The Structural Basis for Coordinating Oogenesis and Folliculogenesis

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Maria Cristina Guglielmo and David F. Albertini

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

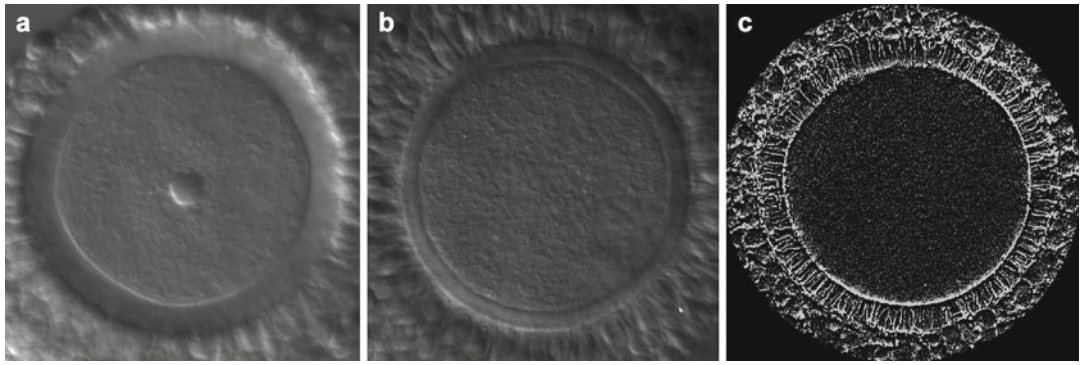

Fig. 5.1 Illustrations of transzonal projections (TZPs) under conventional bright field Nomarski optics (**a**, **b**) and after labeling with rhodamine phalloidin to accentuate details using confocal and image sharpening software. (**c**) All fixed samples represent GV stage oocytes

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),

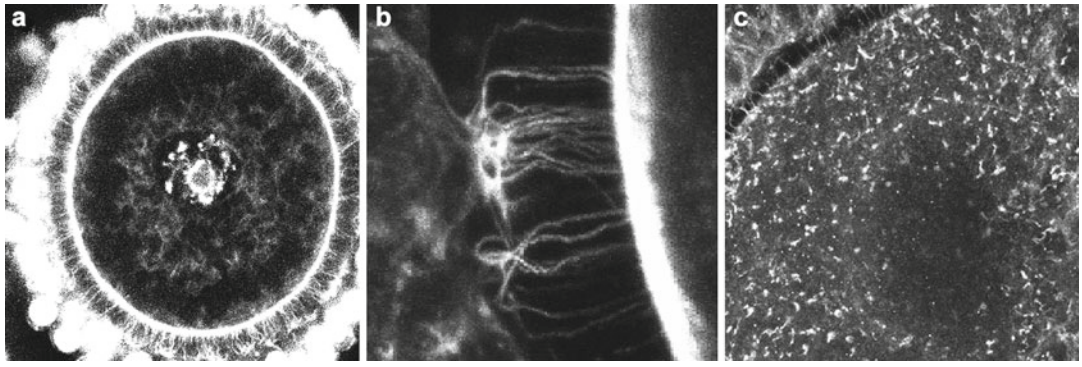

Fig. 5.2 Examples of TZPs from rat (**a**) and bovine (**b**, **c**). GV stage oocytes processed for confocal microscopy after fixation and labeling with rhodamine phalloidin to enhance detection of F-actin. Note in A that nuclear chromatin has been labeled with Hoechst 33258 and a system of actin filaments 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**)

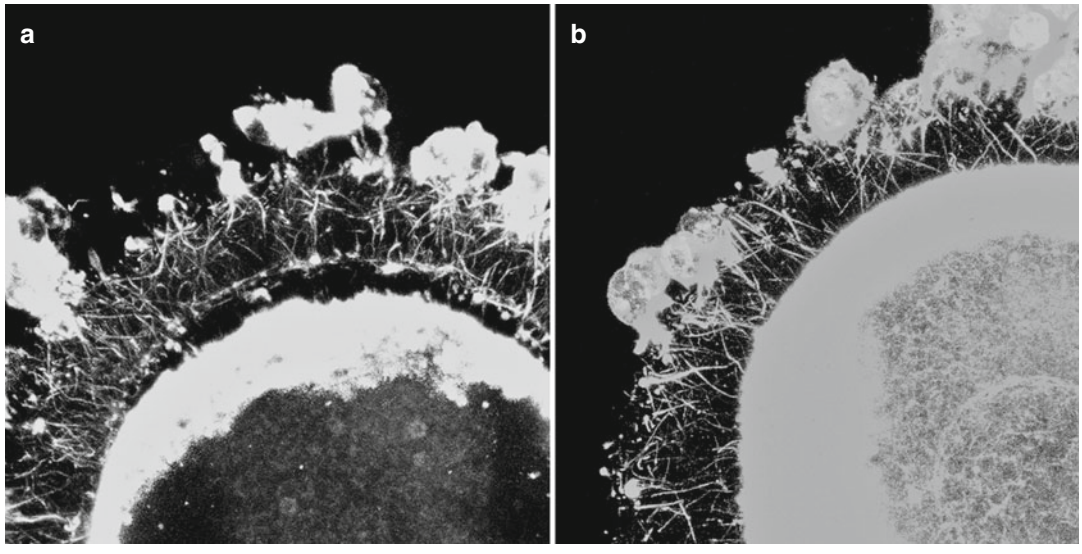

Fig. 5.3 Confocal microscopy images of intact human cumulus-oocyte complexes. In (**a**), a cumulus-oocyte complex labeled to detect actin is illustrated. It is important to note the presence of a dense network of TZPs from some corona cells that extends into the perivitelline space. In (**b**), a projection 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,

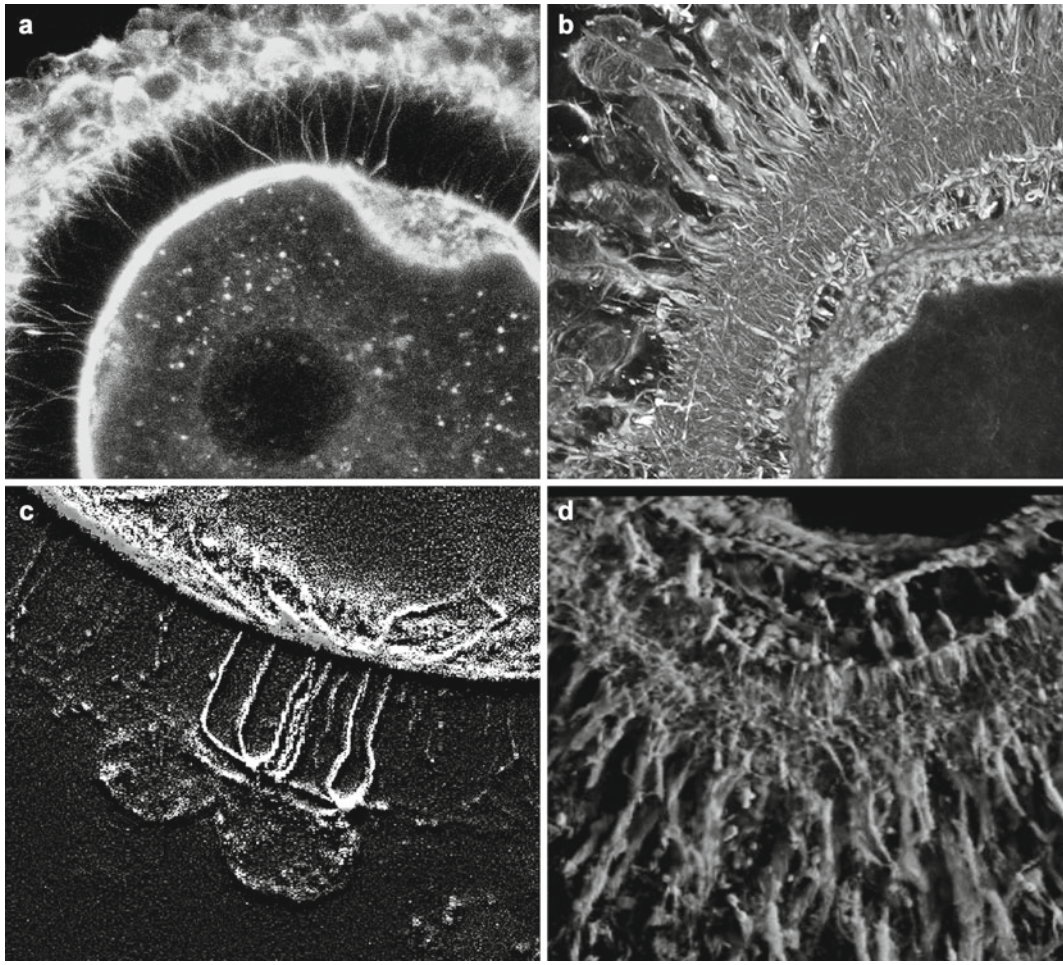

Fig. 5.4 Projections of Z stacks from bovine GV stage (**a**, **c**) and equine metaphase-2 (**b**, **d**) oocytes fixed and labeled with rhodamine phalloidin. Note oolemmal infolding at sites of TZP retraction (**a**) and dense network of TZPs from corona cells that are dilated in the perivitelline 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

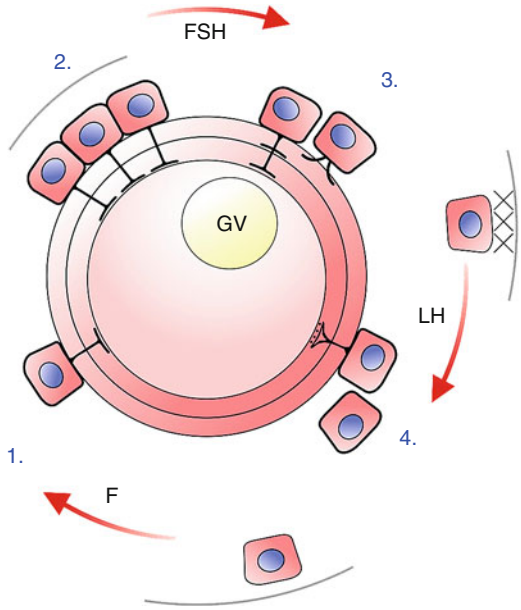

Fig. 5.5 Schematic illustrating patterns of TZP organization in response to *FSH* or *LH*. Prior to FSH stimulation, it is proposed that TZPs are oriented and stable around the oocyte, but after FSH stimulation, a reorganization takes place as a result of granulosa cells entering the cell cycle. TZPs 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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