well as the oocyte cortex [33]. Contact of the sperm aster with the cortex is thought to generate the force necessary to propel the male pronucleus to the center of the oocyte [140]. Microtubule association with the surface of the female pronucleus would in turn facilitate the migration of the female toward the male pronucleus that completes the fertilization process [33]. Thus, the paternally inherited centrosome plays a pivotal role in establishing and supporting the microtubule-based motility within the zygote. A relevant aspect of the disassembly of the sperm aster is the mandatory presence of maternal proteasomes. The ubiquitin-proteasome pathway is the main cytosolic proteolytic system responsible for the regulated substrate-specific degradation of most cellular proteins in eukaryotic cells [141]. The mechanism by which the complex structure of the sperm tail connecting piece disintegrates to release the proximal centriole after sperm entry into the oocyte is not fully understood, but the proteasomes of the sperm and the oocyte are implicated [142–144]. Release of a functional sperm centriole that acts as a zygote microtubule-organizing center relies on selective maternal proteasomal proteolysis.

Formation and Dynamics of Pronuclei

A striking feature of the cell cycle is the separation of the nuclear and cytoplasmic compartments. With either IVF or ICSI, the assembly of the nuclear envelope (NE) after fertilization starts with membrane-free chromatin organized in two distinct entities: a set of maternal chromosomes and the sperm nucleus [145, 146]. Subsequent steps mirror ones described during NE assembly in somatic cells: (1) membrane vesicles assemble

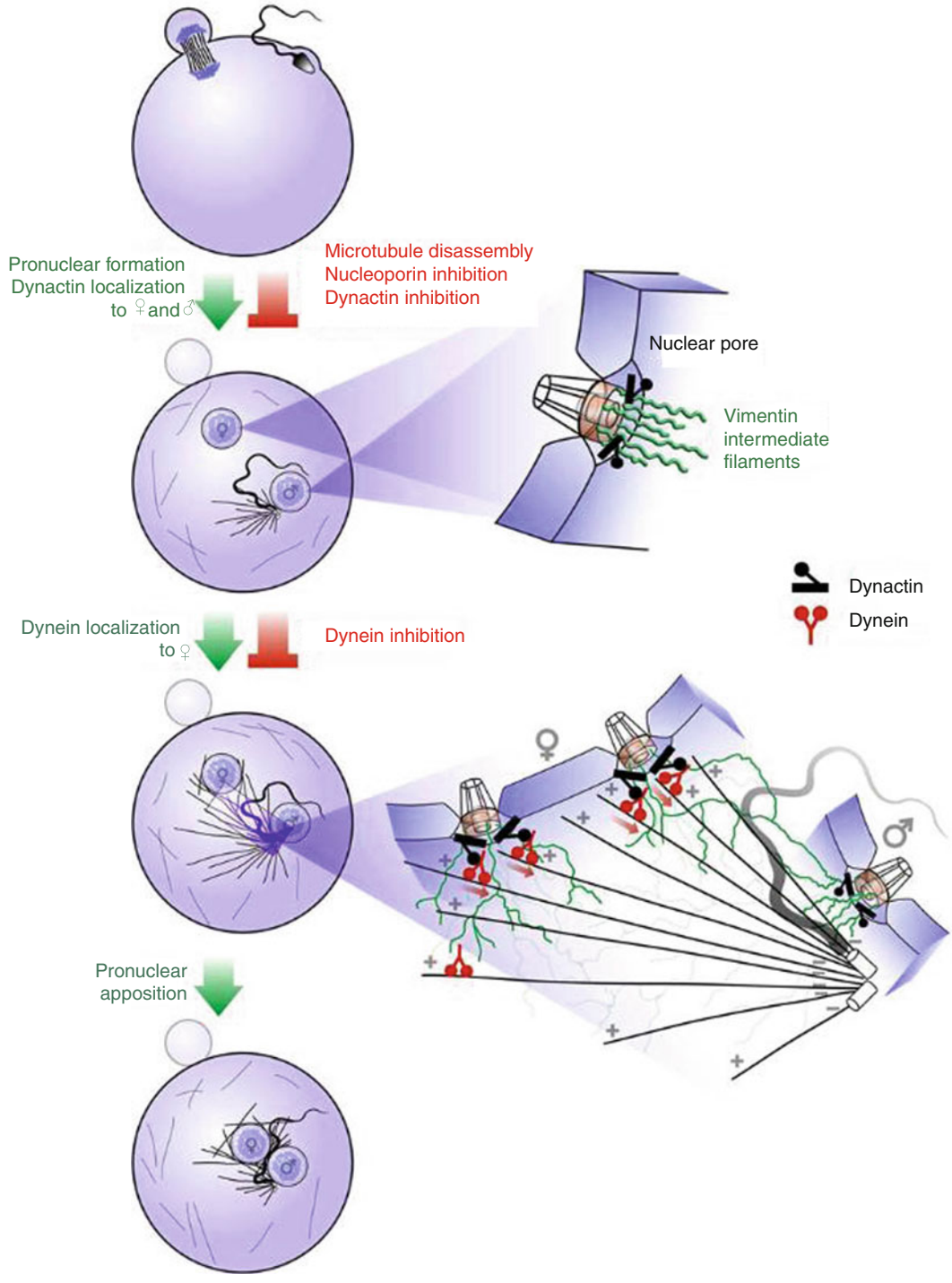
and fuse to form a continuous NE around forming female and male pronuclei ; (2) the nuclear pore complexes (NPC), assembled from the specific set of glycoproteins called nucleoporins, are incorporated into the NE and provide the channels for bidirectional nucleocytoplasmic trafficking between the newly established nuclear and cytoplasmic compartments; and (3) nuclear lamins are imported into the nucleus and form the scaffold of nuclear lamina underneath the NE.

Although the assembly and fusion of membrane vesicles during NE formation have been studied extensively in somatic cells and in a cell-free system [147], little is known about the pathways leading to the formation of NPC on the zygotic NE, especially in mammalian models. Previous studies in bovine have demonstrated that the fertilizing sperm triggers the assembly of oocyte annulate lamellae (AL), in parallel with NPC insertion into NE [146]. Similar results have been obtained in humans [12] where AL (the cytoplasmic stacks of NPC) were studied using a nucleoporin-specific antibody. During fertilization arrest at 2PN stage, the NPC assembly was disrupted, whereas AL were clustered in the zygote cytoplasm into large sheaths. This was accompanied by the lack of NPC incorporation into the nuclear envelope, suggesting that aberrant assembly of NPC and AL coincides with early developmental failure in humans.

Pronuclear migration and apposition (the events concluding the fertilization process) are followed by the breakdown of pronuclear envelopes, the formation of a mitotic spindle, and the first interactions between the maternal and paternal DNA. As it was previously discussed, the molecular pathway by which the microtubule-organizing center recruits maternal components to form a fully developed sperm aster is slowly becoming better understood in mammalian zygotes [148]. Oocyte's molecular motors appear to have prominent roles in spindle assembly and maintenance, with molecules such as dynein/dynactin, minus-end-directed motor protein HSET, and NuMA crucial for preserving proper spindle structure and function [149]. Hall et al. [150] characterized the developmental potential of aged oocytes by studying essential microtubule proteins involved in meiotic and mitotic division. RT-PCR in single oocytes was performed to determine expression of oocyte-specific genes and microtubule markers [nuclear mitotic arrest (*NuMA*), minus-end-directed motor protein *HSET,* and the microtubule kinesin motor protein *Eg5*]. Immunocytochemical analysis revealed that many oocytes displayed aberrant expression of NuMA and Eg5 and had disrupted meiotic spindles and tetrapolar mitotic spindles. The authors concluded that aberrant maternal proteins can cause mitotic spindle defects that may contribute to poor embryo development.

Contrarily to rodents, bovine, nonhuman primate, and human zygotes rely upon sperm asters to mediate genomic union [151, 152]. Another maternal contribution to pronuclear apposition and normal spindle formation is the requirement for molecular motors dynein and dynactin, as demonstrated in bovine and nonhuman primate models [153]. Given the known interactions among microtubules, motor proteins, intermediate filaments, and nuclear pore complexes, Payne et al. [153] suggested that dynein and dynactin bind to nucleoporins and vimentin at the cytoplasmic surface of the female pronuclear envelope to mediate pronuclear migration along sperm aster microtubules. A model has been proposed for pronuclear assembly, motility, and union with dynactin, nucleoporins, and vimentin, interacting together with microtubules and dynein (Fig. 21.5). Nuclear pore complex assembly (accumulated as annulate lamellae (AL) in the oocyte cytoplasm) and insertion into the envelopes of newly forming pronuclei bring dynactin and vimentin filaments (all from the ooplasm) to the cytoplasmic face of nuclear pores, where they interact as a macromolecular complex.

The apposition of both pronuclei depends upon sperm aster-independent microtubules, appearing within the ooplasm after oocyte activation and contacting the surfaces of the pronuclei during their assembly. The sperm aster, meanwhile, concomitantly develops as a focused set of microtubules radiating out from the centrosome, now serving as the dominant microtubule-organizing center in the zygote. Enlargement of the sperm aster extends the microtubule plus ends away


Pronuclear formation
Dynactin localization
to ♀ and ♂
Microtubule disassembly
Nucleoporin inhibition
Dynactin inhibition
Nuclear pore
Vimentin
intermediate
filaments
Dynactin
Dynein
Dynein localization
to ♀
Dynein inhibition
Pronuclear
apposition
♀
♂

from the male pronucleus, some of which then reach the female pronuclear envelope. Growth of the sperm aster toward the cortex could deliver dynein to the surface of the female pronucleus on these microtubule plus ends, allowing it to bind to dynactin and vimentin at nuclear pores and activate its motor activity. The dynein-dynactin complex would then be able to transport the female pronucleus to the sperm aster minus ends, culminating in pronuclear apposition [153].

Understanding these protein interactions may shed light on certain cases of clinical idiopathic infertility in which inseminated oocytes arrest in development after the pronuclei fail to unite [6, 12]. As it has been mentioned before, in approximately 6 % of human fertilization failures discarded after IVF-ICSI, the sperm aster shows abnormal morphology within the zygote [144]. Both incomplete assembly and disarrayed organization of sperm aster microtubules would compromise the association of dynein with the female pronucleus [153]. Should microtubules fail to bind to the female pronucleus, preventing dynein from localizing to its surface, genomic union would be unsuccessful. Dynactin associates with nucleoporins and vimentin at the surfaces of both pronuclei upon their formation and throughout the fertilization cell cycle. Dynactin concentration around zygotic pronuclei, meanwhile, resembles its localization to prophase nuclear envelopes in somatic cells, where it is thought to facilitate nuclear envelope breakdown [154, 155]. Dynein, in contrast, depends upon sperm aster microtubules to associate to the female pronucleus where it interacts with dynactin, nucleoporins, and vimentin to facilitate pronuclear motility. To our knowledge, neither dynein nor dynactin has previously been reported to associate with interphase nuclear envelopes during mammalian fertilization. Because migrating pronuclei are in S phase, dynactin concentration around pronuclear envelopes is likely to persist throughout zygotic interphase, revealing a unique functional association during fertilization. The interaction between dynactin and nuclear envelope proteins common to both pronuclei, together with the spatial distribution of dynein to these proteins at the female pronucleus, may ensure successful genomic union that completes mammalian fertilization.

Concluding Remarks

The oocyte becomes fertilizable during the protracted and complex developmental journey of oocyte growth, prematuration, maturation, and fertilization. Any exogenous factors with negative

Fig. 21.5 A model for pronuclear assembly, motility, and union. Sperm entry activates the mature oocyte, leading to second polar body extrusion (*top*, first polar body not shown). Formation of pronuclei is accompanied by sperm aster-independent microtubules (second from *top*, *left*), which bring dynactin (*black rectangle* with side arm) and vimentin filaments (*squiggly green lines*) to the cytoplasmic face of the nuclear pore complex (*basket structure*) (enlargement, *upper right*). Disassembly of microtubules and inhibition of nucleoporins and dynactin block proper complex formation at the outer surfaces of both pronuclei. Growth of sperm aster microtubules, nucleated by the centrosome attached to the male pronucleus, extends microtubule plus ends away from the male pronucleus, some of which then reach the female pronuclear surface (second from *bottom*, *left*). These microtubule plus ends could deliver dynein (*red wishbone*) preferentially to the surface of the female pronucleus, allowing dynein to bind to dynactin and vimentin at nuclear pores, and enabling the dynein-dynactin complex to transport the female pronucleus to the minus ends along the sperm aster (enlargement, *lower right*). The inhibition of either dynactin or dynein blocks migration and prevents apposition (*bottom*) (Reproduced/adapted with permission from Payne et al. [153])

influences (such as an inappropriate follicular environment) or intrinsic oocyte abnormality will compromise the fertilizing potential of a subset, or the entire cohort, of retrieved oocytes. During its development, the oocyte becomes equipped with complete cellular/molecular machinery that proves adequate for successful fertilization. We do not yet fully grasp all of the events underlying fertilization failure, and the list of maternal determinants that ensure fertilization success is expected to grow with additional research. Despite extensive research in the area of human reproductive biology, much is still to be understood at the cellular level of how oocytes control the assembly and disassembly of sperm's components. Understanding the role of the cytoskeleton during ICSI is an important step to improve fertilization. The sperm contributions to fertilization failure also merit further attention.

During any routine IVF or ICSI cycle, there is a small proportion of oocytes that remain unfertilized. An inability to become fertilized may stem from an immaturity (at the nuclear, cytoplasmic, and/or membrane level) in some of the retrieved oocytes. Extensive and important changes occur in the oocyte during pre- and post-ovulatory moments; indeed, the temporal window within which a human oocyte would possess maximal fertilization competence remains to be established. Once precisely defined in vivo (while the oocyte remains in the follicle) and in vitro (while awaiting sperm addition), we will be in a better position to improve the efficiency of the technique, thereby helping reduce the occurrences of fertilization failure in the laboratory.

There are also the unusual cases of total fertilization failure, including the particularly challenging situations when a complete failure recurs across multiple cycles. In these cases, diagnostic efforts (including a thorough evaluation of both gametes) prove helpful, particularly when it comes to managing the patients effectively. Once again, an augmented understanding of oocyte determinants is imperative given that further evaluations have attributed unexplained cases of complete fertilization failures as due to an oocyte-intrinsic abnormality [115, 156]. In instances of elevated fertilization failure, evaluating the cause underlying the failure would help detect inherent oocyte problems, which may include ones described herein or other yet unknown factors. As a result, targeted treatment strategies may be envisaged, and their efficacy and safety tested.

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Maternal Diet, Oocyte Nutrition and Metabolism, and Offspring Health

22

Miguel A. Velazquez and Tom P. Fleming

Abstract

Malnutrition (i.e., undernutrition and overnutrition) is a worldwide phenomenon that can affect mammalian oocyte developmental competence following fertilization, compromising the establishment of pregnancy. However, live birth is usually possible during maternal malnutrition even in extreme cases of undernutrition (i.e., anorexia nervosa) and overnutrition (i.e., morbid obesity). Several epidemiological and clinical studies in humans and experimental animal models have demonstrated that in utero development under nutritional stress can program the development of noncommunicable diseases (NCD) in adult life (e.g., diabetes, metabolic syndrome). Of further significance is the fact that malnutrition can also program the development of NCD in adulthood via changes on oocyte physiology before conception. This chapter focuses on the available evidence supporting this latter hypothesis.

Keywords

Maternal nutrition • Oocyte metabolism • Offspring health

Introduction

The oocyte is the largest cell of the body, and unlike the spermatozoon, its role in the generation of mammalian offspring is essential [1]. Although challenged by some [2–4], the prevailing dogma is that postnatal ovaries of mammalian species are set with a fixed and nonrenewing pool of primordial oocytes [5]. These primordial oocytes are at the diplotene stage of meiosis I and are surrounded by a single layer of flattened epithelial cells (i.e., pre-granulosa cells), forming a so-called primordial follicle [6, 7]. Primordial follicles remain in a

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quiescent state until activated to grow. When primordial follicles are activated to grow, their single layer of flattened granulosa cells turns into a cuboidal shape, leading to the formation of primary follicles [7, 8]. Primary follicles further progress into successive preantral and antral stages during folliculogenesis [9]. Most of the primary follicles entering the growth phase will end up atretic, and only a limited number of follicles will be recruited to achieve the antral stages [10]. From this cohort of growing antral follicles, one (i.e., monovulatory species such as women, cows, and mares) or several follicles (i.e., polyovulatory species such as pigs, rats, and mice) will be selected to ovulate [11–16].

During follicular growth from the primordial to the preovulatory stage, the oocyte increases its size, develops more layers of granulosa cells, and acquires both theca interna and externa layers [17–19]. As oocyte growth proceeds, there is also an increase and reorganization of cytoplasmic organelles [20–22] along with synthesis and storage of mRNAs and proteins [23–25]. These cellular and molecular changes in the oocyte of the growing follicle are controlled by the interaction between endocrine (e.g., gonadotropins, metabolites, and metabolic hormones) and paracrine/autocrine (e.g., growth factors) pathways in both monovulatory and polyovulatory species [26]. The resultant ovarian follicular microenvironment from these interactive pathways determines the developmental competence of the oocyte. Hence, unperturbed oocyte development during folliculogenesis is essential for successful early embryo development, fetal growth, and offspring health [24, 27–29].

The endocrine and paracrine/endocrine pathways controlling the follicular milieu in which the oocyte develops are affected by several environmental factors, including nutrition [30]. A large number of studies have shown that imbalances in the maternal diet during pregnancy can impair preimplantation embryo viability, fetal development, and offspring health [31–37]. However, data are also available outlining the influence of maternal malnutrition on embryonic, fetal, and postnatal development via effects on oocyte quality. This chapter will focus on the effect of malnutrition on the ability of the oocyte to achieve pregnancy. The long-term effects of the nutritional in vivo microenvironment of the oocyte on offspring health will also be addressed.

Is the Study of Undernutrition and Oocyte Developmental Competence Only Relevant for Countries with Developing Economies?

Undernutrition is the result of deficient bioavailability of one or more macro- and micronutrients caused by decreased dietary intake, increased nutritional requirements or losses, or impaired ability to absorb or utilize nutrients [38, 39]. Nutritional requirements are not being fulfilled in a significant percentage of the population from countries with developing and emerging economies [40], and extreme cases of starvation are still present in the twenty-first century [41]. However, deficient intake of nutrients can occur in individuals living in developed countries. Indeed, underweight pregnant women are not uncommon in countries with very high human development index [42–46]. Even in the current superpower country, a significant proportion of homeless pregnant women experience undernutrition [47]. Adolescent girls in developed countries can also experience undernutrition when trying to keep up with the thin appearance fashion trend promoted by the media [48, 49]. In fact, some studies have found that thinness is more prevalent than obesity among adolescent girls [50, 51]. A recent survey indicated that a notably thin figure as an ideal body shape is a perception also present in young women [52]. This drive for thinness is usually accomplished by reducing food intake with dieting rather than increasing physical activity, which in some cases can reach extreme levels, leading to serious eating disorders [48, 53, 54]. The possible effects of undernutrition on offspring health via oocyte developmental competence in this population sector are relevant as pregnancies in adolescent girls are common [55–58], and patients with eating disorders (e.g., anorexia nervosa, bulimia nervosa) can conceive

and deliver a child [59, 60], even in unplanned pregnancies [61].

Undernutrition and the Ability of the Oocyte to Achieve the Early Stages of Pregnancy

Excessive undernutrition can stop ovulation in humans [62]. Nutritional-induced anovulation has been replicated in other species including pigs [63], cattle [64, 65], and mice [66]. However, anovulation is not always present in women experiencing severe undernutrition, and their oocytes are capable of achieving fertilization [59, 61]. Nevertheless, women with anorexia nervosa and bulimia nervosa are more likely to experience two or more miscarriages than the general population [67], suggesting that oocyte developmental competence may be compromised to a certain extent by undernutrition. Studies in monovulatory mammalian species have illustrated the effects of low nutritional intake on the ability of the oocyte to develop into a blastocyst. For example, the percentage of in vivo embryos in the stage of expanded blastocyst was reduced in non-superovulated ewes fed 0.5 of their maintenance nutritional requirements (M) compared with control animals fed 1.5 M for 14 days before mating [68]. In an in vitro fertilization (IVF) model, oocytes from ewes underfed (60 % of control maintenance diet) for 8 weeks before oocyte collection displayed reduced rates of cleavage and blastocyst formation [69]. In contrast, the ability of the oocyte to develop into a preimplantation embryo in polyovulatory species seems to be less affected by nutritional deficits. For instance, the capacity to attain the blastocyst stage in vitro of in vivo-derived two-cell embryos collected from mice fed with a low-protein diet during the pre-mating period was not different from that of females feeding on a medium-protein diet [70]. Pigs with restricted feed intake before puberty (i.e., 33 % less than that of an ad libitum diet) did not show a significant increase in early pregnancy loss [71]. Likewise, nutritional restrictions in gilts during estrous cycles preceding mating [72, 73] or in primiparous sows before breeding [74] did not limit progress to the blastocyst stage. However, total food deprivation can impair oocyte competence as shown by reduced blastocyst formation in does subjected to 72 h fasting [75].

Effect of Low Body Composition on the Ability of the Oocyte to Achieve the Early Stages of Pregnancy

Body condition score (BCS) is a common subjective method used to estimate fat reserves in domestic species including cattle [76], sheep [77], goats [78], pigs [79], horses [80], dogs [81], and cats [82]. In sheep, both the number and percentage of blastocysts formed in vitro have been positively correlated with body condition score (BCS) [69]. A low BCS is a common characteristic of high-producing dairy cows experiencing negative energy balance (NEB) during the postpartum period [83]. It is believed that this period of NEB is due to the lack of dietary intake capacity to cover energy demands for high milk yield imposed by intense genetic selection, resulting in mobilization of fat reserves and reduction in BCS [84]. Bovine oocytes collected during the period of NEB can display reduced morphological quality [85]. Moreover, oocytes recovered from dairy cows with a low BCS shown a reduced in vitro cleavage and blastocyst formation rate compared to oocytes collected from cows with a good BCS [86].

In humans, the ratio of body weight to height, known as the body mass index (BMI), is a measurement (kg/m^2) used to allocate individuals that are underweight (18.4), overweight (25–29.9), and obese (≥30) in relation to a reference "healthy" BMI (18.5–24.9) according to the World Health Organization [87, 88]. Studies with IVF patients have revealed that women with a low BMI may produce oocytes with reduced morphological quality [89], but the capacity to achieve in vitro fertilization and early embryonic stages, and the chances of pregnancy following embryo transfer, seem not to differ from women with normal BMI [89–96]. Nevertheless, recent data showed a nonsignificant trend toward lower odds of fertilization [97] and clinical pregnancy [97, 98]

in underweight women compared with normal weight control IVF patients. Moreover, underweight women displayed a significant reduction in the number of in vitro-produced embryos compared with women with normal BMI [99].

Is the Study of Overnutrition and Oocyte Developmental Competence Only Relevant for Countries with Developed Economies?

Overnutrition is a form of malnutrition that arises when bioavailability of one or more macro- and micronutrients exceeds the amounts necessary for normal physiological activity and metabolism, which commonly leads to overweight or obesity [100]. World statistics indicate that more than 30 % of the adult population is either overweight or obese, and if the current trend continues, this value will rise to 57.8 % by 2030 [101]. However, this is not a problem only present in countries with advanced economies. The prevalence of obesity and metabolic syndrome in some Latin America countries is similar or even higher than in developed countries [102, 103]. Similarly, obesity has become a public health concern in most countries of the Eastern Mediterranean region [104]. In fact, 80 % of deaths caused by nutrition-related chronic diseases occurs in countries with developing economies, and a large proportion of these deaths are associated to overweight and obesity [105].

Overnutrition and the Ability of the Oocyte to Achieve the Early Stages of Pregnancy

Overnutrition can cause anovulation in both humans [106] and animals [107]. Although obese women can ovulate and achieve pregnancy spontaneously [43, 108], or after induction of ovulation [109], they are often at greater risk of pregnancy complications, including fetal and neonatal death [42, 45, 110–112]. Several studies have provided evidence that oocyte competence is usually impaired in overfed individuals. Adamiak et al. [113] using ovum pick-up/in vitro embryo production (OPU/IVEP) demonstrated that heifers in good BCS consuming 2.0 M had a cumulative reduction in blastocyst yields compared to oocyte donors fed 1.0 M. The same group showed that bovine donors with low BCS consuming a diet high in starch and palm oil fatty acids yielded oocytes with reduced capacity to achieve the blastocyst stage. This high consumption of carbohydrates and fatty acids, however, did not affect blastocyst yields in oocyte donors with moderate BCS [114]. In another OPU/IVEP study, bovine in vitro blastocyst production in superstimulated oocyte donors started to decline after a continuous experimental overfeeding regime for 16 weeks [115]. The overfed donors showed an improvement in in vitro embryo production when they were switched to restricted feeding [115]. A similar scenario has been found in mice, in which in vivo fertilization rates were reduced in females fed a high-fat diet [107]. In vivo-derived zygotes from mice fed a high-fat diet also displayed a reduced ability to reach the blastocyst stage during in vitro culture [116]. Likewise, in vivo exposure of oocytes to a microenvironment high in omega-3 polyunsaturated fatty acids during in vivo fertilization impaired zygote morphology and decreased developmental ability to the blastocyst stage [117]. In humans, impaired cumulus-oocyte complex morphology has been associated with elevated fatty acid concentrations in ovarian follicular fluid [118].

High intake of proteins leading to high ammonia and urea concentrations in systemic circulation and reproductive tract can also affect negatively oocyte developmental competence [119]. For instance, the proportion of oocytes that developed to the blastocyst stage during in vitro culture was reduced in bovine oocyte donors fed diets that increased ammonia concentration in plasma and ovarian follicular fluid [120]. Oocytes recovered from heifers with high plasma urea concentrations showed lower rates of cleavage and blastocyst formation during in vitro embryo production [121]. Recently, Ferreira et al. [122] reported a decreased hatching rate of in vitro-produced blastocysts derived from oocytes collected from heifers supplemented with urea

compared to non-supplemented oocyte donors. These findings could be relevant for humans, as women consuming a diet with high content of protein showed increased blood urea concentrations [123], and a strong positive correlation between blood and follicular fluid urea concentrations has been documented in IVF patients [124]. High-protein diets have been used as an intervention for weight loss in patients with the polycystic ovary syndrome (PCOS) [125].

Interestingly, studies with ruminants suggest that overnutrition seems to be more detrimental than undernutrition for oocyte developmental competence. For instance, morphological oocyte quality was better in ewes offered a low diet (0.5 M) than in ewes fed ad libitum [126]. Superovulated heifers fed ad libitum for 100 days before superovulation produced less viable embryos than animals on a restricted diet [127]. Oocytes recovered from heifers under concentrate supplementation (high-energy source) had reduced ability to develop to the blastocyst stage in vitro compared to oocytes collected from animals consuming silage or hay [128]. Ewes fed 0.5 M until the day of artificial insemination produced more viable morulae than animals fed with 2.3 M [129]. When these in vivo-produced morulae were cultured in vitro for 72 h, the in vitro development to the blastocyst stage was also higher for the underfed group [129]. Oocytes from sheep fed 2.0 times maintenance energy requirements (MER) and supplemented with urea had lower cleavage rate and blastocyst production in vitro than ewes supplemented with urea but under 0.5 MER [130]. Likewise, in a recent IVF study, ewes fed with 1.5 M showed a reduced blastocyst production compared to animals fed with 0.5 M [131].

Farm pigs seem to be an exception to this detrimental effect of overnutrition on oocyte developmental competence. In superstimulated gilts, in vivo oocyte maturation was not affected by a high intake of energy [132]. A high intake of lysine was not associated with parameters of in vitro maturation in sows [133]. In fact, a high plane of nutrition during the pre-mating period did not affect [134] or improve pig embryo survival [135, 136]. Accordingly, gilts subjected to a high plane of nutrition for 19 days showed a higher proportion of oocytes reaching the metaphase II (MII) status following in vitro maturation compared to gilts under a maintenance diet [137]. The proportion of oocytes maturing to MII in vitro was also reduced in gilts subjected to restricted feeding (2.2–2.5 M) compared to those on a high plane of nutrition (3.0–3.5 M) [138]. Similarly, compared to a maintenance-energy diet, gilts fed a high-energy diet yielded a higher proportion of oocytes showing cumulus expansion and reaching the MII state after in vitro culture [139]. The reason for this phenomenon is unknown, but it could be related to the use of nutritional requirement guidelines that are suboptimal for sows used in modern pig production systems, which are the result of years of intensive genetic selection for high prolificacy [140] and, therefore, with greater nutritional requirements than their less-prolific ancestors. Whatever the reason, it is clear that extrapolation of data generated in farm pigs for the creation of conceptual models on oocyte quality [141], especially those relevant for human malnutrition, should be done with caution.

Effect of High Body Composition on the Ability of the Oocyte to Achieve the Early Stages of Pregnancy

High BCS has been associated with impaired bovine oocyte developmental competence in vitro [113]. Likewise, repeat breeder heifers are usually associated with a high BCS [142]. In women undergoing IVF treatment, high BMI can decrease fertilization rates and increase the incidence of early pregnancy loss and miscarriage, leading to a reduction in live birth rate [91–93, 97–99, 143–148]. In contrast, several studies reported that high maternal BMI did not affect pregnancy outcome in IVF patients [90, 95, 149–153]. In an oocyte donation model, it was found that human embryo recipients with a high BMI did not show differences in pregnancy outcome compared with embryo recipients with normal BMI [154]. Since young healthy women provided the oocytes, possible detrimental effects of high BMI on oocyte

quality were excluded, suggesting that the uterine microenvironment of embryo recipients with a high BMI is not a determinant factor for IVF pregnancy success when a good quality embryo is transferred [154]. Accordingly, implantation and pregnancy rates are not impaired in IVF patients with high BMI when embryos considered to be of high quality are transferred [155]. On the other hand, oocyte donation studies have also reported a higher risk of spontaneous abortion [144] and poor pregnancy rates [156] in women with high BMI. It has also been reported that high maternal BMI did not affect the morphological quality of in vitro-produced blastocysts, but still it decreased pregnancy and live birth rates after embryo transfer [94]. These latter studies suggested that the adverse effects of high BMI on pregnancy IVF outcome can be also exerted in the uterus following transfer [94]. However, morphological evaluation is not an accurate method to determine embryo quality [157, 158]. An embryo with good morphological appearance may have a compromised developmental competence. Under in vivo conditions, it is known that malnutrition can affect both the oviductal [159, 160] and uterine microenvironments [161], and development of the early embryo critically depends on the luminal fluid present in the oviduct and uterus [162–166]. Hence, the detrimental impact of overnutrition cannot only be infringed at the oocyte level during folliculogenesis but also during embryo development in the oviduct and uterus.

The conflicting results in studies addressing the effects of obesity on human IVF treatment using BMI measurements are probably related to the population sample and the cutting point used to define obesity [167, 168]. It also has to be considered that BMI is not a calculation of the percentage of body fat and the estimation of body fatness it provides is poor, as it cannot assess body fat compartmentalization (i.e., subcutaneous vs. visceral fat) [87, 169]. Inconsistencies among studies could also be due to the lack of control over critical variables that can influence human IVF outcome such as age, dietary intake, lifestyle (smoking, drinking), and the BMI and fertility of the male partner [97, 153]. Interestingly, in a recent study analyzing data comprising over 700,000 IVF cycles from 120 IVF units covering a 12-year period, Kupka et al. [170] found that implantation rates in IVF cycles are affected positively when the male partner is obese and the female partner has a normal BMI.

Effect of the Nutritional Microenvironment of the Oocyte on Offspring Health

The above-discussed information clearly shows that oocytes that develop under a microenvironment of malnutrition can achieve pregnancy, and although some pregnancies can be lost (Fig. 22.1), a successful delivery to term is usually possible, even in extreme cases of undernutrition [59, 60] and overnutrition [171, 172]. It seems that some individuals can adapt better than others to nutritional stress and are able to maintain their fertility [173]. However, data from epidemiological and clinical studies in humans and experimental trials in animals have shown that malnutrition during pregnancy can program the development of noncommunicable diseases (NCD) such as obesity, diabetes, cardiovascular conditions, and metabolic syndrome in adult life [34, 174–177]. This information has been the basis for the creation of the concept of developmental origins of health and disease (DOHaD). This adverse programming can be induced during the periconceptional period (i.e., from folliculogenesis to implantation) [178], affecting both the oocyte and oviductal-uterine embryo development [34, 175]. Although this nutritional programming of NCD in adulthood can be exerted in the oviduct and uterus during preimplantation embryo development, there is evidence showing that these effects can also be induced solely in the ovary, during folliculogenesis (Fig. 22.2). For example, mice under an isocaloric low-protein diet prior to natural mating produced male and female offsprings with elevated systolic blood pressure and attenuated in vitro vasoreactivity of mesenteric arteries to vasodilators [179]. In this nutritional model, the kidney weight of female offspring was reduced, but the number of glomeruli was

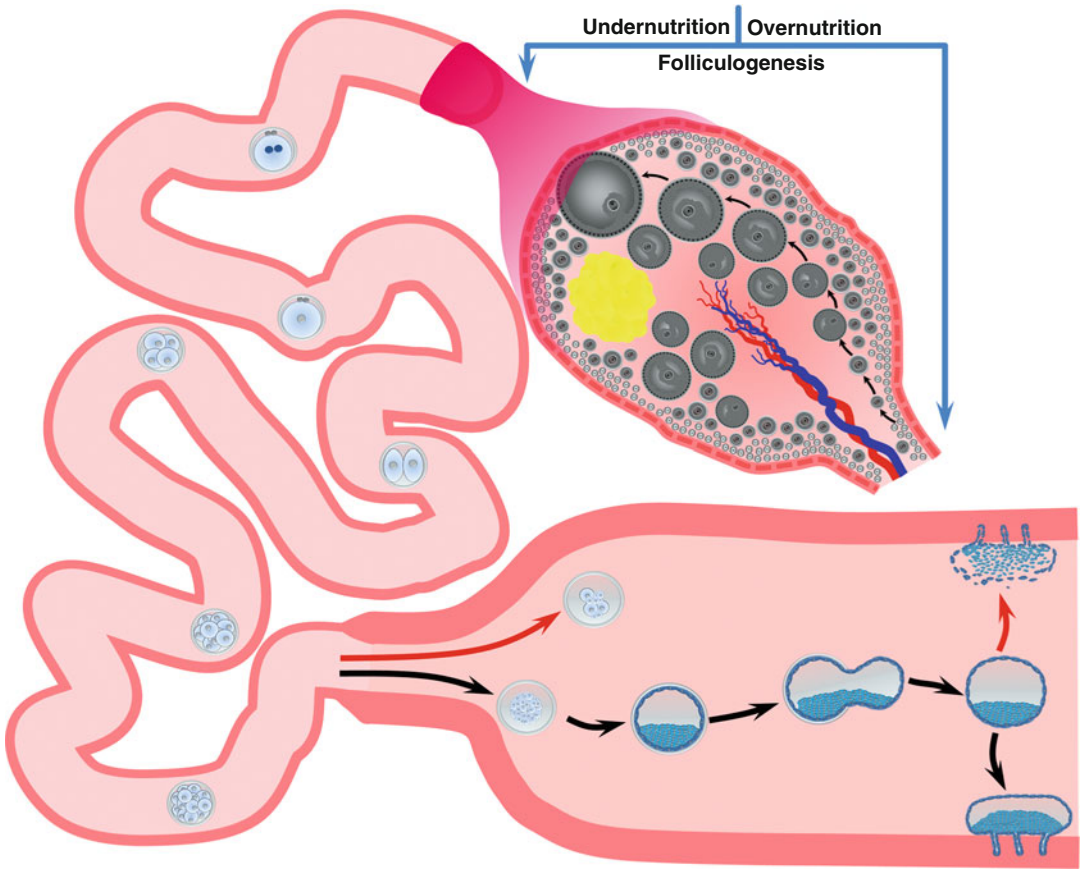

Fig. 22.1 Malnutrition can affect the quality of the oocyte during folliculogenesis, impairing its ability to reach the blastocyst stage or to sustain pregnancy following implantation (*red arrows*). However, oocytes subjected to nutritional stress are often capable of achieving a successful pregnancy (*black arrows*)

increased. This putative compensatory response to maternal undernutrition resulted in correlations between systolic blood pressure and morphological variables such as glomerular number, kidney to body weight ratio, and heart to body weight ratio not seen in offspring from mothers fed with normal levels of protein [179]. Similarly, female offspring from ewes experiencing undernutrition (i.e., 0.5 M) for 30 days before conception displayed attenuated vasodilatation in left descending coronary artery, left internal thoracic artery, and third-order femoral artery [180]. The altered repertoire of cardiovascular and blood vessel function induced in these models of undernutrition is a known contributory factor for the development of cardiovascular disease [181]. Furthermore, maternal protein undernutrition before conception in mouse also caused abnormal anxiety-related behavior in the resultant offspring when subjected to open-field tests [179]. Likewise, ewes undernourished for 60 days before mating gave birth to lambs that display fewer attempts to escape when subjected to an isolation stress test compared to controls [182]. The implications of these alterations in behavioral reactions for human development are unknown, but they could be significant. For instance, it is known that Holocaust survivors subjected to malnutrition and related stress can develop long-term alterations in metabolism [183], and their offspring can display higher levels of distress, including higher anxiety levels, lower self-esteem, and relational

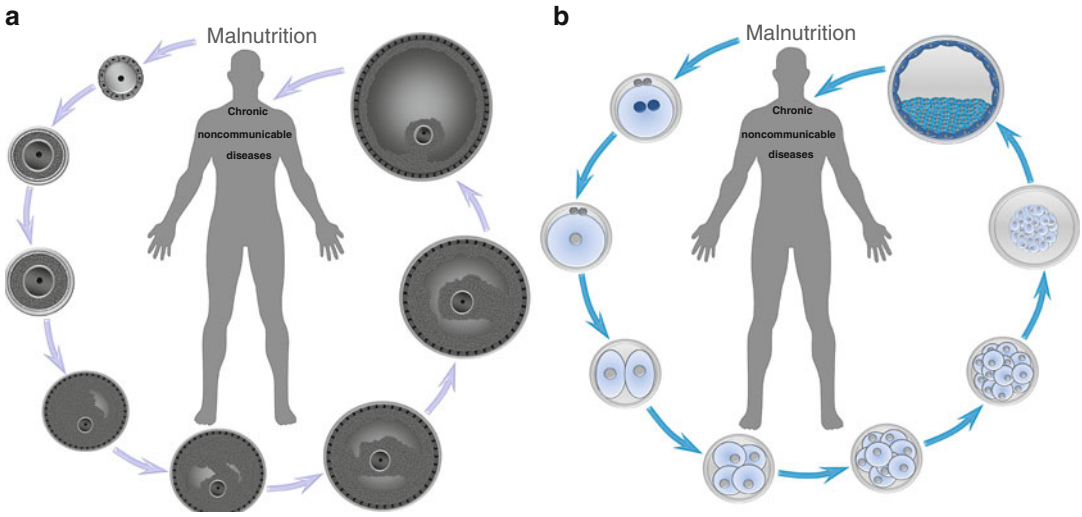

Fig. 22.2 Malnutrition during folliculogenesis (**a**) and preimplantation embryo development (**b**) can program the development of noncommunicable chronic diseases in adult life

ambivalence [184, 185], which can lead to a greater use of psychotropic medication [186].

In a model of diet-induced obesity before conception, 6-month-old offspring from obese mice were heavier and had greater visceral adipose tissue and increased adipose tissue/body mass ratio than controls [187]. Compared to their lean control counterparts, male obese offspring showed elevated blood concentrations of insulin, triglycerides, free fatty acids, adiponectin, and C-reactive protein (CRP); whereas female obese offspring did not have high levels of insulin, adiponectin, and CRP but displayed increased concentrations of cholesterol, leptin, triglycerides, free fatty acids, and soluble intercellular adhesion molecular-1 (sICAM-1) [187]. The impaired secretion pattern of these blood analytes has been associated with the metabolic syndrome and/or cardiovascular disease [188–191]. A similar scenario has been found in ewes, where dams with a high BCS and fed ad libitum (1.7–1.9 MER) for 4 months before conception produced female lambs with increased total fat mass at 4 months of age [192]. These animal studies gave partial support to the findings from recent studies in humans indicating that children born from mothers that were overweight or obese before pregnancy have a higher risk of becoming overweight or obese during adolescence [193–195].

The effects of malnutrition before conception have been detected during the fetal period as well. Accordingly, methylation of proopiomelanocortin and glucocorticoid receptor was reduced in the hypothalamus of fetuses (day 135 of gestation) from ewes subjected to undernutrition for 60 days before conception, suggesting a possible alteration in the regulation of food intake [196]. With the same model, it was found that undernourished ewes developed higher insulin sensitivity at 65 days of pregnancy [197], associated with lower growth rate of fetuses in late pregnancy [197, 198] compared with well-nourished control counterparts. Interestingly, non-overweight women losing weight before pregnancy have a higher risk of having small for gestational age newborns [199].

Mechanisms Underlying the Effects of Malnutrition on Oocyte Developmental Competence

Malnutrition and Endocrine and Paracrine Mediators

Both undernutrition and overnutrition can affect circulating concentrations of metabolites and metabolic hormones capable of affecting oocyte

physiology (Tables 22.1 and 22.2). However, circulating concentrations of blood constituents do not always reflect changes in ovarian follicular fluid (FF) concentrations during nutritional trials. This is especially true for blood analytes with endocrine and paracrine action. For example, concentrations of insulin-like growth factor-1 (IGF1), a peptide essential for mammalian ovarian activity [225], were affected in systemic circulation but not in FF of cattle subjected to short-term fasting [202]. In ewes, IGF1 concentrations in FF were unaffected by body condition or level of feed intake despite significant treatment differences in systemic concentrations [226]. An opposite scenario was found during fat supplementation in cows, in which IGF1 concentrations were increased in FF but not in serum [219]. Similarly, concentrations of IGF1 were increased

Table 22.1 Effects of nutritional conditions on selected systemic metabolic hormones in female mammals with capacity to affect oocyte physiology

Blood constituent	Effect	Species – nutritional model	Reference
IGF1	↑	Pigs – high plane of nutrition	[134, 137, 138]
	↑	Cattle – high BCS	[161, 200]
	↑	Pigs – high-energy diet	[139]
	↑	Pigs – starch diet	[134]
	↑	Humans – high BMI	[201]
	↓	Pigs – low-energy diet	[139]
	↓	Cattle – fasting	[202]
	↓	Cattle – negative-energy balance	[203]
	↓	Cattle – low plane of nutrition	[204]
	↓	Cattle – low BCS	[113]
	↓	Ewes – fasting	[205]
Insulin	↑	Pigs – high plane of nutrition	[137]
	↑	Buffaloes – high-energy diet	[206]
	↑	Pigs – high-energy diet	[139]
	↑	Pigs – starch diet	[207]
	↑	Cattle – starch diet	[114]
	↑	Cattle – high BCS	[204]
	↑	Cattle – high plane of nutrition	[113]
	↑	Ewes – high plane of nutrition	[208]
	↓	Mice – low protein	[209]
	↓	Pigs – low-energy diet	[139]
	↓	Buffaloes – low-energy diet	[206]
	↓	Ewes – fasting	[205]
Leptin	↑	Pigs – high plane of nutrition	[134, 137]
	↑	Cattle – high BCS	[161]
	↑	Buffaloes – high-energy diet	[206]
	↑	Human – high BMI	[201, 210–212]
	↑	Human – high-fat mass	[213]
	↑	Ewes – high plane of nutrition	[208]
	↓	Buffaloes – low-energy diet	[206]
	↓	Cattle – low plane of nutrition	[204]
	↓	Cattle – low BCS	[113]
	↓	Ewes – fasting	[205]
Adiponectin	↓	Humans – high BMI	[214]
	↑	Humans – fish-based diet	[215]
	↑	Mice – low-energy diet	[216]

↑ increase, ↓ decrease, *BCS* body condition score, *BMI* body mass index

Table 22.2 Effects of nutritional conditions on selected systemic metabolites in female mammals with capacity to affect oocyte physiology

Blood constituent	Effect	Species – nutritional model	Reference
Glucose	↑	Buffaloes – high-energy diet	[206]
	↑	Pigs – starch diet	[207]
	↑	Ewes – high plane of nutrition,	[208]
	↓	Pigs – high plane of nutrition	[137]
	↓	Mice – fasting	[66]
	↓	Mice – low protein	[209]
	↓	Buffaloes – low-energy diet	[206]
	↓	Cattle – negative-energy balance	[203]
Urea	↑	Cattle – high-protein diet	[217]
	↑	Human – high-protein diet	[218]
Cholesterol	↑	Buffaloes – high-energy diet	[206]
	↑	Cattle – fat supplementation	[219]
	↑	Mice – high-fat diet	[187]
	↑	Rabbits – high-fat and high-cholesterol diet	[220]
	↓	Buffaloes – low-energy diet	[206]
ß-Hydroxy-butyrate	↑	Cattle – negative-energy balance	[203]
	↑	Humans – high-protein diet	[221]
	↑	Humans – fasting	[222]
Nonesterified fatty acids	↑	Cattle – negative-energy balance	[203]
	↑	Ewes – low-energy diet	[223]
	↑	Humans – high-carbohydrate diet	[224]

↑ increase, ↓ decrease, *BCS* body condition score, *BMI* body mass index

in FF but not in systemic circulation of buffaloes fed with different levels of energy [206]. This emphasizes the importance of measuring analytes in blood and FF in experiment trials addressing the effect of malnutrition on ovarian physiology. Several analytes in FF have been reported to be altered during malnutrition, but for most of these FF components, a cause-effect relationship with oocyte developmental competence has not yet been demonstrated (Fig. 22.3). It is important to emphasize that FF components are not only influenced by the amount of nutrients but also by diet composition [241, 242]. Interestingly, some analytes in follicular fluid can be affected in opposite directions under similar models of undernutrition. Moreover, both undernutrition and overnutrition can induce similar fluctuations of some intrafollicular components (Fig. 22.3).

Undernutrition and Oocyte Quality

Cows with low BCS can have a greater proportion of oocytes with impaired morphology such as incomplete or absent cumulus layers, condensed cumulus, or vacuolated cytoplasm [243]. Oocyte morphological quality (i.e., the number of granulosa layers and cytoplasm homogeneity) was also reduced in buffaloes [206] and pigs [139] subjected to low-energy intake compared to animals fed a high-energy diet. Compared with feeding ad libitum, fasting for 48 h in immature female mice reduced the percentages of oocytes with germinal vesicle breakdown and polar body extrusion after in vitro FSH treatment, suggesting a possible detrimental effect on oocyte maturation [66]. Interestingly, large follicles developed only in ovaries from fasting mice, and the follicle-enclosed oocytes were dead [66]. Feed restriction can also decrease the number of neutrophil granulocytes and monocytes-macrophages infiltrating the theca interna of rat preovulatory follicles [244], but their role in oocyte developmental competence is unknown.

Overnutrition and Oocyte Quality

Mice supplemented with urea showed a reduction in the percentage of oocytes of good morphological quality [245]. In a model of diet-induced obesity, the percentage of oocytes

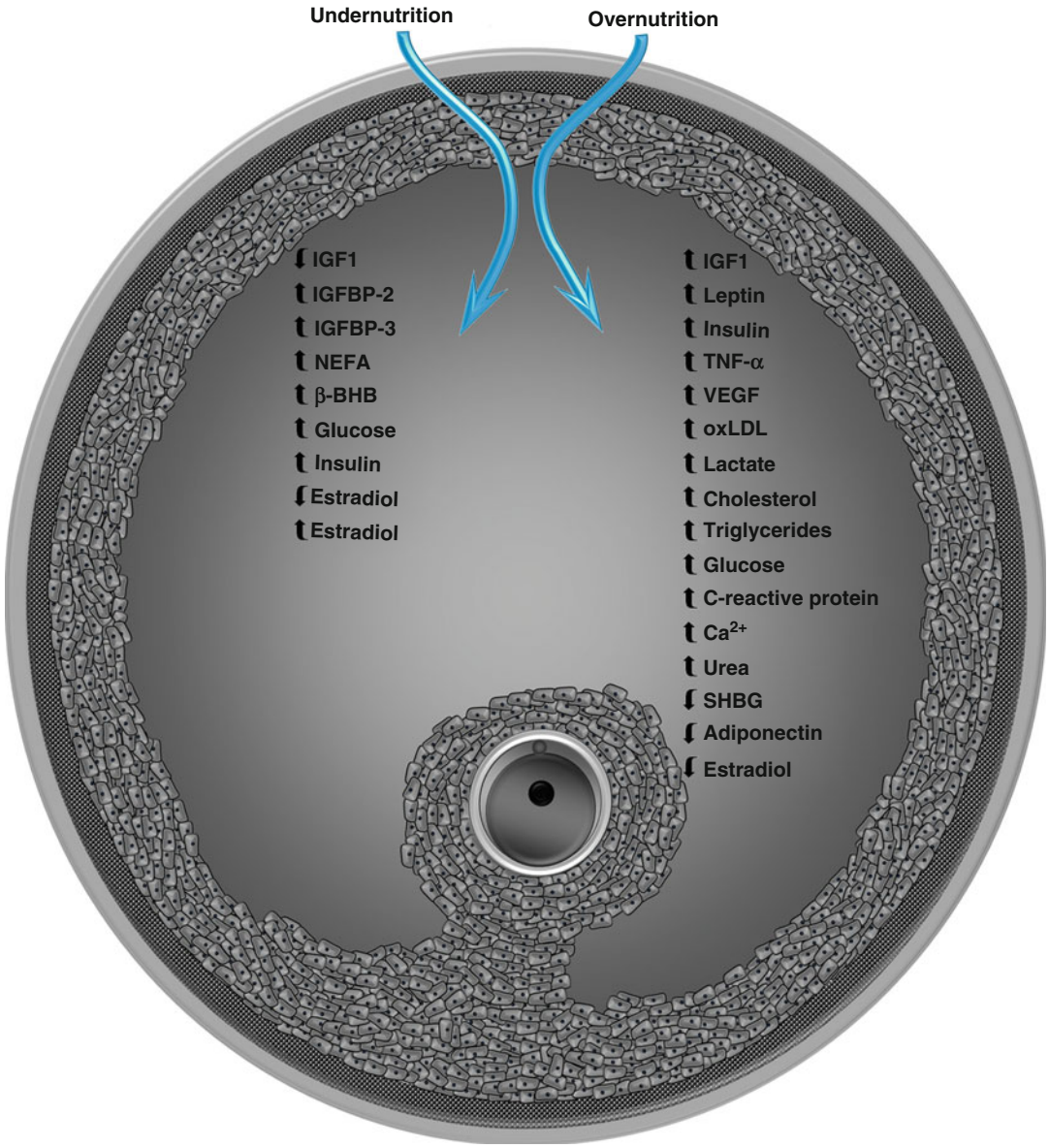

Fig. 22.3 Malnutrition can alter components in ovarian follicular fluid. Data from humans [107, 227–231], cattle [217, 219, 232–236], ewes [237, 238], and pigs [239, 240]

reaching germinal vesicle breakdown was reduced, the follicles presenting apoptosis was increased, and the size of the oocytes was reduced in obese mice compared to lean control counterparts [246]. Likewise, oocyte size was reduced in women undergoing IVF treatment and categorized as obese [247]. In vitro bovine models have shown that development to the blastocyst stage is positively associated with oocyte diameter [248], and failure of the oocyte to reach a proper size has been linked with the inability to undergo meiotic maturation and increased frequency of polyspermic fertilization [249, 250]. Higher lipid content is also a common characteristic of oocytes from obese individuals and is considered a biomarker of

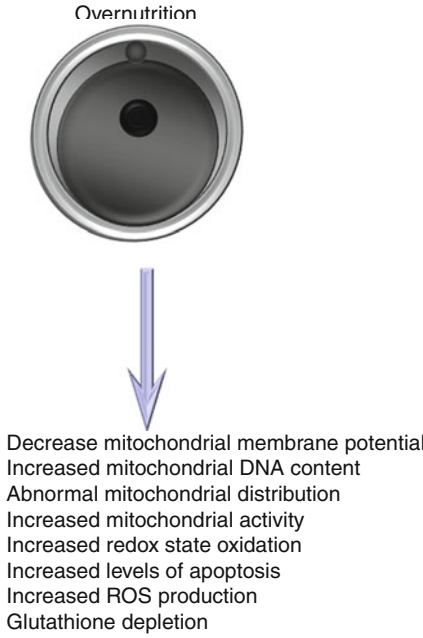

Fig. 22.4 Molecular variables of oocyte quality can be altered by overnutrition [107, 159]

lipotoxicity [107, 142]. Several molecular variables of oocyte quality have been also found to be altered during overnutrition (Fig. 22.4).

Malnutrition and Gene Expression in Oocytes and Granulosa Cells

Transcript expression of several developmentally important genes has been found to be affected by malnutrition in oocytes and granulosa cells. In ewes subjected to undernutrition, genes related to oocyte metabolism were downregulated, whereas in granulosa cells, genes related to follicle development and metabolism were upregulated [251]. A higher expression of genes associated with endoplasmic reticulum stress has been reported in cumulus-oocyte complexes from obese mice and women [107]. Likewise, genes related to insulin signaling were upregulated in obese women compared with moderate-weight women [227]. Nevertheless, the number of genes examined in these studies is minute compared to the several thousand genes involved in oocyte developmental competence [252]. Furthermore, gene expression does not always translate into equal protein expression.

In Vitro Models of Malnutrition

In an in vitro model of cell undernutrition, Sirotkin [253] found that porcine granulosa cells subjected to serum deprivation reduced BAX protein expression and release of progesterone and IGF1, but markers of cell proliferation (proliferating cell nuclear antigen [PCNA] and cyclin B1) remained unaffected. In cattle, oocytes exposed in vitro to nonesterified fatty acids (NEFA) concentrations found in FF of cows in NEB displayed an increased apoptosis of cumulus cells and impaired progression of meiosis with subsequent negative effects on cleavage rate and blastocyst formation [254]. Likewise, concentrations of ß(beta)-hydroxybutyrate (BHB) are increased in cows experiencing NEB, and in vitro oocyte maturation in a high BHB microenvironment resulted in reduced cleavage rate and blastocyst yield [255]. In a more recent experiment, in vitro oocyte maturation with elevated NEFA concentrations resulted in a diminished ability of oocytes to reach the blastocyst stage compared to oocytes matured with physiological concentrations of NEFA [256]. Blastocysts originating from oocytes matured with high NEFA concentrations showed reduced number of cells, increased levels of apoptosis, and upregulation of mRNA transcripts for methyltransferase 3 alpha (DNMT3A), insulin-like growth factor receptor 2 (IGFR2), and solute carrier family 2 (facilitated glucose transporter) member 1 (SLC2A1, also known as GLUT1) [256]. Further analysis of the resultant blastocysts revealed lower consumption of oxygen, pyruvate, and glucose, along with higher consumption of lactate and greater amino acid consumption and production [256]. Studies in cattle and humans reported that granulosa cell survival was reduced after in vitro exposure to high NEFA concentrations, which was associated to increase levels of apoptosis [257, 258]. The effects of NEFA on oocyte physiology are not only relevant for undernutrition but also for obesity [259].

In a different bovine model, in vitro estradiol secretion by granulosa cells from ovarian follicles collected from heifers fed 2 M was higher than granulosa cells from animals offered a maintenance diet [260]. This nutritional effect was observed in small but not in medium-sized ovarian follicles [260]. The effect of high estradiol levels induced by overnutrition on oocyte competence are unclear as ovarian follicular concentrations of estradiol have been associated positively with pregnancy outcome in some but not all human IVF studies [reviewed by 261].

Malnutrition and Cell Number of Blastocysts

Previous research showed that a diet low in protein during the preimplantation period can alter cellular allocation of rat blastocysts [209]. More recently, Mitchell et al. [70] compared high (HPD), medium (MPD), and low (LPD) protein diets during the periconceptional period in mice and found that HPD and LPD blastocysts displayed a reduced number of cells in the inner cell mass (ICM) compared to blastocysts produced in females consuming medium levels of protein. However, the environment the oocyte is exposed to during folliculogenesis can program embryo cell number. Indeed, in vitro-derived blastocysts produced with oocytes collected from heifers with high plasma urea concentrations showed a decrease in the total number of cells [121]. Compared to control lean counterparts, oocytes from obese mice yielded in vitro-derived blastocysts that showed a significant smaller ICM/total cell proportion, due to higher allocation of cells to the trophectoderm and a decrease in the number of cells of the ICM [116].

Conclusion

Malnutrition is affecting people around the world, and although the ability of the oocyte to achieve pregnancy can be decreased by malnutrition, delivery to term is usually possible even in conditions of extreme undernutrition and overnutrition. However, epidemiological and clinical studies in humans have shown that NCD in adult life can be programmed by malnutrition during pregnancy. Furthermore, animal studies have shown that NCD can be also programmed before conception, during folliculogenesis. Still, not much emphasis has been put in researching the effects of malnutrition via oocyte competence. Several cellular and molecular variables of oocyte quality can be altered during malnutrition, but the underlying cause of this developmental programming is far from being totally elucidated, and research has to continue in order to develop strategies for the prevention of NCD.

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Erratum

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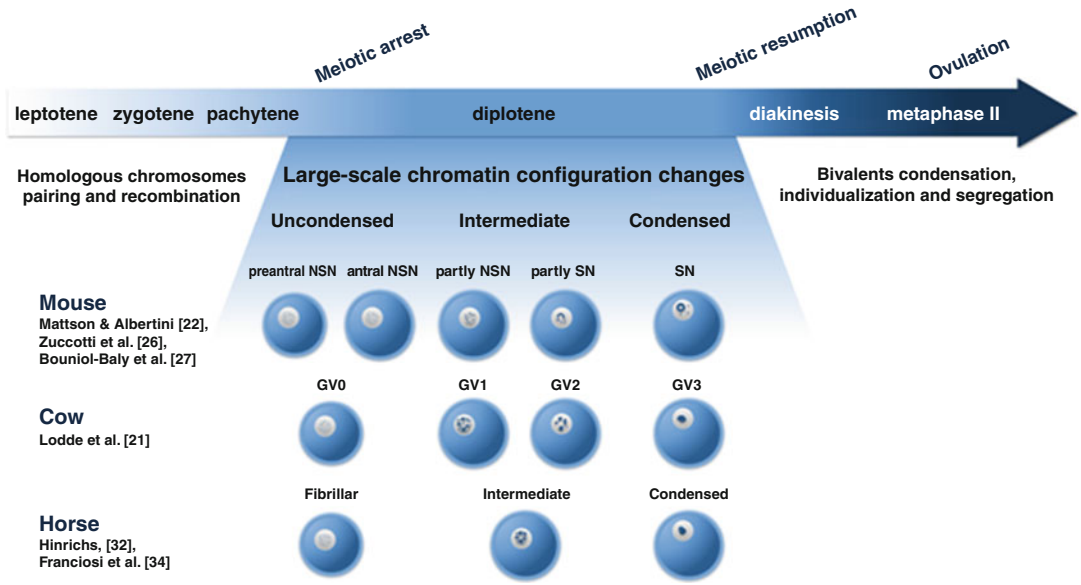
Please find here below the correct figure 7.1.



Further, the figures 8.3 and 8.4 contain mistakes regarding the size represented by the scale bars.

It says “black (white) scale bar = 5 (10 or 20 µm)” and should say 50, 100 and 200 µm.

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