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REGULATION OF GERM CELL DEVELOPMENT BY INTERCELLULAR SIGNALING IN THE MAMMALIAN OVARIAN FOLLICLE

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Abstract

Prior to ovulation, the mammalian oocyte undergoes a process of differentiation within the ovarian follicle that confers on it the ability to give rise to an embryo. Differentiation comprises two phases – growth, during which the oocyte increases >100-fold in volume as it accumulates macromolecules and organelles that will sustain early embryogenesis; and meiotic maturation, during which the oocyte executes the first meiotic division and prepares for the second division. Entry of an oocyte into the growth phase appears to be triggered when the adjacent granulosa cells produce specific growth factors. As the oocyte grows, it elaborates a thick extracellular coat termed the zona pellucida. Nonetheless, cytoplasmic extensions of the adjacent granulosa cells, termed transzonal projections (TZPs), enable them to maintain contact-dependent communication with the oocyte. Via gap junctions located where the TZP tips meet the oocyte membrane, they provide the oocyte with products that sustain its metabolic activity and signals that regulate its differentiation. Conversely, the oocyte secretes diffusible growth factors that regulate proliferation and differentiation of the granulosa cells. Gap junction-permeable products of the granulosa cells prevent precocious initiation of meiotic maturation, and the gap junctions also enable oocyte maturation to begin in response to hormonal signals received by the granulosa cells. Development of the oocyte or the somatic compartment may also be regulated by extracellular vesicles newly identified in follicular fluid and at TZP tips, which could mediate intercellular transfer of macromolecules. Oocyte differentiation thus depends on continuous signaling interactions with the somatic cells of the follicle.

Graphical Abstract

All stages of post-natal oocyte development depend on communication with the neighbouring somatic granulosa cells of the ovarian follicle. Signals sent by the oocyte also regulate differentiation of the granulosa cells and ensure that they provide a healthy environment for the germ cell.

INTRODUCTION

Newborn mammalian females contain an enormous number – ranging from about 20,000 in the mouse (1) to up to one million in humans (2) – of oocytes, each enclosed by a small number of somatic granulosa cells in a structure termed a primordial follicle. Before ovulation, each oocyte undergoes a process of differentiation to generate an egg that can be fertilized and develop as an embryo. The oocyte does not undertake this journey alone. Rather, it relies on support provided by the somatic compartment of the follicle, which provides nutrients that support its metabolic activity and signals that regulate its differentiation. The oocyte is not, however, simply a passive participant in this process. It also sends signals to the somatic cells that regulate their differentiation and help to ensure that they provide the microenvironment that the oocyte needs as it grows and develops. Thus, bi-directional and continuous signaling between the oocyte and somatic compartment of the follicle are essential to produce a healthy egg.

Several characteristics of post-natal oocyte development within the follicle make it especially attractive for experimental study. First, the follicle presents a relatively simple anatomy, consisting of three principal cell types, each occupying a well-defined spatial position. Second, cohorts of primordial follicles regularly enter and complete the growth phase, so the growth and differentiation process can be studied throughout most of the post-natal life of a female. Third, culture systems have been developed that recapitulate much of post-natal oocyte and follicular development. As a result, much has been learned about the signaling mechanisms that control the development of the female germ cell. Here, I review

pathways of communication between the oocyte and the somatic compartment of the ovarian follicle, focusing on work carried out using the mouse as a model system.

POST-NATAL OOCYTE DEVELOPMENT: GROWTH AND MEIOTIC MATURATION

Post-natal oocyte development comprises two phases – a prolonged period of growth within the follicle, followed by a much briefer period known as meiotic maturation that occurs coincident with ovulation (Figure 1). Current evidence indicates that no new functional oocytes are created after birth under physiological conditions (3–6). Instead, the population of oocytes present at birth represents the lifetime endowment of the female.

Oocyte and follicular growth

During growth, which requires 3–4 months in humans and about 3 weeks in mice, the volume of the oocyte increases more than 100-fold. This increase in size reflects the accumulation of messenger (m) RNAs and proteins, as well as organelles such as mitochondria, that are essential for embryonic development after fertilization (7–9). For example, mRNA synthesis falls to an undetectable level in fully grown oocytes and is not fully reactivated until a species-specific stage after fertilization (8, 10), and the embryo does not resume mitochondrial replication until after it has implanted into the uterus. Oocyte growth is also marked by important epigenetic modifications, including methylation of specific DNA sequences that influences their subsequent transcriptional activity (11–14), and by ultrastructural changes that are necessary for embryogenesis (15, 16).

Growth of the oocyte is accompanied by growth and differentiation of the follicle (7, 17, 18). Upon entry into the growth phase, the squamous granulosa cells that characterize the primordial follicle become cuboidal in shape and begin to proliferate mitotically so that they continue to fully cover the expanding surface of the growing oocyte (19, 20). These follicles, now termed primary, are delimited by a basement membrane that lies apposed to the basal side of the granulosa cells. As the granulosa cells continue to proliferate, they generate multiple layers around the oocyte; such follicles are termed secondary. Steroidogenic cells known as the theca are recruited to follicles containing two layers of granulosa cells external to the basement membrane (21). Near the time that the oocyte completes its growth, a fluid-filled cavity termed the antrum appears. The antrum separates the granulosa cells into two populations – the mural granulosa, which line the inner wall of the follicle, and the cumulus granulosa, which surround the oocyte. Under the opposing influences of signals from the oocyte and from extra-follicular sources – notably, follicle-stimulating hormone (FSH) secreted by the pituitary gland, the mural and cumulus granulosa cells express different genes and fulfil different functions (22, 23). Follicles containing a large antrum are termed Graafian and it is these that typically ovulate in response to luteinizing hormone (LH).

Meiotic maturation

The final phase of oocyte development is known as meiotic maturation, and is triggered by the ovulatory release of LH from the pituitary gland (24–27). The oocyte cell cycle, which has been arrested at late diplotene of prophase I since fetal life, resumes and the oocytes

enter M-phase. The chromosomes assemble on a spindle, which is translocated by an actin-dependent process to the cortex where the first meiotic division occurs, segregating one homologue of each pair into the small first polar body (28, 29). The chromosomes remaining in the oocyte then align on a spindle in preparation for the second meiotic division, which is triggered by fertilization. These nuclear events are accompanied by cytoplasmic maturation, notably the translational activation of a subset of previously silent mRNAs and the silencing and in some cases degradation of previously active mRNAs (30–32).

Thus, all aspects of post-natal oocyte development depend on signaling interactions with the granulosa cells of the ovarian follicle. I now turn to a discussion of the different mechanisms of signaling and their specific roles during oocyte development. First, however, it is important to highlight a potential impediment to contact and communication between the oocyte and granulosa cells and how this is bypassed.

TRANSZONAL PROJECTIONS - BRIDGES FOR INTERCELLULAR SIGNALING

Within a primordial follicle, the oocyte and granulosa cells are directly apposed to each other, and adherens and gap junctions link the two cell types. Oocytes express mainly E-cadherin whereas granulosa cells express mainly N-cadherin, and other components of junctional complexes such as nectins are also detectable (33). During its growth, however, the oocyte produces an extracellular coat termed the zona pellucida (34–36). Composed of three or four glycoproteins depending on the species, the zona pellucida is initially assembled in small aggregates or chunks. These subsequently become knit together to form a continuous envelope around the oocyte that separates it from the bodies of the adjacent granulosa cells. The zona pellucida thickens as the oocyte grows, reaching a final size of about 7 μm in mice and 15 μm in humans. In view of the formidable physical barrier that the zona pellucida imposes between the oocyte and granulosa cells, how do the two cell types maintain contact and communication?

Contact between the granulosa cells and oocyte is mediated by structures termed transzonal projections (TZPs) (Figure 2A, B). These thin cytoplasmic filaments, which may be up to about 1 μm in diameter and morphologically resemble filopodia, originate from the granulosa cells and extend to the oocyte where they contact the plasma membrane (Figure 2C, D) (29, 37). TZPs that project from the cumulus granulosa cells in the layer immediately adjacent to the zona pellucida are easiest to trace morphologically or using markers (29, 38, 39) and likely constitute the bulk of the population. However, there is no a priori reason to suppose that TZPs do not project from more distal granulosa cells also (40). Electron micrographs reveal that the foot of the TZP is frequently enlarged and cradled by exvaginations of the oocyte plasma membrane (39, 41). TZPs have also been observed to penetrate deeply into invaginations of the oocyte membrane (42). These structural features increase the area of membrane contact between the two cell types and potentially facilitate increased communication. The density of the TZPs surrounding a fully grown oocyte (Figure 2A) suggests that many can emanate from a single granulosa cell (42), and it appears that several TZPs may emerge from a single point of origin or node (Figure 2B). TZPs were

first described more than a century ago (43) and physically couple the granulosa cells to the oocyte in all mammals studied including humans (38, 39, 44–47). Analogous structures have also been identified in a wide range of non-mammalian species including frog, chicken and starfish (48–50). Thus, the principle that filopodia-like structures mediate contact between the somatic compartment of the follicle and the oocyte appears to be highly evolutionarily conserved.

As the sole vehicle of contact between the granulosa cells and the oocyte, TZPs serve two key functions (Figure 2E). First, they enable contact-dependent communication between the cells. The tips of the TZPs (though not necessarily all TZPs) harbor gap junctions and possibly also membrane-associated growth factors (29, 37). As discussed below, oocyte development depends crucially on communication mediated through these pathways. Second, they are essential to maintain adhesion between the two cell types, which is probably necessary to keep the granulosa-oocyte complex intact. This function is likely mediated at least in part by adherens junctions that have been identified at the points of membrane contact (33). Experimental support for the role of adherens junctions was recently obtained. Proline-rich tyrosine kinase (PTK) 2 regulates the assembly of adherens junctions and has been immunologically localized in foci at the plasma membrane of oocytes (51). Although it is not known whether these foci are located where the TZP tips meet the oocyte plasma membrane, oocyte-specific deletion of *Ptk2* reduces the number of TZPs by about one-third. Moreover, this decrease in the number of TZPs in the absence of PTK2 in the oocyte is accompanied by a decrease in gap junctional coupling (51). Thus, the cell-communication and cell-adhesion functions of the TZPs are tightly coupled.

Two types of TZPs have been identified. Early studies using electron microscopy revealed multiple parallel filaments along the length of the TZPs, suggesting that cytoskeletal elements provide a structural backbone (38). Most TZPs appear to possess an actin-rich cytoskeleton; thus phalloidin, which binds to and stabilizes polymerized actin, is routinely used to mark the TZPs that project to an oocyte (29, 41, 46, 51, 52). The actin cytoskeleton further reinforces the similarity between TZPs and filopodia (53). TZPs containing a tubulin backbone have also been detected. These appear to be less abundant than the actin-rich TZPs; however, the relative proportions of the two have not been reported. Nor is it known whether both actin- and tubulin-rich TZPs may project from an individual granulosa cell. It has been proposed that the tubulin-rich TZPs mediate cell adhesion whereas the actin-rich TZPs mediate cell communication (29). There is not yet direct evidence to support this attractive model, and the impaired gap junctional communication in oocyte-specific knockout of *Ptk2* (51) implies that adherens-type junctions are required to stably attach TZPs containing gap junctions to the oocyte.

The origin of the TZPs remains mysterious. At least two non-exclusive mechanisms may be envisioned (Figure 2F). TZPs may represent sites of attachment that exist between the oocyte and adjacent granulosa cells before the zona pellucida becomes assembled around the oocyte (36, 43). According to this ‘stretching’ model, as the zona pellucida is deposited and pushes the bodies of the granulosa cells away from the oocyte, the two cells remain attached at the original points of contact (see Figure 2C). The subsequent thickening of the zona pellucida elongates this cytoplasmic finger, thereby generating the TZPs. Alternatively,

TZPs may arise after the zona pellucida has been deposited. According to this ‘pushing’ model, TZPs are elaborated by the granulosa cells and grow towards the oocyte. This model is consistent with the idea that TZPs are specialized filopodia that are generated by the granulosa cells, possibly in response to signals sent by the oocyte.

No direct proof yet supports either model. Several lines of evidence nonetheless indicate that as oocytes grow, new TZPs are generated by the granulosa cells. First, gap junctional communication between the oocyte and adjacent granulosa cells increases during oocyte growth (54, 55). A simple explanation for this observation is that as oocytes grow, the number of TZPs increases in parallel with the increase in the number of granulosa cells adjacent to the oocyte. Alternatively, the gap junctional plaques on existing TZPs could become larger thereby enabling increased coupling. Second, the number of TZPs was reported to increase in parallel with oocyte growth in an electron-microscopic study of human follicular development (42). Third, when cryopreserved follicles were subsequently grown in culture, the number of TZPs increased, as assessed by an increase in the birefringence of the zona pellucida (52). These results favor the model that the granulosa cells surrounding growing oocytes generate new TZPs. Precise quantification of the number of TZPs associated with oocytes at different stages of growth and analysis of TZP dynamics during growth in vitro should clarify the mechanism by which they are generated.

If the granulosa cells surrounding growing oocytes generate new TZPs, many questions about this process remain to be answered. What signals induce their formation and why do they grow towards the oocyte? Intriguingly, in mice lacking growth-differentiation factor (GDF) 9, a growth factor produced by the oocyte, the number of TZPs is reduced and they frequently lie parallel rather than perpendicular to the oocyte surface (56). These results hint at a role for the oocyte in promoting TZP formation. What role is played by gonadotropins, which regulate the differentiation and function of the granulosa cells? A larger number of TZPs are associated with the oocytes of mice lacking *Fshb*, encoding the essential β -subunit of FSH, than with wild-type oocytes (57). Moreover, when either wild-type or *Fshb*^{-/-} mice were ‘primed’ by injection of an FSH analogue prior to collection of cumulus cell-oocyte complexes, the density of TZPs was reduced. These results suggest that FSH antagonizes the formation of TZPs. Identifying the molecular pathways that link FSH to TZPs and how the reduced number of TZPs impact the developmental progression of the oocyte are important issues for future work to address.

CELL SIGNALLING AT THE INITIATION OF OOCYTE GROWTH

Shortly after birth in the mouse, a cohort of primordial follicles and their enclosed oocytes enter the growth phase while the remaining follicles remain at the primordial follicle stage. Although the oocytes of this cohort are not subsequently ovulated, presumably because the female is not yet sexually mature, they are an invaluable resource to dissect the signaling pathways that control entry into the growth phase. The first morphological sign that an oocyte has entered the growth phase is a morphological remodeling of the granulosa cells from a squamous to cuboidal shape. This may reflect (re-)entry of the granulosa cells into the mitotic cell cycle, as they must proliferate in order to continue to fully cover the

expanding surface of the growing oocyte. It also suggests that the granulosa cells may be the source of the signal that initiates oocyte growth.

Indeed, multiple lines of evidence suggest that granulosa cells trigger oocyte growth by activating phosphatidylinositol 3-kinase (PI3-kinase) signaling in the germ cell (Figure 3). When *Pten*, whose activity inhibits the pathway, is deleted from oocytes within primordial follicles, most begin to grow shortly after birth (58, 59). This is accompanied by an increase in phosphorylation of AKT, suggesting that PI3-kinase signaling has been activated. Similarly, deletion of *Tsc1* or *Tsc2*, which also inhibit PI3-kinase signaling, in oocytes of primordial follicles also causes most oocytes to begin to grow (60, 61). *Tsc2* deletion was associated with an increase in phosphorylation in ribosomal protein S6, as predicted; curiously, however, this was not observed when *Tsc1* was deleted. Importantly, in all three cases, the granulosa cells often did not assume the cuboidal morphology characteristic of primary and later-stage follicles, but instead retained the squamous morphology of primordial follicles. This is strong evidence that activating PI3-kinase signaling within the oocyte is sufficient to initiate its growth, even when the granulosa cells of the follicle remain in the primordial state. Two inferences may be drawn from these observations. First, PI3-kinase signaling promotes oocyte growth directly, rather than indirectly such as by causing the oocyte to send a signal to the granulosa cells that induces them to send a growth-promoting signal to the oocyte. Second, oocyte growth per se does not trigger granulosa cell proliferation, although factors produced by growing oocytes might play a role.

These experiments convincingly show that activating PI3-kinase signaling in the oocyte can cause it to start growing, but is it the physiological mechanism? And if so, how does it become activated under normal conditions? Kit ligand (KITL) is constitutively expressed by the granulosa cells (62, 63) and interacts with the Kit receptor (KIT) expressed by oocytes (64, 65). This ligand-receptor pair has long been suspected to play a key role in multiple aspects of germ cell development. Because KIT signaling is required for pre-natal development of female germ cells (66), mutants typically possess very few oocytes at birth, making it challenging to study potential post-natal functions. The advent of the *Cre-lox* strategy, enabling genes to be modified in specific cells at specific stages of differentiation, has been an especially valuable boon to untangling the multiple roles of KIT signaling during oocyte development.

A constitutively active form of KIT (D318V), corresponding to the most common activating mutation in human germ cell tumors, was engineered and targeted to oocytes using a *Vasa*-driven *Cre* (67). Strikingly, almost all oocytes of primordial follicles began to grow shortly after birth. Phosphorylated AKT could be detected in these oocytes, confirming that PI3-kinase signaling had been activated. The surrounding granulosa cells in most cases remained squamous, however, indicating that the somatic compartment of the follicle remained in a primordial state. Perhaps more crucial are experiments in which KIT signaling in oocytes was ablated, by generating a mutant that lacks the exon encoding the kinase domain (67). Even though granulosa cells of primordial follicles became cuboidal, the enclosed oocytes failed to grow. This strongly suggest that the granulosa cells generated the growth-promoting signal, but the KIT-deficient oocytes were unable to respond.

Complementary experiments targeting the granulosa cells support this model. When *Tsc1* was deleted from the granulosa cells, thereby activating PI3-kinase signaling in these cells, KITL expression in the granulosa cells was increased and most oocytes began to grow (68). As would be expected given that they are the source of the growth-promoting signal, the granulosa cells of the growing follicles became cuboidal. Conversely, deletion of *Rptor* in the granulosa cells, which would decrease protein synthesis, prevented the squamous-cuboidal transition in these cells as well as the initiation of oocyte growth (68). Taken together, these experiments suggest that KITL produced by the granulosa cells in the primordial follicle triggers growth of the enclosed oocyte (Figure 3).

These findings also raise many questions for further study. Differential splicing of its mRNA generates two isoforms of KITL – a soluble form and a membrane-associated form that has a higher bioactivity. Although increased production of KITL may be the trigger that initiates growth, it is also plausible that a switch from soluble to membrane-associated forms plays a role. The source and the nature of the signal that increases protein synthesis in the granulosa cells of one primordial follicle but not those of its neighbor remains unknown. A clue may lie in observations that, when ovary-like structures have been generated by aggregating germ cells and granulosa cells, many of the follicles in these reconstituted ovaries rapidly initiate growth (69, 70). This strongly suggests that the regulated entry of primordial follicles into the growth pool depends on cellular interactions in the intact ovary that are not recapitulated in the de novo-generated structures. Finally, the transcription factor, FOXO3, translocates from the nucleus to the cytoplasm at an early stage of growth and genetic deletion of *Foxo3* cause most oocytes in primordial follicles to begin to grow (59, 71). Similarly, deletion of the oocyte-specific transcription factor *Sohlh2* or oocyte-specific deletion of transcription factor *Lhx8* also trigger initiation of oocyte growth, apparently independently of signals from the granulosa cells (72–74). These results indicate that PI3-kinase signaling in the oocyte somehow decreases the activity these transcription factors, but the nature of the link remains to be defined. Alternatively, it may be that oocyte growth can be triggered through independent pathways.

CELL SIGNALLING DURING OOCYTE GROWTH

Signals from the granulosa cells play an essential role not only in initiating oocyte growth but also in sustaining it. This was clearly demonstrated when granulosa-oocyte complexes (GOCs) were isolated from ovarian follicles and cultured intact or following physical separation of the two cell types (75). Oocytes within intact GOCs continued to grow, whereas oocytes not in direct contact with the granulosa cells grew little or not at all, even when the granulosa cells were provided in co-culture. Subsequent studies showed that the rate of growth of an oocyte was proportional to the number of granulosa cells that were present in the GOC containing that oocyte (54). These results highlight the indispensable role played by the granulosa cells as well as the crucial importance of physical contact between the two cell types.

Gap junctions

The best understood function of the granulosa cells is to transfer essential nutrients and signals to the oocyte via the gap junctions that link the two cell types (65, 76). Gap junctions are intercellular channels composed of transmembrane proteins termed connexins that permit the exchange of molecules up to about 1 kDa between the coupled cells (77, 78). Oligomers of six connexins form hemi-channels, and the docking of hemi-channels of adjacent cells generates the gap junction. Many individual gap junctions may cluster in one region of the plasma membrane, generating a structure termed a plaque. Mammalian connexins are encoded by approximately 20 genes, and the subtype(s) of connexin present in a gap junction can affect its properties and thus the type or efficiency of intercellular communication that it supports. Although many gap junctions contain only a single connexin subtype, there also exist heterotypic junctions where the two hemi-channels contain different connexins and heteromeric hemi-channels where the hexamer contains different connexins. Not all connexins can interact to form heteromers or heterotypic junctions, but the potential combination of different connexins within one gap junction may permit additional diversity in their signaling properties.

Both oocytes and granulosa cells express numerous connexin genes; however, GJA4 (connexin-37) is the principal essential connexin identified in oocytes, whereas GJA1 (connexin-43) predominates in the granulosa cells (79–82). Deletion of either gene severely compromises oocyte development. In mice lacking *Gja4*, no gap junctional coupling is detectable between the oocyte and granulosa cells (82). The oocytes grow to only about half the volume of wild-type oocytes and they fail to acquire a property termed meiotic competence, defined as the ability to undergo meiotic maturation when removed from the follicle and incubated in vitro (83, 84). When wild-type oocytes are enclosed by *Gja4*^{−/−} granulosa cells, however, they grow apparently normally (84). Thus, GJA4 is essential in oocytes but not in granulosa cells for gap junctional coupling between the two cell types and for normal oocyte development. Intriguingly, the granulosa cells of *Gja4*^{−/−} mice become luteinized and begin to secrete progesterone (82), a process that normally occurs only after ovulation. This suggests that oocyte-granulosa cell coupling is also required to maintain normal granulosa cell physiology.

In mice lacking *Gja1*, granulosa-granulosa cell coupling is severely reduced and the granulosa cells are compromised as shown by their failure to generate the normal multilaminar structure around the growing oocyte (81). Even though coupling between the oocyte and adjacent granulosa cell is retained, the oocyte does not develop normally. They reach less than half the volume of wild-type oocytes, generate only a thin zona pellucida, lack specific ultrastructural features including fibrous lattices and cortical granules, and do not acquire meiotic competence (81, 84). When *Gja1*^{−/−} oocytes are enclosed by wild-type granulosa cells, however, they grow apparently normally (84). These results indicate that gap junctional communication mediated through GJA1 between the granulosa cells is required for normal oocyte development, with the caveat that such experiments cannot rule out an essential GJA1 function not related to gap junctions. In any case, these results illustrate the general principle that, when the normal physiology of the granulosa cells is disrupted, oocytes cannot develop normally.

A point that remains unresolved is the identity of the granulosa cell connexin that establishes gap junctions with the oocyte. Immunofluorescence using subtype-specific antibodies reveal GJA1 at the interface between granulosa cells as expected, but not between the granulosa cells and the oocyte (85), and gap junctional communication with the oocyte is retained when *Gja1* is deleted from granulosa cells (81). These observations suggest that, whereas granulosa cells use GJA1 to assemble gap junctions with other granulosa cells, they use a different connexin, likely GJA4, to assemble those with the oocyte. This suggests that GJA1 and GJA4 are selectively trafficked to different locations within the granulosa cells; alternatively, homotypic junctions may be formed or maintained more efficiently than heterotypic junctions. Whether GJA4 confers a particular function to the granulosa cell-oocyte gap junctions also remains unknown. However, wild-type oocytes develop apparently normally when reaggregated with granulosa cells that lack *Gja4* (84), as do oocytes in mice that express *Gja1* under the control of the *Gja4* promoter (86). These results suggest that GJA4 does not confer an essential property to the gap junctions linking the granulosa cells to the oocyte.

What do the granulosa cells provide to the growing oocyte via the gap junctions (Figure 4)? Oocytes are unable to efficiently utilize glucose as an energy source (87), probably because several key enzymes in the glycolytic pathway are expressed at relatively low levels (88). These enzymes are present at a much higher concentration, however, in cumulus granulosa cells (and presumably the granulosa cells of preantral follicles). Thus, the granulosa cells are able to metabolize glucose to pyruvate, which could then be transmitted via gap junctions to the oocyte, where it serves as an energy substrate. Similarly, oocytes are unable to efficiently take up certain amino acids – for example, they lack *Slc38a3*, encoding an alanine transporter – and rely on the granulosa cells to provide them via gap junctions (89). The granulosa cells of preantral follicles also provide a function via gap junctions that enables the oocyte to regulate its pH in response to environmental stress (90). The granulosa cells may also provide cholesterol to the oocytes, which express the biosynthetic enzymes at relatively low levels (91). Cholesterol presumably is not transported through gap junctions but might be transferred at the sites where the plasma membranes of the TZPs and oocyte are sufficiently closely apposed. Conceptually, the extensive gap junctional coupling between the oocyte and granulosa cells, as well as between the granulosa cells themselves, may be considered to be a mechanism that, by generating an enormous syncytial-like structure, greatly increases the effective plasma membrane surface area that the oocyte can exploit to obtain essential nutrients from extracellular sources (92).

Growth factors

Oocytes that are physically separated from the granulosa cells grow very little or not at all, even when the two cell types are co-cultured. In contrast, although oocytes lacking *Gja1* lack detectable gap junctional communication with the granulosa cells, they reach a final diameter of ~50 μm , representing a >50-fold increase in volume (83, 84). This suggests that not only gap junctional communication but also other cell contact-dependent signals from the granulosa cells promote oocyte growth. These putative signals have not been identified; however, several lines of evidence implicate KITL. The granulosa cells of pre-antral follicle, which contain growing oocytes, express both *Kitl1* and *Kitl2* (93–95). Addition of soluble

KITL to culture medium promoted an increase in the rate of growth of granulosa cell-free oocytes (96, 97). Additionally, when oocytes from which the zona pellucida had been removed were incubated on monolayers of fibroblasts expressing the membrane-bound KITL2, their growth was robustly stimulated and this effect was suppressed by addition of a function-blocking KIT antibody or inhibitors of PI3-kinase signaling (97). It is worth noting that only a small region of the spherical oocyte would have been in physical contact with the KITL-expressing fibroblasts. Deletion of *Kit* from growing oocytes, which could be achieved using the *Zp3-Cre* mouse, would directly test a role for KIT signaling during oocyte growth. Finally, it is intriguing that expression of both *Kitl1* and *Kitl2* are substantially decreased in the granulosa cells of antral follicles, whose oocytes have reached full-size, and fully grown but not growing oocytes are able to suppress *Kitl1* and *Kitl2* expression (94, 95, 98). These results imply that there is an additional pathway by which fully grown oocytes are able to suppress growth-promoting signals from the granulosa cells.

Signaling from the oocyte to the granulosa cells

The developing oocyte not only receives and responds to signals from the surrounding cells, it also sends signals that regulate differentiation of the somatic compartment of the follicle. This was hinted at many years ago by the observation that when the oocyte was removed from the follicle, the granulosa cells rapidly underwent luteinization, which normally occurs only after ovulation has expelled the oocyte from the follicle (99). This probably reflects at least in part the activity of the oocyte to suppress expression in the granulosa cells of the *Lhcgr* gene encoding the LH receptor (100). Specific signals sent by the oocyte to the granulosa cells via gap junctions have not yet been identified. Factors secreted by the oocyte do, however, regulate the differentiation of the granulosa cells and even the thecal cells (21, 87, 101).

GDF9 and bone morphogenetic protein (BMP) 15 are closely related members of the transforming growth factor (TGF) β superfamily. Like other family members, GDF9 and BMP15 are secreted as dimeric pro-peptides, and removal of the inhibitory N-terminal pro-domains by membrane-associated furin-like proteases generates the mature biologically active form (102, 103). The dimeric pro-peptides may also be active (103). The receptor for each is a heteromeric complex comprising type I and type II serine-threonine kinases. Ligand binding to the BMPR2 type II receptor triggers recruitment and phosphorylation of a type I receptor, which in turn phosphorylates SMAD2/3 (GDF9) or SMAD1/5/8 (BMP15). The phosphorylated forms associate with the so-called common SMAD4, and the heterodimeric SMAD is translocated to the nucleus where it can regulate transcription of target genes. GDF9 and BMP15 can also form heterodimers whose signaling activity is much greater than that of the homodimers (102, 103). However, the relative proportions of homomeric and heterodimeric forms in vivo has not been reported.

In situ hybridization and immunohistochemical studies indicate that the oocyte is the major source of both GDF9 and BMP15 (104–106). The mRNAs for both factors increase when oocytes begin to grow and GDF9 protein is detectable in oocytes of primary follicles and all subsequent stages of folliculogenesis. In the mouse, BMP15 may not be produced in large amounts until near the time of ovulation, possibly due to KITL-mediated inhibition of

production (107). Some studies have reported expression of *Gdf9* and *Bmp15* in granulosa cells as well (108). Female mice lacking *Gdf9* are anovulatory and sterile (109). The granulosa cells fail to proliferate normally and do not generate more than a single layer around the growing oocyte. This proliferative defect is due to increased production of inhibin in the *Gdf9*^{-/-} females, because the granulosa cells of *Gdf9*^{-/-}; *Inha*^{-/-} females proliferate apparently normally (110, 111).

The follicles of *Gdf9*^{-/-} females also fail to acquire a thecal layer (21, 109, 112). These cells arise from a progenitor cell population in the ovarian mesenchyme and can be identified by the expression of *Gli1*, encoding a transcription factor (21). Ligands of the Hedgehog pathway secreted by the granulosa cells induce expression of *Gli1* in the thecal cell precursors, and GDF9 promotes production of these ligands. Thus, GDF9 directs differentiation not only of the granulosa cells but also, albeit indirectly, of the thecal cells. It may be noted that this effect of GDF9 is manifested in ovaries near the time of birth. As ovaries at this stage would not contain growing follicles, this result indicates that non-growing oocytes also produce GDF9.

The oocytes of *Gdf9*^{-/-} females are able to grow and, possibly due to a 30-fold increase in KITL production by the GDF9-deprived granulosa cells (111, 112), reach a final size significantly larger than wild-type oocytes. Nonetheless, these oocytes are abnormal. In prepuberal mice, a large fraction of the growing oocytes fail to achieve meiotic competence and oocytes of both prepuberal and adult mice show specific ultrastructural abnormalities, including an apparently disorganized cytoskeleton (56, 109). These abnormalities cannot be attributed to an autocrine effect of GDF9 on the oocyte, because deletion of *Smad4* within the oocyte has no phenotypic effect (113). Rather, it appears the GDF9-deprived granulosa cells fail to interact appropriately with the oocyte. In support of this, the TZPs of *Gdf9*^{-/-} mice are reduced in number and are frequently oriented parallel rather than perpendicular to the oocyte surface. In view of the rescue of granulosa cell proliferation defect in *Gdf9*^{-/-}; *Inha*^{-/-} mice, it would be interesting to test whether the TZPs of these individuals recover their normal orientation and whether their oocytes develop normally.

Bmp15^{-/-} female mice show a mild impairment of fertility, which is exacerbated in the *Bmp15*^{-/-}; *Gdf9*^{+/-} females, suggesting that SMAD2/3-dependent signaling may be dominant in this species (114, 115). Sheep homozygous for inactivating mutations of either gene are infertile. In contrast, heterozygous females carrying one mutant allele of either gene show enhanced fecundity, which may be linked to a change in the relative proportion of homo- and heterodimeric forms (103). Mutations in these genes have also been linked to infertility in humans (108). Thus, the functions of GDF9 and BMP15 during follicular development are evolutionarily conserved.

Remarkably, the oocyte-derived paracrine factors (ODPF), through their effects on the granulosa cells, also promote development of the oocyte itself. As discussed above, the granulosa cells metabolize glucose to generate pyruvate that they transfer to the oocyte. Removing the oocyte from the cumulus-oocyte complex (COC), in a procedure termed oocyctomy (OOX), leads to a decrease both in the amount of mRNAs encoding key enzymes in the glycolytic pathway and in the rate of glycolysis in the remaining cumulus-

cell shell (88, 116). Importantly, incubating the OOX shells with fully grown oocytes restores both the quantities of the affected mRNAs and glycolytic activity. These mRNAs and glycolytic activity are also reduced in the cumulus granulosa cells of *Bmp15*^{-/-}; *Gdf9*^{+/-} mice, and incubating OOX shells in the presence of BMP15 and fibroblastic growth factor (FGF) 8B restores mRNA levels and glycolysis (116). Thus, ODPFs stimulate the granulosa cells to manufacture the pyruvate that they transfer to the oocyte.

Similarly, in both *Bmp15*^{-/-}; *Gdf9*^{+/-} mice and OOX shells of wild-type mice, the quantities of mRNAs encoding enzymes required for cholesterol synthesis are reduced in the cumulus granulosa cells and cholesterol synthesis is impaired, and these effects can be rescued fully and partially, respectively, by co-culture of the OOX shells with fully grown oocytes (91). Similarly, expression of the amino acid transporter, *Slc38a3*, and uptake of alanine by the cumulus granulosa is reduced in OOX shells and these defects can be rescued by co-incubation of the cumulus granulosa cells with fully grown oocytes (117). Thus, the oocyte produces and secretes factors that stimulate the neighbouring granulosa cells to produce molecular nutrients that they in turn provide to the oocyte.

CELL SIGNALING DURING MEIOTIC MATURATION

As discussed above, oocytes of primordial follicles are arrested at prophase I of meiosis and they remain so until they have completed growth and the pre-ovulatory LH surge triggers meiotic maturation. When oocytes at mid-growth phase are removed from the follicle and placed in culture, however, they will undergo maturation in the absence of LH. This result indicates that some property of the follicular environment actively prevents these meiotically competent oocytes from undergoing maturation until the appropriate LH-mediated signal is received. This inhibitory action requires contact between the cumulus and mural granulosa cells, because when the cumulus-oocyte complex was microsurgically detached from the mural granulosa cells within an intact antral follicle, the oocyte underwent maturation (118). Blocking gap junctional activity pharmacologically or by injecting peptides or antibodies targeting either GJA1 or GJA4 into the antrum also induces maturation of follicle-enclosed oocytes (119–121). These results imply that the maturation-inhibiting signal requires the mural granulosa cells and gap junctional communication to reach the oocyte.

Maturation is initiated by a drop in cyclic AMP within the oocyte, which enables the dephosphorylation and activation of CDK1 that drives entry into M-phase (25) (Figure 5). A plausible early hypothesis was that the gap junctions allowed cAMP to pass from the granulosa cells to the oocyte. Subsequent studies showed that cAMP, which is generated by the adenylyl cyclase activity of a GPR3-activated G_s-protein, produced by the oocyte itself is required to maintain high intracellular cAMP (122, 123). Although these experiments do not entirely rule out a role for cAMP provided by the granulosa cells, it appears that the key inhibitory molecule provided by these cells is cGMP. This inhibits the cAMP-specific phosphodiesterase (PDE) 3A within the oocyte and thus maintains a high concentration of cAMP (122).

cGMP is synthesized by the membrane-associated guanylyl cyclase natriuretic peptide receptor (NPR2). NPR2 is not detectable in the oocyte, but is abundantly expressed in the

cumulus granulosa cells and the mural granulosa cells adjacent to the antrum (124). Consistent with this pattern of expression, GDF9 alone or in combination with BMP15 stimulates the expression of *Npr2* (124). Moreover, these ODPFs also increase expression in the cumulus granulosa cells of inosine monophosphate dehydrogenase, which is required to generate the substrate for cGMP synthesis. Experiments using fluorescent cGMP sensors have shown that the concentration of cGMP is uniform throughout the oocyte-granulosa cell compartment in antral follicles (125). As the mural granulosa cells are located far from the oocyte, this highlights the efficiency with which gap junctions can permit cGMP to be delivered from remote sites of synthesis to the oocyte.

NPR2 is activated by the C-type natriuretic peptide (NPPC or CNP). *Nppc* mRNA is detectable only in the mural granulosa cells in antral follicles (124). It is released extracellularly and diffuses through the follicular fluid to activate NPR2 receptors on both mural and cumulus granulosa cells. The restriction of NPPC synthesis to the mural granulosa cells likely explains why oocytes, even within COCs, undergo maturation when removed from the follicle. As the mural granulosa cells that supply the NPPC are no longer present, synthesis of cGMP stops; when the concentration of cGMP within the oocyte falls below a threshold, PDE3A becomes activated. Consistent with this, addition of NPPC to the culture medium inhibits maturation of cumulus-enclosed but not cumulus-free oocytes, and this inhibition requires gap junctional communication (120, 122, 124, 126). These results also indicate that, in vitro, the cumulus granulosa cells can make sufficient cGMP to prevent oocyte maturation.

Although LH triggers meiotic maturation, its receptors are not expressed by the oocyte or even by the cumulus granulosa cells that surround it. Instead, they are found on the mural granulosa cells (as well as on the thecal cells). Thus, the signal must be relayed from the mural granulosa cells to the oocyte. The binding of LH to its mural granulosa cell receptors triggers the release of epidermal growth factor-related peptides - amphiregulin, epiregulin, and beta-cellulin - that diffuse through the antrum and bind to EGF receptors located on both the mural and cumulus granulosa cells (26). Following application of LH to follicles in vitro, the cGMP concentration within the granulosa-oocyte compartment rapidly falls (125). This decrease is first seen in the mural granulosa cells and is then detected in the cumulus granulosa cells and oocyte. The fall in cGMP reflects both decreased synthesis, owing to dephosphorylation of NPR2 (127, 128) and reduced production of NPPC (126, 129, 130), and to increased hydrolysis, owing to phosphorylation of PDE5 (131).

It remains to be fully resolved whether the decreased activity of NPR2 and decreased production of *Nppc* are triggered by LH directly or indirectly via activation of EGFR, and recent work suggests that both pathways play key roles (26, 122, 127, 129). Regardless of the specific pathways involved, gap junctions play a crucial role in the rapid decrease in cGMP. When gap junctions are pharmacologically inhibited, addition of LH provokes a rapid fall in cGMP in the mural granulosa cells but a much slower decline in the cumulus cells (125). This suggests that gap junctions permit a rapid flow of cGMP from the cumulus granulosa cells, and also from the oocyte outward to the mural granulosa cells. Later during maturation, gap junctional communication is lost between the two cell types. This may be due both to phosphorylation-mediated changes in the properties of the gap junctions and to

displacement of the granulosa cells away from the oocyte during cumulus layer expansion (132). Gap junctions thus mediate not only meiotic arrest, but also meiotic resumption.

Recent work has uncovered an additional regulatory role for the cumulus granulosa cells during maturation. It has long been known that a subset of mRNAs in the oocyte become translationally activated during maturation (30). Addition of amphiregulin (or EGF) to cumulus-enclosed oocytes but not cumulus-free oocytes in vitro further boosts the translational activity of specific mRNAs (133). Moreover, oocytes of *Areg*^{-/-} mice show a reduced ability to develop as embryos, suggesting that this increased translational activity is developmentally important. As oocytes do not detectably express the EGF receptor, these results indicate that its activation in the granulosa cells induces them to send a signal to the oocyte that upregulates translation of specific mRNAs. Although this signal remains to be identified, EGF-receptor activation in the cumulus granulosa cells triggers phosphorylation of AKT in the oocyte, and pharmacological inhibition of PI3-kinase signaling (which would affect signaling in both cumulus granulosa and the oocyte) blocks the translational activation. EGF receptor ligands also did not further increase translation of the reporter mRNAs in oocytes in which *Pten*, which antagonizes PI3-kinase signaling, had been deleted – presumably because the pathway had become constitutively activated. This evidence suggests that the signal may be a growth factor that is mechanistically coupled to PI3-kinase signaling.

Coincident with oocyte maturation, the cumulus granulosa cells secrete an extracellular matrix that separates the cumulus cells from each other and pushes them away from the oocyte. Termed expansion of the cumulus layer, this process terminates contact between the granulosa cells and contributes to the loss of contact between the oocyte and the granulosa cells. Expansion is triggered by the EGFR ligands that are released by the mural granulosa cells in response to LH. When the oocyte is microsurgically removed from COCs obtained from pre-ovulatory follicles, however, the remaining shell of cumulus cells is unable to undergo expansion (134, 135). Co-culture of the shells with oocytes or with oocyte-conditioned medium, however, restores the ability to expand via a SMAD2/3-dependent pathway (134–136). Thus, oocytes secrete an expansion-enabling factor.

Subsequent work has revealed that cumulus expansion is impaired in *Bmp15*^{-/-} and in *Bmp15*^{-/-}; *Gdf9*^{+/-} mice (114, 115). Moreover, addition of GDF9 and/or BMP15 to culture medium can induce cumulus expansion as well as upregulation in the cumulus cells of genes implicated in this process (102–104, 114). More broadly, as exemplified by their activity to suppress expression of the LH receptor, the oocyte-derived factors promote differentiation towards the cumulus cell phenotype and antagonize the mural granulosa cell phenotype (22, 137, 138). This is due in part to their activity to upregulate MTOR signaling in the cumulus cells (23). Recent work has identified additional growth factors that are secreted by oocytes during maturation, following translational activation of their encoding mRNAs. Specifically, interleukin-7 (IL7) secreted by maturing oocytes enhances proliferation of the cumulus cells (139). Identifying additional functions for IL7 and other factor secreted by maturing oocytes is an important goal of future research.

NEW HORIZONS - EXTRACELLULAR VESICLES

Recent studies have uncovered a new and unanticipated means by which cells within the follicle may communicate. Analyses of fluids from a range of biological sources have revealed the presence of small membrane-bound structures known as extracellular vesicles (EV). Among these, two main types based on their size and origin have been identified so far (140–142). Microvesicles range in diameter from 100–1000 nm in diameter and are budded off at the plasma membrane. Exosomes are smaller, ranging from 50–200 nm in diameter. They arise from endocytotic invaginations within the late endosome compartment, which generate multivesicular bodies (MVBs) containing multiple exosomes. Fusion of MVBs with the plasma membrane releases the exosomes into the extracellular space. They are often described together as EV, reflecting the difficulty in assigning the origin of a vesicle after it has been released from the cell.

Recent studies have identified EVs in ovarian follicular fluid (Figure 6) (143–149). Like those of other origins, follicular EVs contain mRNAs, miRNAs and proteins, suggesting a potential role for these structures in intrafollicular communication (140, 141). Several strategies have been employed to test this idea. In one approach, EVs have been loaded with a fluorescent marker and then either co-incubated with granulosa cells or injected directly into the follicle. The granulosa or follicular cells then have been examined to see whether they contain fluorescent particles within the cytoplasm (144, 147, 149). Both the *in vitro* and the *in vivo* tests have confirmed that the EVs derived from follicular fluid can fuse with granulosa cells, establishing their potential to carry information between cells.

To address the function of EVs, it has been tested whether EVs can alter physiology or behavior of the granulosa cells. As discussed above, the cumulus cell layer undergoes expansion at the time of ovulation, owing to the secretion of a jelly-like matrix by the cumulus cells. Strikingly, follicular EVs were found to induce cumulus expansion *in vitro* as well as increases in the amount of mRNAs that are upregulated during expansion *in vivo*. However, the effects were considerably attenuated compared to the response to the EGFR ligands that are the physiological trigger (147). Additionally, it has been shown that mixing EVs with granulosa cells leads to an increase in the granulosa cell content of miRNAs that are abundant in the EVs (143, 149); conversely, the quantities of putative mRNA targets of EV-enriched miRNAs are decreased (143, 149). This suggests that EVs could deliver miRNAs to target cells and thereby regulate gene expression post-transcriptionally. Finally, it has been shown that the density of EVs in the follicular fluid decreases as the antrum increases in volume and that the content of the EVs differs between small and large follicles (144, 146, 149). These data suggest that the generation and composition of EVs could be developmentally regulated.

Unexpectedly, EVs may also allow macromolecular transfer not just between somatic cells within the follicle but also between the cumulus cells and the oocyte (Figure 6). Electron microscopy has revealed structures that are morphologically similar to EVs at the tips of TZPs, adjacent to the oocyte plasma membrane (41, 150). These results are especially intriguing in view of live-cell imaging that reveals mRNAs travelling along the TZPs towards their tips. Strikingly, after incubation of wild-type oocytes on a monolayer of EGFP-

expressing cumulus granulosa cells, *Egfp* mRNA has been detected in the oocytes (41). mRNAs could not pass through gap junctions, but could be packaged into EVs that are budded from the tip of the TZPs and by this mechanism delivered to the oocyte.

While these results provide tantalizing hints that EVs might be a new player in the signaling network within the growing follicle, many important questions remain to be answered. If EVs carry specific signaling molecules, it would be expected that EVs from a non-follicular source should be unable to trigger the same biological effects. Indirect evidence supporting this idea has been presented (147), but definitive experiments remain to be done. It also remains unclear what fraction of the cells in the follicle can be reached by the EVs – for example, are the inner layers of cumulus cells around the oocyte accessible? Furthermore, although there are differences in the molecular content of EVs, from small and large follicles (144, 146), a mechanism by which (for example) specific miRNAs, mRNAs or proteins could be packaged in the EVs has not been demonstrated. Most importantly, we do not yet know whether the biomaterials delivered by exosomes play an essential role in the physiological function of their target cells. Because the growing follicle is a well-defined system comprising a small number of spatially segregated and easily distinguished cell types, such studies should be feasible.

CONCLUSION

The anatomically simple structure of the follicle and its accessibility for experimental studies have allowed us to gain considerable insight into the signaling interactions with the somatic compartment of the follicle that support and direct differentiation of the oocyte. Not surprisingly, this knowledge has identified new black boxes and generated new questions to be pursued. As discussed earlier, we do not know why one primordial follicle begins to grow while its neighbor does not. What is the nature of the signal that increases expression of KITL in the granulosa cells of a primordial follicle, and do other signals contribute to triggering growth? Once the oocyte has begun to grow, we know that Kit signaling can promote further growth, but not whether it plays this role under physiological conditions. If not KITL, what is the contact-dependent, gap junction-independent signal that granulosa cells provide that sustains oocyte growth? Although TZPs are the only physical bridge that enables contact-dependent communication between the growing oocyte and its follicular environment, we know almost nothing of their origin. Nor do we know whether they are dynamic structures, that might be continually extended and retracted, and it is not certain how many types of TZP exist that might serve different functions. LH acting on the mural granulosa cells triggers meiotic maturation of the oocyte, but the relative roles of signaling directly through its receptor or indirectly through EGFR activity remain to be clarified. Finally, the role of extracellular vesicles in regulating differentiation of the somatic follicular cells and the oocyte needs to be experimentally defined. Importantly, the answers to these questions will likely benefit human reproductive health. While it has long been known that disease and aging can reduce oocyte quality, the underlying mechanisms remain largely obscure. It is plausible that in some cases the root cause lies in impaired signaling between the oocyte and its follicular microenvironment. A more profound understanding of this complex and continuing interaction should enable therapeutic strategies to be designed and implemented to preserve fertility in women.

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OOCYTE AND FOLLICULAR GROWTH

MEIOTIC MATURATION

Figure 1. Post-natal oocyte and follicular development

(A) The arrangement of the principal cell types of the follicle at different stages of oocyte and follicular growth is shown. Each primordial follicle contains one oocyte enclosed by a small number of squamous granulosa cells. The first morphological sign that a follicle and its oocyte have entered the growth phase is a transition of the granulosa from a squamous to cuboidal morphology. As the oocyte grows, the cuboidal granulosa cells proliferate so that they continue to cover the surface of the oocyte. Continued proliferation of the granulosa cells generates a second layer, defining the follicle as secondary. Thecal cells are recruited around the exterior of the follicle, and are separated from the granulosa cells by a basement membrane. As the follicle continues to grow, a fluid-filled cavity termed the antrum appears. This divides the granulosa into mural and cumulus subpopulations, which express different genes and follow different fates. Even though the follicle will continue to increase in size, growth of the oocyte slows or stops at the antral stage. This transition is accompanied by transcriptional arrest and a change in the degree of condensation and spatial arrangement of the DNA in the oocyte nucleus, from a non-surrounded nucleolus (NSN) to a surrounded nucleolus (SN) configuration, as shown in the oocyte in the pre-ovulatory follicle. The fully grown oocyte contains, in addition to actively translated mRNAs, products including stored translationally inactive mRNAs and mitochondria that are required during meiotic maturation or early embryogenesis. (B) The nuclear events of meiotic maturation are highlighted. The membrane surrounding the nucleus (germinal vesicle) breaks down and the chromosomes condense and align on the spindle, which becomes translocated to the

periphery of the oocytes. The first meiotic division segregates homologous chromosomes, and one set is discarded in the first polar body. The chromosomes remaining in the oocyte then align on a second spindle. Meiosis becomes arrested at metaphase II until fertilization activates the egg. During maturation, the cumulus granulosa cells secrete a matrix that separates them from each other and from the oocyte and physical contact between the two cell types is terminated. Modified from (17) with permission.

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Figure 2. Transzonal projections

(A) A dense forest of TZPs, indicated by the arrow, projects from the cumulus granulosa cells to the oocyte. Phalloidin (green) stains their actin-rich cytoskeleton as well as the cortex of the oocyte. Nuclei are stained using DAPI (blue). Bar = 25 μ m.

(B) Higher-power magnification shows that several TZPs often appear to emerge from a single locus (arrows).

(C) Electron-micrograph showing an oocyte at an early stage of growth, as indicated by the thin zona pellucida (zp), and an incipient TZP (arrow) projecting from a granulosa cell. Bar = 500 nm.

(D) Electron-micrograph showing an oocyte at a later stage of growth and several narrow and elongated TZPs (one marked by arrow). Bar = 500 nm

(E) Cartoon depicting interactions between TZP tips and oocyte plasma membrane. The TZPs are anchored by adherens and possibly other types of junctions. Gap junctions permit the passage of molecules up to 1 kDa. The TZP-oocyte interface is presumably also the site of contact between membrane-associated growth factors and their membrane-associated receptors.

(F) Two non-exclusive models of how TZPs may form. Upper: Stretching model. (1) Prior to deposition of the zona, the plasma membranes of the oocyte (pink) and adjacent granulosa cells (light brown) are in physical contact at numerous sites (two shown per granulosa here

for simplicity). (2) Deposition of the zona pellucida (light blue) pushes the bodies of the granulosa cells away from the oocyte, but the cells remain connected at the original points of contact. (3) As the oocyte continues to grow and the zona pellucida thickens, the cytoplasmic strands of the granulosa cell elongate to produce TZPs. New granulosa cells (dark brown) are born to enable a continuous layer to be maintained around the expanding oocyte surface. (4) Because granulosa cells born after the deposition of the zona pellucida have never been in direct contact with the oocyte, they do not generate TZPs. Hence, the number of TZPs does not increase as oocytes grow. Lower: Pushing model. (1, 2) Deposition of the zona pellucida prevents physical contact the oocyte and surrounding granulosa cells. (3) Granulosa cells elaborate filopodia-like structures the extend towards the oocyte. New granulosa cells (dark brown) are generated as the oocyte surface expands. (4). The newborn granulosa cells elaborate new TZPs. Hence, the number of TZPs increases as oocytes grow.

Figure 3. Initiation of oocyte and follicular growth

(A) Schematic representation of the canonical PI3-kinase signaling pathway.

(B) Possible signaling pathway that activates oocyte growth. Oocytes in primordial follicles are enclosed by squamous granulosa cells. Unknown signals trigger an increase in protein synthesis in the granulosa cells and a transition to a cuboidal morphology. This may reflect entry into the mitotic cell cycle. The granulosa cells may increase production of KITL; another possibility is that synthesis of the more bioactive membrane-bound form becomes favored. The consequent activation of KIT signaling within the oocyte increases protein synthesis, thus driving an increase in cell size.

Figure 4. Communication between the growing oocyte and adjacent granulosa cells

(A) Gap junctions enable the granulosa cells to transfer pyruvate, nucleotides and amino acids to the oocyte. The granulosa cells also provide the oocyte with cholesterol, which might be transferred between cells where the plasma membranes lie in close apposition. It is unknown whether the oocyte supplies essential factors to the granulosa cells via gap junctions.

(B) Granulosa cells produce KITL whereas oocytes express the KIT membrane receptor. The membrane-associated form of KITL promotes oocyte growth more efficiently than soluble KITL. Oocytes secrete the TGF β family members, GDF9 and BMP15, as well as FGF8B, which activate receptors on the granulosa cell plasma membranes.

Figure 5. Regulation of meiotic maturation

(A) Before the activation of the LH receptor (LHCGR), the mural granulosa cells produce and release NPPC, which activates its receptor, NPR2, located on both mural and cumulus granulosa cells. Active NPR2 generates cGMP, which diffuses through gap junctions and reaches a concentration in the oocyte that is sufficient to inhibit phosphodiesterase 3A. This allows the concentration of cAMP, produced by the oocyte, to remain high thereby maintaining CDK1 in a hyper-phosphorylated inactive form.

(B) Binding of LH to LHCGR triggers the release of ligands from the mural granulosa cells that activate EGFR on the mural and cumulus granulosa cells. cGMP levels within the granulosa cells fall due both to inhibition of its synthesis owing to dephosphorylation of NPR2 and (later) reduced production of NPPC and to increased hydrolysis owing to activation by phosphorylation of PDE5A in the granulosa cells. The relative contribution of LHCGR-mediated and EGFR-mediated signaling to these events remains to be fully elucidated. As the concentration of cGMP within the granulosa cells falls, it flows out of the oocyte to equalize its concentration throughout the granulosa cell-oocyte compartment. The decreased cGMP in the oocyte permits PDE3A to become active and hydrolyze cAMP, enabling dephosphorylation and activation of CDK1. Modified from (17) with permission.

Figure 6. New mechanisms of intrafollicular communication: extracellular vesicles

Extracellular vesicles (EV) are present in follicular fluid and represent a potential mechanism by which macromolecules including mRNA and miRNA could be transferred between cells. Structures resembling EV have also been detected at the tips of TZPs and could permit similar transfer from cumulus granulosa cells to the oocyte. Modified from (151) with permission.