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Regulation of Mammalian Oocyte Meiosis by Intercellular Communication Within the Ovarian Follicle

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Abstract

Meiotic progression in mammalian preovulatory follicles is controlled by the granulosa cells around the oocyte. Cyclic GMP (cGMP) generated in the granulosa cells diffuses through gap junctions into the oocyte, maintaining meiotic prophase arrest. Luteinizing hormone then acts on receptors in outer granulosa cells to rapidly decrease cGMP. This occurs by two complementary pathways: cGMP production is decreased by dephosphorylation and inactivation of the NPR2 guanylyl cyclase, and cGMP hydrolysis is increased by activation of the PDE5 phosphodiesterase. The cGMP decrease in the granulosa cells results in rapid cGMP diffusion out of the oocyte, initiating meiotic resumption. Additional, more slowly developing mechanisms involving paracrine signaling by extracellular peptides (C-type natriuretic peptide and EGF receptor ligands) maintain the low level of cGMP in the oocyte. These coordinated signaling pathways ensure a fail-safe system to prepare the oocyte for fertilization and reproductive success.

Keywords

oocyte meiosis; ovarian follicle; luteinizing hormone; intercellular communication; cyclic GMP; gap junctions

INTRODUCTION

Meiosis, which reduces the number of homologous chromosomes from two to one, allows egg and sperm genomes to combine at fertilization. In mammalian oocytes, meiosis starts during embryogenesis and concludes at fertilization, months later in mice, and years later in humans (1). The oocyte's DNA duplicates, and recombination occurs between homologous chromosomes (Figure 1). Meiosis then pauses at the early stage, which is characterized by an intact nuclear envelope and nucleolus as well as partially condensed chromatin. Oocytes remain in this state until after puberty, when during each reproductive cycle, follicles grow to the preovulatory stage and are then stimulated by luteinizing hormone (LH) from the pituitary, which acts on the outer granulosa cells to restart meiosis. LH signaling also causes

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ovulation, releasing the oocyte from its follicle such that it can enter the oviduct where fertilization occurs.

This review discusses the remarkable regulatory system in mammalian preovulatory follicles that maintains meiotic prophase arrrest and how this system is reversed by LH to cause meiosis to resume. Both events, maintaining arrest and restarting meiosis, involve signaling from granulosa cells to the oocyte through gap junctions and the extracellular space. Signaling within the oocyte was reviewed elsewhere (1–5) and is considered only briefly here.

OVARIAN FOLLICLE STRUCTURE

Ovaries contain follicles of various sizes. The smallest follicles, called primordial, consist of a single layer of granulosa cells around the oocyte. The largest follicles, called preovulatory or Graafian, consist of approximately 2–3 layers of inner granulosa cells, called cumulus cells, and approximately 5–10 outer layers of cells called mural granulosa cells (Figure 2). Gap junctions connect all of the granulosa cells in a functional syncytium, and the cumulus cells also form gap junctions with the oocyte (see 6). The cumulus cells are attached to one region of the mural layer, but elsewhere the two types of granulosa cells are separated by a space called the antrum, filled with follicular fluid. The cumulus cells form a pseudostratified epithelium, with cells from the first, second, and even third layers extending processes through the extracellular coat of the oocyte, the zona pellucida (7). These processes form gap junctions with the oocyte (8), comprised primarily of connexin 37 (6, 9, 10), although some contribution of connexin 43 from the cumulus cells is possible (11, 12). Elsewhere in the follicle, the gap junctions are composed of primarily connexin 43 (6, 9, 10). The localization of these connexins and some of the other signaling proteins that regulate meiotic arrest and resumption in preovulatory follicles is shown schematically in Figure 3.

The outer layers of mural granulosa cells form another pseudostratified epithelium, extending processes to the extracellular matrix forming the basal lamina (13, 14). These processes may facilitate communication with the theca cells and blood vessels outside of the basal lamina, although gap junctions do not form connections across the basal lamina. The theca cells initiate the production of steroids important for follicle development, and the blood vessels, along with their many other functions, deliver LH from the pituitary (Figure 3). We use the term follicle to include just the granulosa cells and oocyte, although it is sometimes used to include the theca as well. Figure 4 shows a photograph of a mouse preovulatory follicle (and a schematic diagram of its cellular structure is illustrated later in Figure 5).

Among mammals, the size of the preovulatory follicle is proportional to the size of the animal, ranging in diameter from approximately 400 μm in a mouse to more than 10 cm in a rhinoceros (15). The diameter of a human preovulatory follicle is approximately 2 cm. This proportionality probably arises because larger animals need more estrogen, which comes from the follicle. The number of cell layers lining the basal lamina and surrounding the oocyte is similar across species; follicle size variation is due to differences in the size of the

antrum. The diameter of the oocyte in preovulatory follicles varies little among different species, ranging from ~70 to ~120 μm (16).

The prophase-arrested oocyte contains a large nucleus, also known as a germinal vesicle, with a prominent nucleolus. The first obvious sign of meiotic resumption in response to LH is the breakdown of the nuclear envelope and disappearance of the nucleolus. In mice, this begins ~2–3 h after LH exposure, and the first meiotic division occurs at ~10–12 h (17). In larger animals, the time between LH receptor activation and nuclear envelope breakdown is longer (18), ~18 h in women, based on the limited information that is available (19).

During the first meiotic division, one set of chromosomes is discarded in a tiny cell called a polar body, leaving the other set in the oocyte (Figure 1). This division reduces the complement of chromosomes remaining in the oocyte from the diploid (2N) to the haploid (N) number. The remaining chromosomes assemble on the second meiotic spindle, but then meiosis pauses again. At this point, the oocyte (now also called an egg), is ovulated from the follicle and enters the oviduct. Meiosis remains arrested at second metaphase until fertilization causes separation of the chromatids and formation of a second polar body. This leaves one copy of each oocyte-derived gene in the egg, such that the egg genome can combine with the sperm genome to create a new individual.

MEIOTIC PROPHASE ARREST

Dependence of Prophase Arrest in the Preovulatory Follicle on the Mural Granulosa Cells

During the months or years before a follicle reaches the preovulatory stage, prophase arrest is maintained by the oocyte itself and does not rely on signals from the surrounding follicle. In part, this is thought to be due to limiting levels of cyclin-dependent kinase 1 (CDK1, CDC2), which is required for the progression from prophase to metaphase (20–23). When present in sufficient amounts and when activated by dephosphorylation and binding to its cyclin partner, CDK1 is proposed to cause nuclear envelope breakdown by controlling signaling pathways that trigger the disassembly of nuclear lamins (23) and nuclear pores (24). CDK1 activity also causes chromosomes to condense (25).

As the follicle grows to its full size and approaches the preovulatory stage, the oocyte increases its expression of CDK1 as well as other proteins needed to resume meiosis. Nevertheless, the oocyte remains arrested in prophase as long as it resides within the follicle. If the cumulusoocyte complex is removed from a preovulatory follicle, meiosis resumes spontaneously (26). This phenomenon is observed in all mammalian species examined, including humans (27). Within 1–2 h after the isolation of a mouse oocyte or cumulus-oocyte complex, the nuclear envelope breaks down, and meiosis proceeds to second metaphase. The ability of the isolated oocyte to resume meiosis develops gradually as the follicle grows (28).

Meiosis-inhibitory signals from the mural granulosa cells travel to the oocyte both through gap junctions and through the follicular fluid. Gap junction inhibitors such as carbenoxolone cause meiosis to resume in preovulatory follicles (6, 29–31), as do peptides or antibodies that specifically block either connexin 37 or connexin 43 (6, 31). These findings indicate

that the transmission of the inhibitory signal from the mural granulosa cells to the oocyte requires gap junctions between the cumulus cells and oocyte (connexin 37), between mural granulosa cells, and between the mural and cumulus cells (connexin 43). Incubating isolated cumulus-enclosed oocytes in follicular fluid also partially inhibits meiotic resumption, indicating the presence of an extracellular inhibitor (32).

Dependence of Prophase Arrest in the Preovulatory Follicle on Cyclic AMP Produced in the Oocyte

Meiotic resumption in isolated oocytes can be suppressed by addition of a membrane permeant, hydrolysis-resistant form of 3′,5′-cyclic AMP (cAMP) (33), or a cAMP phosphodiesterase inhibitor (34). Thus, it was initially thought that meiotic arrest in the follicle might be due to cAMP diffusing from the granulosa cells into the oocyte (35). However, early studies also showed that the oocyte itself could produce cAMP (35). Testing these concepts became possible with the development of techniques for imaging and injecting the oocyte within the intact living follicle (36), allowing investigation of physiological functions that depend on intercellular signaling.

Evidence that the oocyte itself is an essential source of inhibitory cAMP came from experiments showing that inhibition of the G_s G protein in follicle-enclosed oocytes causes meiotic resumption. G_s, which stimulates adenylyl cyclase that converts ATP to cAMP, was inhibited by injection of a function-blocking antibody (36) or a dominant negative form of G_s (37). Because these proteins cannot pass through gap junctions into the granulosa cells, it can be concluded that the oocyte is the original source of cAMP. Furthermore, any cAMP that might enter the oocyte through gap junctions from granulosa cells is insufficient to maintain meiotic arrest. Injection of a G_s-inhibitory antibody and/or dominant negative G_s also causes meiotic resumption in oocytes of fish and frogs (37, 38) and humans (39), indicating that this mechanism is conserved among vertebrate oocytes.

The requirement of G_s for maintaining arrest also suggests that G_s is kept active via a transmembrane G protein–coupled receptor present in the oocyte. Indeed, this receptor was identified from a list of the G-protein–coupled receptors in a mouse oocyte cDNA library (40). Of the ~1000 potential candidate receptors, only 15 were present in this particular oocyte library, and of these, two were known to activate G_s, the β-adrenergic receptor and the orphan receptor GPR3. This latter receptor was previously characterized as a constitutive activator of G_s (41). Because β-adrenergic agonists do not maintain meiotic arrest in isolated oocytes (LM Mehlmann, LA Jaffe, unpublished data), GPR3 was the prime suspect. *Gpr3* mRNA is highly enriched in the mouse oocyte, with levels 10 times greater than detected in surrounding granulosa cells (40) (Figure 3).

Loss of function studies in mice established that GPR3 activates G_s and maintains meiotic arrest in the oocyte, as depicted in Figure 5. Oocytes isolated from *Gpr3*$^{-/-}$ mice exhibit a loss of G_s activity (42), and meiosis resumes spontaneously in preovulatory follicles of *Gpr3*$^{-/-}$ mice (40, 43). These phenotypes are rescued by injection of *Gpr3* mRNA into follicle-enclosed oocytes (40, 42). Similar to the genetic knockout, knockdown of *Gpr3* by siRNA injection into wild type follicle-enclosed oocytes initiates resumption of meiosis (44). GPR3 also maintains meiotic arrest in porcine oocytes (45). In rats, the corresponding

and functional receptor is the closely related GPR12 (46). GPR3, but not GPR12, is present in human oocytes, but the function of GPR3 in maintaining meiotic arrest was not tested (39). To date, no endogenous agonists were definitively identified for GPR3 or GPR12, consistent with evidence that these receptors are constitutively active. The GPR3–G_s system produces cAMP in the oocyte by activating adenylyl cyclase AC3 (47).

Targets of cAMP in the Oocyte

Early studies showed that cAMP acts through protein kinase A (PKA) to maintain meiotic arrest (48). Through multiple steps, PKA maintains the CDK1 kinase in an inactive form (4). Although oocytes within preovulatory follicles have sufficient amounts of CDK1 to proceed to metaphase, they remain arrested in prophase, due primarily to phosphorylation of CDK1 on sites that inhibit its activity. PKA phosphorylates and activates the kinase WEE1B that then phosphorylates and inactivates CDK1 (49). PKA also phosphorylates the phosphatase CDC25B that dephosphorylates CDK1. Phosphorylated CDC25B is sequestered in the cytoplasm by the 14-3-3 protein and prevented from entering the nucleus (50, 51). By keeping CDC25B out of the nucleus, cAMP signaling maintains nuclear CDK1 in a phosphorylated and inactive state. In turn, nuclear envelope breakdown and chromosome condensation are unable to proceed. Thus, through the PKA-dependent activation of WEE1B and inhibition of CDC25B, high levels of oocyte cAMP help to limit CDK1 activity and meiotic arrest. Additional PKA substrates may be important as well; in *Xenopus* oocytes, the PKA substrate ARPP19 was recently identified as an essential regulator of CDK1 activity and meiotic resumption (52).

cAMP also suppresses cytoplasmic changes in the oocyte. These include the transformation of an interphase microtubule network into microtubule asters (53) and the development of mechanisms that release calcium from the endoplasmic reticulum (54) and prevent polyspermy (55). Similar to the progression of meiosis, these cytoplasmic responses are probably suppressed by inhibiting CDK1 kinase activity, although this hypothesis remains to be tested.

Dependence of Prophase Arrest in the Preovulatory Follicle on Cyclic GMP Produced in the Granulosa Cells

The finding that the oocyte is the source of inhibitory cAMP raises the question as to what role(s) granulosa cells play in maintaining prophase arrest. Earlier studies showed that cAMP in the mouse oocyte decreases when it is removed from the follicle (56–58), indicating that the granulosa cells participate in regulating oocyte cAMP. Several mechanisims have been considered to explain this observation.

The first mechanism proposes that granulosa cells synthesize and secrete an agonist that keeps GPR3 active in the oocyte, thus keeping G_s active and increasing cAMP. However, G_s activity fails to change when the oocyte is removed from the follicle (42). Another possibility is that additional cAMP enters the oocyte from the granulosa cells through gap junctions, adding to levels already being produced in the oocyte. Whether the concentration of free cAMP in the granulosa cells is high enough to provide a source of cAMP for diffusion into the oocyte is unknown. To test whether cAMP from the granulosa cells

contributes to maintaining meiotic arrest, it would be informative to specifically lower cAMP in the granulosa cells and determine if this causes meiosis to resume. At present, it can only be concluded that cAMP diffusion from the granulosa cells into the oocyte is not sufficient to maintain meiotic arrest in the absence of GPR3/ G_s-mediated production of cAMP in the oocyte.

An alternative hypothesis, which ultimately proved to be correct, suggested that granulosa cells provide an inhibitor of cAMP phosphodiesterase activity, thus maintaing high cAMP levels in the oocyte. Another cyclic nucleotide, cGMP, was found to inhibit cAMP phosphodiesterase activity in oocyte lysates (59), and removing the oocyte from the follicle lowered cGMP in the oocyte (58). These findings were consistent with the hypothesis that cGMP from granulosa cells diffuses through gap junctions into the oocyte, where it inhibits cAMP hydrolysis and maintains meiotic arrest (58, 60). It was subsequently established that the predominant cAMP phosphodiesterase in the oocyte is PDE3A (61–63) (Figures 3 and 5), an enzyme that is competitively inhibited by cGMP (63, 64).

This hypothesis was tested by injecting follicle-enclosed oocytes with Förster resonance energy transfer (FRET) sensors for cAMP and cGMP, allowing quantitative measurements of cyclic nucleotide concentrations in a physiologically intact system (65). Injecting oocytes with a specific cGMP phosphodiesterase, PDE9, decreases cGMP and leads to resumption of meiosis. However, this effect could be blocked by milrinone, an inhibitor of PDE3A, supporting the conclusion that lowering cGMP caused meiosis to resume by relieving the inhibition of PDE3A. The concentration of cGMP in follicle-enclosed oocytes was measured to be ~900 nM, which is sufficient to competitively inhibit PDE3A at the ~700 nM cAMP level in the oocyte (63, 65). Based on the finding that inhibiting follicular gap junctions decreased cGMP in the oocyte and is accompanied by resumption of meiosis, it was concluded that this inhibitory cGMP comes from granulosa cells and enters the oocyte through gap junctions (65).

Production of cGMP in the Granulosa Cells by the NPR2 Guanylyl Cyclase

cGMP is produced in the granulosa cells by the guanylyl cyclase natriuretic peptide receptor 2 (NPR2, also called guanylyl cyclase B) (66). NPR2 mRNA and protein are present in both the mural granulosa cells and cumulus cells, but not in the oocyte or theca cells (66–69) (Figure 3). The concentration of *Npr2* mRNA is nearly two times higher in the cumulus versus mural granulosa cells. Within mural cells, *Npr2* levels are higher near the antrum, consistent with a report demonstrating that signals from the oocyte increase *Npr2* (66). However, in terms of the total mass of NPR2 protein, ~97% of the NPR2 in the follicle resides in mural cells, correlating with the much larger volume of the mural compartment (70). By imaging follicles from mice expressing a global cGMP sensor, cGMP concentrations are seen to be uniform throughout mural granulosa cells, cumulus cells, and the oocyte; this results from cGMP diffusion through gap junctions (71) (Figures 4 and 5).

NPR2 is activated by the 22-amino acid C-type natriuretic peptide (CNP, also called NPPC) that is produced in mural granulosa cells and released into the extracellular space (Figure 3) (66). CNP diffuses in the follicular fluid and activates NPR2 in both cumulus and mural granulosa cells. Loss-of-function mutations in *Npr2* or *Nppc* result in spontaneous meiotic

resumption in preovulatory follicle-enclosed oocytes (66, 69, 72). *Npr2*-deficient follicles also contain less cGMP (72). As would be predicted, female *Npr2* mutant mice are infertile (72). Human females with loss-of-function mutations in *NPR2* are fertile when heterozygous, but the fertility of homozygous females has not been investigated (73).

Application of CNP (10–100 nM) to cumulus-oocyte complexes from mice (66, 74), pigs (75, 76), cows (77), and domestic cats (78) inhibits meiotic resumption. CNP does not inhibit meiotic resumption in isolated oocytes (66, 75, 76) because oocytes do not express NPR2 (66). A second peptide, porcine B-type natriuretic peptide (BNP), was also shown to activate NPR2 and inhibit meiotic resumption in porcine cumulus-oocyte complexes (76). Porcine BNP is unique in this regard because BNP of other species does not activate NPR2 (79). Meiotic inhibition by CNP (and porcine BNP) is consistent with early evidence that a small peptide (~2 kDa) in follicular fluid partially inhibits meiotic resumption in porcine cumulus-oocyte complexes (32).

The CNP concentration in follicular fluid isolated from human preovulatory follicles is ~100 nM (74), and the combined CNP and BNP concentration in porcine follicular fluid is ~130 nM (76). This concentration of CNP and BNP is similar to that required to maintain meiotic arrest in ~75% of porcine cumulus-oocyte complexes (76). However, when hamster cumulus-oocyte complexes are dislodged from the follicle wall, but remain within the follicular fluid, meiosis resumes (80). This suggests that sufficient cGMP levels are maintained in the cumulus-oocyte complex only if the complex is attached to mural cells. Likewise, when connexin 43 junctions are inhibited, meiosis resumes, illustrating the importance of gap junction–mediated signals from the mural cells (31). It is possible that local CNP concentrations are higher in the extracellular space between the multiple layers of mural granulosa cells, where CNP is produced, than in the follicular fluid surrounding the cumulus cells (see Figure 5). If so, the cGMP concentration in the oocyte could be maintained at a higher level if gap junctions between the mural cells and cumulus cells are intact.

Activity of the enzyme inosine monophosphate dehydrogenase (IMPDH) is also essential for maintaining meiotic arrest. Specific IMPDH inhibitors induce resumption of meiosis when injected into mice (81) or applied to CNP-arrested cumulus oocyte complexes (82). IMPDH catalyses the conversion of IMP to guanylyl substrates for NPR2 production of cGMP (82).

RELEASE OF MEIOTIC PROPHASE ARREST BY LUTEINIZING HORMONE

Signaling Across a Tissue

A long-standing mystery is how LH signals through its receptor in the outer granulosa cells and then remotely initiates resumption of meioisis in the oocyte that is located up to 10 cell layers away. Understanding of this long distance signaling in meiotic resumption might also shed light on hormonal signaling in other avascular tissues, such as the growth plate of developing bone (83). LH receptors are expressed almost exclusively in mural granulosa and in theca cells with little or no presence in cumulus cells or the oocyte (14, 84–90). In rat and mouse preovulatory follicles, LH receptors are enriched in the outer layers of mural granulosa cells (14, 84, 85, 87, 89, 91) (Figure 3). Although in most conditions LH receptors

are also expressed in theca cells, they are absent in the theca cells of preovulatory follicles from gonadotropin-primed immature rats (84), and yet these follicles are able to resume meiosis following LH stimulation. Furthermore, in granulosa-oocyte complexes derived by culturing preantral follicles from which both the basal lamina and most of the theca cells are removed, LH signaling can also stimulate meiotic resumption (92). Collectively, these observations indicate that LH receptors in the granulosa cells rather than theca cells mediate meiotic resumption.

In fish and amphibians, LH receptors are located in the somatic cells surrounding the oocyte. However, in these vertebrate species, LH conveys the meiosis–inducing signal by release of a steroid that acts on the oocyte (93, 94). This has raised the question of whether a steroid-mediated process also restarts meiosis in mammalian follicles; however, published studies indicate that the answer is no (95).

Other mechanisms accounting for this long-distance signaling included the possibilities that LH signaling reduces endogenous GPR3 agonists or elevates GPR3 antagonists. Either one of these changes would decrease G_s activity in the oocyte, resulting in less cAMP production. However, G_s activity in the oocyte is unchanged by LH signaling (96). Likewise, inhibition of the G_i G protein by pertussis toxin fails to prevent meiotic resumption in the mouse oocyte (97), unlike results reported for starfish, where an agonist from the follicle cells (1-methyladenine in starfish) causes meiotic resumption by activating G_i in the oocyte (98).

Following the recognition that cGMP could inhibit cAMP hydrolysis in the oocyte, it was proposed that lowering oocyte cGMP could mediate LH signaling of meiotic resumption and that this could occur either by closing gap junctions or by decreasing cGMP in granulosa cells (60). As discussed below, the development of methods for measuring and manipulating cyclic nucleotide levels in individual compartments of live follicles has allowed testing of this hypothesis. These studies established that one important component of the signaling that reinitiates meiosis is a rapid decrease in cGMP in the outer granulosa cells where LH receptors are located. As a consequence, cGMP diffuses out of the oocyte down its concentration gradient (Figure 5).

Signaling to the interior of the follicle is also accomplished by slower processes involving changes in the amounts of peptides that are released by the mural cells and that diffuse through the extracellular space to act on the cumulus cells. In particular, as discussed below, LH signaling decreases the production of the NPR2 agonist CNP (74) and increases the production of epidermal growth factor receptor (EGFR) agonists, which act to lower cGMP (99). Regulation of gap junction permeability is yet another component in this complex system.

LH signaling begins with the activation of a G-protein–coupled receptor that is coupled primarily to G_s, thus increasing cAMP production by adenylyl cyclase in the granulosa cells (100). This seems paradoxical, as an increase in cAMP in the granulosa cells leads to a decrease in cAMP in the oocyte. We return to this paradox later but first discuss the cyclic nucleotide changes in the oocyte that result from LH signaling. We then discuss the

intercellular signaling mechanisms in the follicle that accomplish these changes in the oocyte and finally consider how G-protein signaling in the outer layers of mural granulosa cells initiates these events.

The Luteinizing Hormone–Induced Decreases in cAMP and cGMP in Follicle-Enclosed Oocytes

The effects of LH on cAMP and cGMP in follicle-enclosed oocytes were initially measured by rapidly removing the oocytes from the follicles; immunoassays showed that LH causes a decrease in both cAMP (30, 56) and cGMP (101). Such measurements were complicated by the fall in cyclic nucleotides that results from oocyte isolation from the follicle, but nevertheless, LH-induced changes were detected. Later, when it became possible to microinject cyclic nucleotide FRET sensors into follicle-enclosed oocytes, avoiding the need to isolate the oocytes to make measurements, larger changes were seen (65). By 1 h after LH treatment, cAMP decreases from ~700 to ~100 nM, and cGMP decreases from ~900 nM to ~40 nM. Both cAMP and cGMP stay low in the oocyte for at least 5 h. Based on these measurements, the decrease in cGMP in the oocyte would increase PDE3A hydrolysis of cAMP by ~5 times, thus decreasing cAMP (63, 65).

The Luteinizing Hormone–Induced Decrease in cGMP in the Granulosa Cells

The LH-induced decrease in cGMP in the oocyte is accompanied by a decrease in cGMP in rat ovaries (102) and in isolated preovulatory follicles from mice, rats, and hamsters (63, 65, 101, 103), as measured by immunoassays. LH also causes cGMP to decrease in luteinized human granulosa cells (104). Although cGMP concentrations cannot be precisely determined from measurements of follicle cGMP content, due to a lack of precise information about cytosolic volume, estimated concentrations for mouse follicles are ~1–4 μM before LH application and ~100 nM at 1 h afterward (65, 71, 105). cGMP stays at a low level for at least 5 h (103–105). Considering the limitations of each method of measurement, these values from immunoassays of whole follicles are quite similar to the FRET measurements from follicle-enclosed oocytes, both before and after LH treatment.

Using mouse follicles that transgenically express a FRET sensor for cGMP and imaging with a confocal microscope, it was seen directly that prior to LH application, the concentration of cGMP is similar throughout the mural granulosa, cumulus cells, and oocyte (71). Time-lapse recordings of cGMP levels using follicles expressing the FRET sensor (Figure 4) showed that within 1 min of LH application, cGMP starts to decrease in the outer 25 μm of the follicle where LH receptors are located (71). Within this region, a plateau value is reached after ~10 min. In the cumulus cells, the cGMP decrease starts with a delay of ~5 min, and in the oocyte, it starts with a delay of ~7 min. By 20 min after LH application, the cGMP concentration is at a uniformly low level throughout the follicle. These results show that the LH-induced decrease in cGMP occurs first in the outer mural granulosa cells, then in the cumulus cells, and finally in the oocyte.

When gap junction permeability in the follicle is inhibited by carbenoxolone, cGMP stays high in both the mural granulosa cells and cumulus cells, where the NPR2 guanylyl cyclase is located. However in the oocyte, which lacks NPR2, cGMP decreases (71). In the presence

of carbenoxolone, measurements at 20 min after LH application show a decrease in cGMP in the mural cells where the LH receptors are located, but not in the cumulus cells. However, by 2 h, cGMP decreases in the cumulus cells as well. These results support the conclusion that the initial LH-induced cGMP decrease in the cumulus cells is due to cGMP diffusion outward into the mural cells by way of gap junctions (Figure 5), but that later, cGMP is maintained at a low level in the cumulus cells due to gap junction-independent signaling.

The Luteinizing Hormone–Induced Decrease in Synthesis of cGMP by Natriuretic Peptide Receptor 2

Early studies investigating the LH-induced cGMP decrease in follicles explored the possibilities that either cGMP synthesis or cGMP hydrolysis was changed (106, 107). Although variable results were obtained at that time, it is now established that the rapid LH-induced cGMP decrease in the follicle results from both a decrease in cGMP synthesis by NPR2 (103, 108) and an increase in cGMP hydrolysis by the phosphodiesterase PDE5 (109) (Figure 5). These two changes are complementary. Whether LH also increases cGMP efflux was not investigated. This section discusses the decrease in NPR2 activity and its regulation by dephosphorylation of NPR2. The regulation of PDE5 is discussed in a subsequent section.

LH treatment of either mouse or rat follicles reduces NPR2 activity by ~50% by 20–30 min (103, 108). This rapid decrease in activity, as measured in the whole follicle, is indicative of a decrease in activity in the mural granulosa cells, as these account for ~95% of the follicle volume. Two to three hours later, NPR2 activity also decreases in the cumulus cells (108), most likely mediated by paracrine signaling by way of the EGFR, which is discussed in a later section.

The rapid decrease in NPR2 activity occurs without any change in the amount of the NPR2 protein or CNP, although CNP decreases later (103, 108). Instead, the activity decrease is caused by the dephosphorylation of seven juxtamembrane serines and threonines of NPR2 (70, 103). Inhibitor studies indicate that the responsible phosphatase is in the phosphoprotein phosphatase (PPP) family, and together with expression analysis, they point to PP1, PP2A, or PP6 as likely candidates for mediating LH-induced NPR2 dephosphorylation (103).

Because of direct effects on the oocyte, phosphatase inhibitors are not suitable for testing whether NPR2 dephosphorylation is required for LH-induced meiotic resumption. Instead, this question was addressed using mice in which the seven juxtamembrane serines and threonines of NPR2 were replaced with the phosphomimetic amino acid glutamate (NPR2-7E) (70). In these follicles, LH treatment does not reduce NPR2 activity, and the LH-induced decrease in cGMP is attenuated (70). As a result, meiotic resumption in NPR2-7E follicles is delayed by ~5 h (70).

However, meiotic resumption does eventually occur (70). This may be related to a slow decrease in gap junction permeability between the oocyte and cumulus cells, first detected at 5–6 h after LH receptor activation (110, 111), which could isolate the oocyte from the source of inhibitory cGMP. One cause of the permeability decrease could be the secretion of an extracellular matrix by cumulus cells that begins at ~6 h after LH activation and results in an

apparent separation of these cells (111). Another likely contributor to the eventual resumption of meiosis is that the CNP content of the ovary decreases starting ~2 h after LH receptor activation (104, 108). This would decrease NPR2 activity and lower cGMP levels, even with the NPR2-7E mutation. By ~3 h, *Npr2* transcripts decrease as well (68, 69).

Although NPR2 dephosphorylation is required for the normal time course of meiotic resumption, NPR2-7E female mice are fertile (70). This indicates that despite the delay in meiotic resumption, there appears to be time for completion of the first meiotic division prior to fertilization. However, it is unknown whether the mutations cause subfertility.

How activation of the LH receptor leads to NPR2 dephosphorylation and inactivation is poorly understood. One question is whether these mechanisms involve the activation of a phosphatase or the inhibition of a kinase (or both). LH signaling, via PKA, phosphorylates and activates a regulatory subunit of the PP2A phosphatase in rat granulosa cells (112), indicating that PKA activation of PP2A could be a factor leading to NPR2 dephosphorylation. EGFR signaling, discussed below, also contributes to LH-induced NPR2 dephosphorylation, based on the finding that EGFR agonist-induced nuclear envelope breakdown is delayed in NPR2-7E mice (70).

The Luteinizing Hormone–Induced Increase in Hydrolysis of cGMP by Phosphodiesterase 5

Although the rapid LH-induced decrease in NPR2 guanylyl cyclase activity does not occur when NPR2 dephosphorylation is prevented, a partial decrease in cGMP, insufficient to trigger meiotic resumption, still occurs (70, 103). These findings suggest that LH signaling might also increase the activity of a cGMP phosphodiesterase.

Several cGMP-hydrolyzing phosphodiesterases are expressed in granulosa cells of mice and rats (109). Among these, PDE5 is of particular interest because its activity is increased by phosphorylation by PKA (113, 114), and LH signaling activates PKA (115). Indeed, PDE5 in rat follicles is phosphorylated within 10 min of LH treatment and remains phosphorylated for at least 4 h (109). As a result, LH treatment increases the cGMP-hydrolytic activity of PDE5 by ~70%. These events are dependent on PKA activity but independent of EGFR kinase activity (109), which as discussed below, increases in response to LH signaling. The phosphorylation and activation of PDE5 would act, along with the dephosphorylation and inactivation of NPR2, to lower follicle cGMP levels in response to LH (109) (Figure 5).

PDE1A, a phosphodiesterase that is activated by Ca^{2+}/calmodulin, is also expressed in rat and mouse granulosa cells (109), but whether PDE1A activity is increased by LH is unknown. If LH signaling increases cytosolic free Ca^{2+}, an increase in PDE1A activity would follow. Increases in cytosolic Ca^{2+} are detected when LH is applied to isolated porcine granulosa cells (116), and EGFR agonists, which increase in response to LH, cause Ca^{2+} to increase in mouse cumulus cells (117). However, it is unknown if LH signaling causes Ca^{2+} to increase in intact follicles.

cGMP phosphodiesterase activity is required to achieve the low cGMP levels that trigger meiotic resumption (63). However, there are some differences between species in the

particular phosphodiesterases that are required. PDE5 inhibitors delay LH-induced meiotic resumption in mouse follicles by several hours (63), indicating that PDE5 activity is responsible for most of the cGMP decrease. In contrast, in rat follicles, both PDE1 and PDE5 must be inhibited to maximally inhibit meiotic resumption. However, it is unknown whether the contribution of PDE1 stems from its steady-state activity in the granulosa cells or whether LH increases its activity by increasing free Ca^{2+}.

Contribution of the Decrease in C-Type Natriuretic Peptide to the Luteinizing Hormone–Induced cGMP Decrease and Meiotic Resumption

A slower response to LH that contributes to maintaining cGMP at low levels is a decrease in the NPR2 small peptide agonist CNP. This decrease was measured in mouse ovaries (74, 104, 108), rat follicles (103), and human and porcine follicular fluid (74, 76). In mouse ovaries, the decrease is not seen until 2 h after LH receptor stimulation, when CNP content has decreased to about half of its initial value (104, 108). Thus, the fall in CNP is too slow to account for the rapid decrease in cGMP in the follicle (71) but could be important for maintaining cGMP at a low level at later times.

CNP would diffuse through the extracellular space of the follicle, such that a fall in CNP production in the mural cells would result in a fall in CNP not only around the mural cells but also around the cumulus cells (Figure 5). At 36 h after LH receptor activation in porcine follicles, at which time most oocytes have undergone nuclear envelope breakdown, the concentration of CNP plus BNP has decreased to ~20 nM (76). When incubated with 20 nM CNP or BNP, ~50% of isolated cumulus-oocyte complexes resume meiosis, versus ~25% at the ~130 nM concentration before LH exposure (76). This finding indicates that the decrease in CNP (and BNP) is sufficient to contribute to the decrease in cGMP in response to LH.

The LH-induced decrease in CNP peptide levels is associated with decreased *Nppc* transcripts encoding the precursor protein of CNP (74, 104); signaling pathways leading to the decrease in *Nppc* are not well understood, although EGFR activation appears sufficient but not essential (104). The LH-induced decrease in CNP is also independent of the natriuretic peptide clearance receptor NPR3 (118).

Contribution of Epidermal Growth Factor Receptor Activation to the Luteinizing Hormone–Induced cGMP Decrease and Meiotic Resumption

An important component of the signaling pathway by which LH decreases cGMP in the follicle is the activation of the EGFR by its endogenous agonists epiregulin and amphiregulin. In ovaries of mice (99), rats (119, 120), pigs (121), and humans (122), epiregulin and/or amphiregulin increase in response to LH. These peptides are produced in the mural granulosa cells (99) and act on EGFRs that are located in mural granulosa and cumulus cells but not in the oocyte (99) (Figure 3). Binding to the EGFR causes dimerization and an increase in intrinsic protein-tyrosine kinase activity, resulting in autophosphorylation (123). EGFR phosphorylation in mouse follicles increases within 15 min after LH application and continues to increase over the next 3 h (99, 104, 124).

Activating EGFR in follicles decreases cGMP (63, 71, 104, 105), which is seen first in the mural cells and then in the cumulus cells and oocyte (71). In different studies, EGFR

activation by epiregulin and/or amphiregulin completely or partially recapitulates the LH-induced cGMP decrease. Moreover, inhibiting EGFR kinase activity attenuates the LH-induced cGMP decrease, although the extent of attenuation reported is variable (63, 71, 104, 105, 125).

Activating EGFR in cumulus-oocyte complexes also decreases cGMP in both cumulus cells and oocytes from mice (117) and pigs (121). This supports the concept that endogenous EGFR agonists are released in response to LH receptor activation in the mural granulosa cells and then diffuse through the extracellular space to decrease cGMP in the cumulus cells (Figure 5). Because gap junctions directly connect cumulus cells to the oocyte, the EGFR-induced cGMP decrease in cumulus cells also decreases cGMP in the oocyte. EGFR agonists also act to decrease cGMP in the mural granulosa cells (71), contributing to the decrease in oocyte cGMP through the series of gap junctions connecting these compartments.

Application of EGFR agonists epiregulin and/or amphiregulin to mouse or rat follicles (99, 105, 120, 126), or to pig cumulus-oocyte complexes incubated in CNP (121), causes meiotic resumption. Correspondingly, the use of pharmacological inhibitors of EGFR signaling that target receptor kinase activity or block protein processing of epiregulin and/or amphiregulin delays LH-induced meiotic resumption by at least 4–6 h in mouse follicles (99, 104, 105, 125); some of these follicles eventually resume meiosis (105). Similar findings are also reported for rat follicles (120, 127) and human and pig cumulus-oocyte complexes (121, 122). Thus, in all mammalian species tested, EGFR signaling is a critical mediator of LH signaling.

Mutant mice with minimal EGFR kinase activity also show delayed meiotic resumption in response to LH receptor stimulation (125, 128). However, most oocytes in these mutant mice do eventually resume meiosis, as indicated by the finding that their fertility is only slighly impaired (125, 128) or normal (129, 130). One such mutant line has highly reduced fertility, but nonovarian effects may contribute to this phenotype (128). The lack of a major effect on fertility in *Egfr* mutant mice is presumably due to functionally redundant parallel signaling pathways that provide a fail-safe system for ensuring meiotic resumption.

How LH signaling stimulates EGFR phosphorylation and activation is incompletely understood. Matrix metalloproteases, which release epiregulin and amphiregulin from their precursor proteins, are required, indicating that the activation of the EGFR occurs by an increase in these endogenous agonists (120, 124). Within 1 h, LH signaling increases transcript levels that encode epiregulin and amphiregulin precursor proteins (99). However, because EGFR phosphorylation increases within 15 min of LH receptor activation (104), it is likely that LH signaling also stimulates the cleavage of epiregulin and amphiregulin from preexisting precursor proteins (Figure 5).

Elevating cAMP with forskolin mimics the LH-induced stimulation of EGFR phosphorylation, and the PKA inhibitor H89 partially inhibits EGFR phosphorylation, supporting a role for PKA in part of the signaling pathway (124). PKA stimulates MAP kinase activity (131), which is required for part of the increase in EGFR agonist mRNAs

(132). It is unknown how LH signaling might stimulate matrix metalloproteases or other pathways that activate the EGFR.

How EGFR activity decreases cGMP is also incompletely understood. As discussed above, it contributes to the pathway leading to NPR2 dephosphorylation (70) but not PDE5 phosphorylation (109) (Figure 5). Because EGFR activation can elevate Ca^{2+} in cumulus cells (117), it may also stimulate hydrolysis of cGMP by PDE1.

Luteinizing Hormone Regulation of Gap Junctions in the Follicle

As mentioned above, on a timescale of hours, LH signaling reduces gap junction permeability between the oocyte and cumulus cells by mechanisms that are not certain but may be related to physical separation of the cumulus cells, associated with the secretion of an extracellular matrix between them (110, 111). There are also incompletely understood changes in gap junction structure in the follicle, as detected by freeze fracture electron microscopy (133, 134).

On a more rapid time scale, LH signaling partially inhibits the permeability of the connexin 43 junctions between the granulosa cells; this decrease is due to phosphorylation of connexin 43 by MAP kinase (6, 135). Permeability decreases by 30 min after LH application, reaches a minimum at 1 h, and returns to the original level by 5 h (6, 71). The permeability decrease was detected by use of fluorescent tracers; whether permeability to cGMP and/or cAMP also decreases was not examined. Gap junction permeability between the cumulus cells and oocyte, which is mediated by connexin 37, does not decrease during this period (6).

The function of the decrease in gap junction permeability between the granulosa cells is unknown. It is not necessary for causing meiotic resumption in response to LH, which still occurs with a normal time course when the permeability decrease is prevented by inhibiting connexin 43 phosphorylation by inhibiting MAP kinase (6). Likewise, the gap junction permeability decrease is not sufficient to cause meiotic resumption under conditions in which NPR2 dephosphorylation is prevented (70). If connexin 43 phosphorylation caused a selective decrease in cAMP versus cGMP permeability, this could be beneficial, as discussed below.

Initiation of Luteinizing Hormone Signaling by G Protein Activation, cAMP Elevation, and Protein Kinase A Activation in the Mural Granulosa Cells

In this section, we consider the earliest events in LH signaling and how these initiate the pathways described above. LH signaling begins with the activation of its G-protein–coupled receptor in the mural granulosa cells. The duration of LH signaling is extended by internalization of the receptors after LH binding, such that signaling continues even in the absence of extracellular LH (127, 136). The LH receptor can activate multiple G proteins (137, 138), but most of its effects on meiotic resumption appear to be due to the activation of G_s.

G_s activation in the mural granulosa cells increases cAMP production by adenylyl cyclase (139, 140). This results in an elevation of cAMP in the follicle, as measured by

immunoassays (56, 128, 139, 141, 142), and specifically in the mural granulosa cells, as detected with a FRET sensor (136). cAMP also increases in the cumulus cells, based on immunoassays of isolated cumulus-oocyte complexes, but this was not seen until ~1.5 h after LH application (56). FRET sensor measurements suggest that the cAMP increase in the cumulus cells might occur earlier, but this is uncertain because of the large region of measurement that extended beyond the 2–3 layers of cumulus cells (136).

The LH-induced increase in cAMP in the mural granulosa cells contrasts with the decrease in cAMP in the oocyte. How this transpires is poorly understood, as it might be expected that cAMP would diffuse from the granulosa cells into the oocyte. Both the connexin 43 junctions between the granulosa cells and the connexin 37 junctions at the oocyte surface are permeable to cGMP (71), but some types of gap junctions are more permeable to cGMP than cAMP (143). Connexin 43 junctions are highly permeable to cAMP (144, 145), but cAMP permeability was not investigated for connexin 37. If connexin 37 had low cAMP permeability, this could explain why the granulosa cell cAMP increase is not transmitted to the oocyte. If phosphorylation of connexin 43 selectively lowered cAMP permeability (but not cGMP permeability) between the granulosa cells, that could also contribute to preventing cAMP diffusion into the oocyte. In addition, cAMP phosphodiesterase activity may restrict the elevation of cAMP in the granulosa cells from spreading into the oocyte.

Transiently elevating cAMP by treating follicles with forskolin to activate adenylyl cyclase (127, 141), or elevating cAMP in the granulosa cells with an inhibitor of the cAMP phosphodiesterase PDE4 (61, 136), causes meiotic resumption. The forskolin-induced cAMP increase activates PKA (112, 115), which phosphorylates and activates PDE5 to the same extent as LH stimulation (109) (Figure 5). Forskolin treatment also inactivates NPR2 (JR Egbert, JW Robinson, LR Potter, LA Jaffe, unpublished data). These experiments indicate that elevation of cAMP in the granulosa cells is the primary stimulus that initiates the events that restart meiosis.

Another indirect consequence of PKA activation is the phosphorylation of the EGFR, as indicated by evidence that EGFR phosphorylation is stimulated by forskolin, and LH-induced EGFR phosphorylation is reduced by inhibiting PKA (124) (Figure 5). It is unknown how PKA causes EGFR phosphorylation; it may stimulate the matrix metalloproteases that cleave epiregulin and amphiregulin from their precursors (Figure 5), and/or it may stimulate transcription of the precursors. As discussed above, activation of the EGFR is a component of the pathway by which LH signaling dephosphorylates and inactivates NPR2, which is essential for the normal progression of meiosis. Thus PKA activation is a regulator of multiple pathways by which LH signaling triggers meiotic resumption.

SUMMARY AND FUTURE ISSUES

Mammalian oocytes become arrested in meiotic prophase before birth, and remain arrested as they develop within a follicle that is ultimately comprised of approximately 10 layers of granulosa cells. Meiotic resumption is triggered by the mid-cycle surge in luteinizing hormone that acts on the outermost granulosa cells, but how this signal is transduced to the

oocyte has only recently become clear. Diffusion of cyclic GMP from the granulosa cells to the oocyte through gap junctions maintains meiotic arrest in the later stages of development. Through many redundant and thus fail-safe processes over different time scales, luteinizing hormone decreases cGMP levels in the granulosa cells and oocyte, which coordinates the timing of meiotic resumption with ovulation and fertilization to ensure reproductive success. Understanding these long-distance signaling mechanisms that control oocyte meiosis might also shed light on signaling in other physiological systems.

Although much has been discovered, many questions remain. Some of these outstanding questions are summarized below.

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1. How does LH signaling cause NPR2 dephosphorylation? What kinase phosphorylates NPR2, and is it inhibited by LH signaling? Does LH signaling activate a phosphatase that dephosphorylates NPR2?
2. Does LH signaling increase Ca^{2+} and activate PDE1 in the granulosa cells?
3. How does LH signaling decrease the concentration of CNP?
4. Does LH signaling activate a matrix metalloprotease that releases amphiregulin and epiregulin from their precursors? Does LH signaling also activate the EGFR through an intracellular pathway?
5. How does EGFR signaling lead to NPR2 dephosphorylation? Does EGFR activation decrease cGMP by other mechanisms as well?
6. Are connexin 37 gap junctions more permeable to cGMP than cAMP? Does connexin 43 phosphorylation reduce cGMP and/or cAMP permeability?
7. Are there other pathways that participate in the fail-safe mechanisms that promote the timely resumption of meiosis in mammals, such as the positive stimuli acting in frogs and starfish?
8. Do other avascular tissues, such as the growth plate of developing bone, use similar mechanisms for intercellular communication?

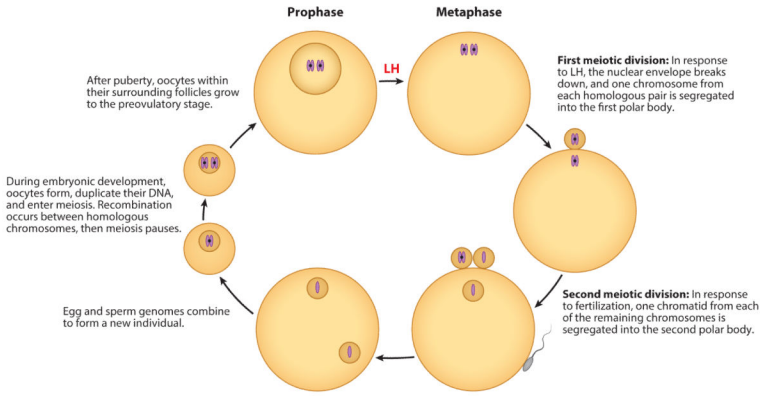

Figure 1.

Life cycle of a mammalian oocyte. The diagram places the prophase-to-metaphase transition in response to luteinizing hormone (LH) into the context of the overall meiotic cell cycle of the oocyte. One set of homologous chromosomes, each composed of two chromatids (*purple*), is shown.

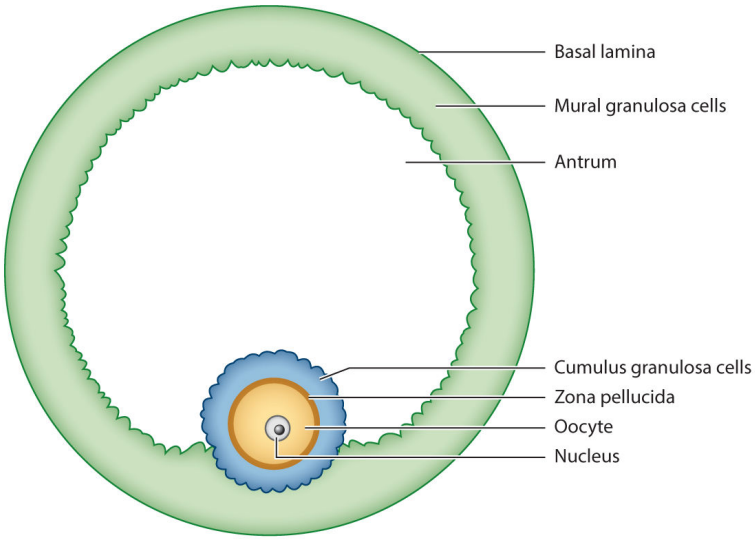

Figure 2.

Tissue layers of a mammalian preovulatory follicle. The oocyte with its prophase-arrested nucleus is surrounded by 2–3 layers of cumulus granulosa cells, which are attached in one region to the 5–10 layers of mural granulosa cells. Elsewhere, a fluid-filled antrum separates the two types of granulosa cells.

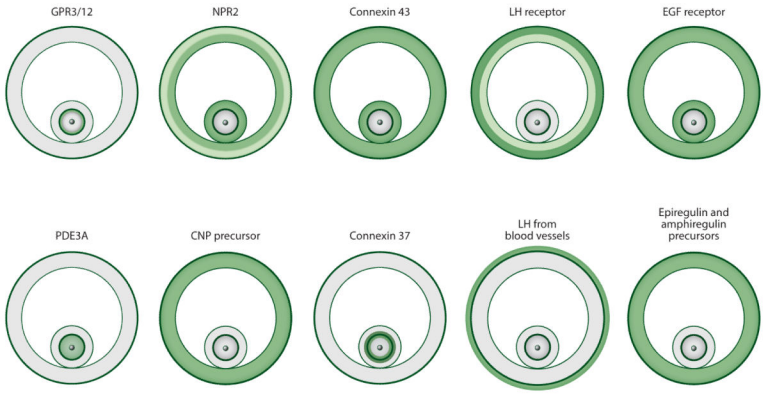

Figure 3.

Localization of some of the signaling proteins that regulate meiotic arrest and resumption in preovulatory follicles. Protein distribution is either determined directly, by ligand binding, immunofluorescence, or Western blotting; or it is inferred from mRNA distribution, by in situ hybridization, or from RT-qPCR. Green indicates the presence of the protein, and lighter green indicates a lesser amount of the protein. White indicates that the protein (or mRNA) was either not detected or detected at a level ≤10% of that elsewhere. Figure is based on data from the following references: CNP precursor (66); connexin 43 (9), connexin 37 (9), EGF receptor (99; LA Jaffe and JR Egbert, unpublished data); epiregulin and amphiregulin precursors (99); GPR3/12 (40, 46); LH from blood vessels (14); LH receptor (84); NPR2 (66, 70); and PDE3A (61, which used a previous nomenclature, PDE3B). Abbreviations: CNP, C-type natriuretic peptide; EGF, epidermal growth factor; LH, luteinizing hormone; NPR2, natriuretic peptide receptor 2; PDE3A, phosphodiesterase 3A.

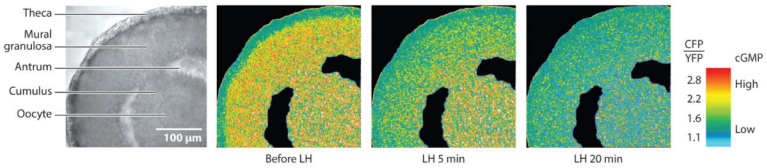

Figure 4.

LH-induced cGMP decreases in a mouse ovarian follicle from a transgenic mouse expressing the cGMP FRET sensor cGi500. A larger CFP/YFP emission ratio indicates higher cGMP. Ratios are color-coded: Yellow indicates high cGMP, and turquoise indicates low cGMP. Before LH perfusion, cGMP is high throughout the follicle but low in the theca cells. At 5 min after LH perfusion, cGMP has decreased in the mural cells but not in the cumulus cells or oocyte. At 20 min, cGMP has decreased throughout the follicle. Figure modified from Reference 71. Abbreviations: CFP, cyan fluorescent protein; cGMP, cyclic GMP; FRET, Förster resonance energy transfer; LH, Luteinizing hormone; YFP, yellow fluorescent protein.

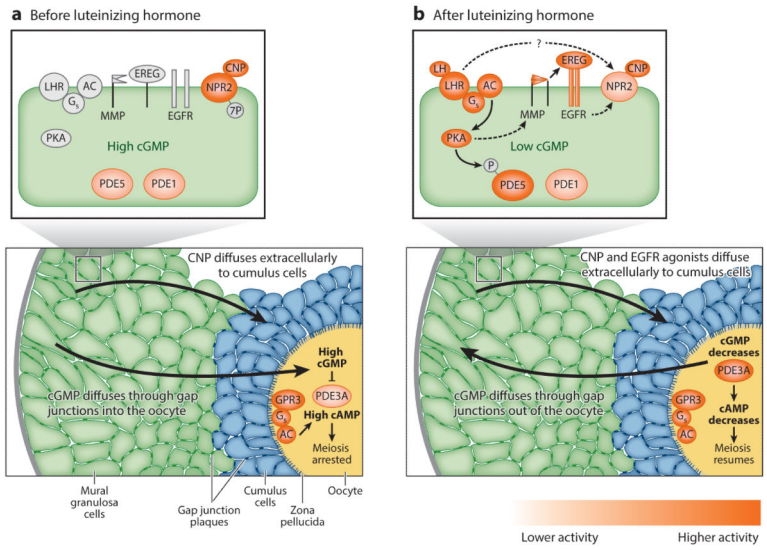

Figure 5.

Working model of signaling pathways that regulate meiotic arrest and resumption in preovulatory follicles. Panel *a* shows a follicle and an expanded view of a mural granulosa cell before LH exposure. Panel *b* depicts events occurring in response to LH. Some of the slower events are not shown, such as the decrease in CNP and the increase in mRNA encoding epiregulin and amphiregulin. Higher levels of enzymatic activity are depicted by darker shades of orange. Abbreviations: AC, adenylyl cyclase; CNP, C-type natriuretic peptide; EGFR, epidermal growth factor receptor; EREG, epiregulin (and amphiregulin); GPR3, G-protein receptor 3; G_S, G_s G protein; LH, luteinizing hormone; LHR, luteinizing hormone receptor; MMP, matrix metalloprotease; NPR2, natriuretic peptide receptor 2; PDE1, 3A, 5, phosphodiesterases; PKA, protein kinase A. Figure modified from Reference 71, with additional details about the phosphodiesterases from Reference 109. The cellular structures were drawn based on electron microscopic images of a mouse follicle from Valentina Baena and Mark Terasaki.