

Regulation of oocyte meiotic maturation by somatic cells

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Abstract In preovulatory follicles, each oocyte is surrounded by numerous layers of cumulus cells, forming the cumulus cell–oocyte complex. An LH surge induces meiotic resumption of the oocyte to progress to metaphase II. Because the expression of LH receptors is not detected in the oocyte and is minimal (negligible) in cumulus cells as compared with granulosa cells, secondary factors from granulosa cells are required to induce the ovulation process. One of the key factors secreted from granulosa cells is an EGF-like factor that activates the EGFR–ERK1/2 pathway in cumulus cells. The activated ERK1/2 pathway is not only involved in gene expression but also essential for the close of gap-junctional communication among cumulus cells and between cumulus cells and the oocyte. Closing gap-junctional communication decreases the amount of cGMP and/or cAMP to transfer into the oocyte, which requires activation of phosphodiesterase type III (PDE3) in the oocyte. PDE3 brakes down cAMP to decrease PKA activity in the oocyte. This decrease in PKA activity induces activation of CDK1 to resume meiosis from the germinal vesicle stage. Thus, the functions of cumulus cells that are regulated by granulosa cell-secreted factors are essential for oocyte meiotic resumption and maturation with developmental competence.

Keywords Cumulus cells · EGF-like factor · Oocyte maturation · Granulosa cells

Introduction

After a surge of LH, oocytes resume meiosis, complete germinal vesicle breakdown (GVBD), and progress to the metaphase II (MII) stage. Because LH receptors (LHCGRs) are dominantly expressed in granulosa cells, but not in cumulus cells or oocytes [1], the LH surge dramatically changes the expression pattern of genes in granulosa cells [2]. Factors secreted from granulosa cells act on cumulus cells to induce meiotic resumption and maturation of the oocyte with developmental competence [3]. Thus, cumulus cells play a critical role in oocyte maturation; however, there is less information about the mechanisms by which cumulus cells differentiate and how these changes in cumulus cells affect oocyte maturation at the molecular level. The mechanisms underlying how cumulus cells regulate oocyte meiotic resumption are the focus of this review.

Dynamic changes in kinase activities in the oocyte

Meiosis of oocytes to the metaphase II stage is dependent on the activation of maturation-promoting factor (MPF), which is composed of p34^{cdc2} kinase (CDK1) and cyclin B [4–6]. The activation of MPF disappears the nuclear membrane and condenses chromosomes to induce meiotic resumption in amphibian oocytes [7, 8] and mammalian oocytes [9–11]. Association with cyclin B is required for activation of CDK1, as well as for dephosphorylation of Thr 14 and Tyr 15 residues [12–14]. The phosphorylation status of these residues is regulated by the activity of two key enzymes: a Wee1 kinase and Cdc25 phosphatase [13–17].

Wee1B, which is a member of the Wee1 kinase family, is selectively expressed in oocytes [18]. Phosphorylation of

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Wee1B by PKA enhances its kinase enzyme activity, which phosphorylates CDK1 in oocytes [18]. Thus, the cAMP-PKA pathway phosphorylates Wee1B to suppress CDK1 activity in order to keep meiotic arrest at the GV stage in mouse oocytes. It has been shown that three Cdc25 families, Cdc25A, Cdc25B and Cdc25C, are expressed in mouse oocytes, whereas only Cdc25B is essential for the induction of meiosis in mice [19]. Interestingly, Cdc25B is also phosphorylated by PKA, and its phosphorylation decreases the enzyme activity of Cdc25B, suggesting that the elevation of cAMP levels activates Wee1B, but decreases Cdc25B activity to markedly increase the phosphorylated inactive form of CDK1 in oocytes arrested at the GV stage (Fig. 1a) [20, 21]. The phosphorylated (active) form of Wee1B is localized in germinal vesicles, whereas the phosphorylated Cdc25B (inactive form) is localized in the cytoplasm. The decrease in cAMP level is a turning point to change the localization of two key enzymes: dephosphorylated Wee1B (inactive form) moves to the cytoplasm, and dephosphorylated Cdc25B (active form) enters the nucleus from the cytoplasm (Fig. 1b).

In pig oocytes, the activation of CDK1 is induced concomitantly with the reduction in cAMP level [22, 23]. To examine the role of decreasing cAMP level in oocytes before GVBD, the cAMP level and CDK1 activity were analyzed in the oocyte after 28 h of culture in the presence of various concentrations of a phosphodiesterase inhibitor, IBMX. Addition of IBMX to the maturation medium produced a significant increase in the cAMP content of oocytes and a significant decline in the proportion of oocytes exhibiting GVBD and CDK1 activity; both results were observed to be dose-dependent. Thus, a fall in cAMP

level is required for activation of CDK1 and then induces meiotic resumption in porcine oocytes, as well as in mouse oocytes. Additionally, Wee1B and Cdc25C are expressed in porcine oocytes and play important roles in the regulation of meiotic resumption [24]. When denuded oocytes were cultured, activation of CDK1 was detected at a much earlier time point as compared with oocytes of COCs [25]. Therefore, cumulus cells regulate oocyte meiotic resumption via a cAMP-dependent mechanism.

Regulation of the cAMP level in oocytes

A recent mutant mice model showed that regulation of the cAMP level in oocytes is dependent on a G protein-coupling receptor family member, GPR3/GPR12 [26, 27]. In *Gpr3* mutant mice, oocyte meiotic resumption was spontaneously exhibited before ovulation stimuli in small antral follicles [26]. Although the endogenous ligands for GPR3 remain unclear, it has been reported that sphingosine 1 phosphate (S1P) is potentially acted as a ligand for GPR3 [27]. When S1P was added to the medium, the cAMP level was increased in oocytes and spontaneous meiotic resumption was suppressed in mice, suggesting that the mouse oocyte produces cAMP to prevent meiotic resumption before ovulation stimuli [27].

Although the oocyte produces cAMP, regulation of the cAMP level in oocytes is dependent on cumulus cells. When oocytes were removed from cumulus cell layers and the denuded oocytes were cultured, the level of cAMP was quickly decreased [28–30]. Recently, Dr. Eppig and collaborators clearly showed that granulosa cells expressed and secreted natriuretic peptide precursor type C (NPPC) and acted on cumulus cells to produce cGMP [31]. In human, follicular fluid contains NPPC in preovulatory follicles, and its level is dramatically decreased after hCG stimulation [32]. cGMP is transferred to oocytes via gap junctional communication. One of the functions of cGMP is to decrease PDE3 activity, which increases the level of cAMP in oocytes [33]. The mechanisms are shown in Fig. 2.

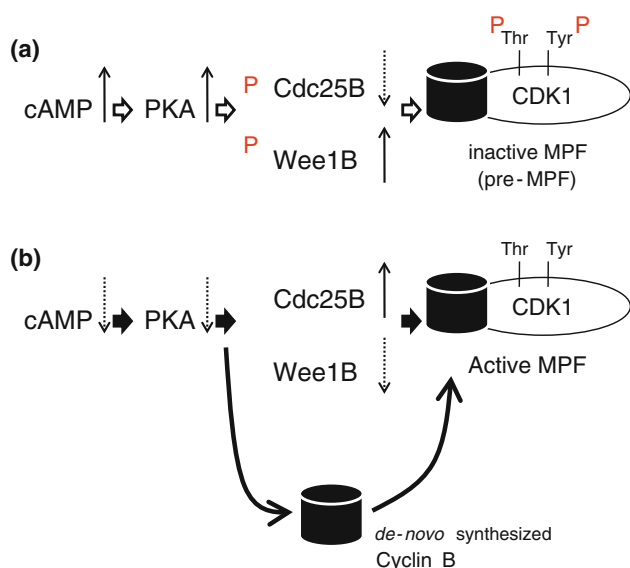


Fig. 1 The regulation of MPF activation by cAMP-PKA dependent manner

Close of gap junctional communication

How is the level of cAMP decreased in oocytes during meiotic maturation? In oocytes, cAMP is changed to 5'AMP before oocytes begin exhibiting meiotic resumption, as described above. In amphibian oocytes, progesterone or insulin-like growth factor 1 (IGF1) activates the PI 3-kinase-PKB pathway that phosphorylates phosphodiesterase type III (PDE3) to increase enzyme activity [34–36]. Activated PDE3 changes cAMP to 5'AMP (Fig. 2).

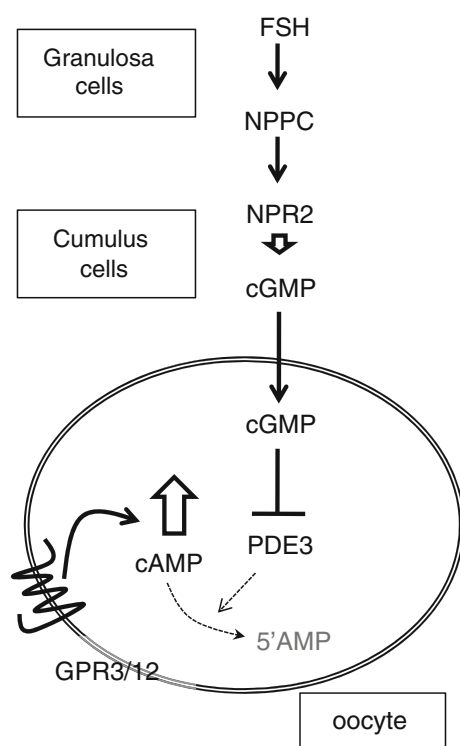


Fig. 2 Model describing the expression and roles of natriuretic peptide precursor type C (*NPPC*) and its receptor, *NPR2* in the maintenance of oocyte meiotic arrest at the GV stage in mice, *NPPC* is secreted from FSH-stimulated granulosa cells, and then acts on cumulus cells to stimulate cGMP production. The cGMP is transferred to oocyte via gap junctional communication, which suppresses *PDE3* activity that converts cAMP to 5'AMP. The cAMP is produced in oocyte via *GPR3/12* dependent manner. The pathways increases cAMP level in oocyte during follicular development stage

The PI 3-kinase–PKB pathway is also activated in mouse oocytes before meiotic resumption [37]. This signaling pathway potentially upregulates *PDE3* enzyme activity in oocytes; however, PI 3-kinase inhibitors do not block meiotic resumption of mouse oocytes [38]. These results suggest that, although the PI 3-kinase–PKB pathway is involved in the cAMP degradation pathway, this regulation is not the primary switch for oocyte maturation but may work as an amplifying loop to decrease cAMP levels during oocyte meiotic resumption in mammals. Other initial factors are required for the initial reduction of cAMP level and the induction of oocyte meiotic resumption.

The other possibility is that the amount of cAMP and/or cGMP transferred from cumulus cells to oocytes is decreased. It has been reported that disruption of gap junctions within cumulus cells induces the meiotic resumption of mouse, rat and porcine oocytes due to blockage of the conduction of meiosis inhibitory signals from the outer layers of cumulus cells to oocytes [39–43]. Gap junctions, the specialized regions in opposite mem-

branes between neighboring cells, are channels that pass low-molecular-weight substances and ions in order to enhance cellular interactions [44]. These channels are formed by hexameric structures consisting of connexin molecules (connexon) in numerous tissues [44]. Porcine ovarian follicles have been reported to express five members of the connexin gene family: connexin-26, connexin-30.3, connexin-32, connexin-43, and connexin-60 [45, 46]. The connexin-43 (Cx-43) protein is dominantly expressed in cumulus cells of porcine COCs [47–50]. Cx-43 is also expressed in bovine cumulus cells of COCs, and Cx-37 selectively consists of gap junctional communications between cumulus cells and oocytes [51].

ERK1/2 is required for the close of gap junctional communication in cumulus cells

Although the ERK1/2 pathway in oocytes is not essential for the resumption of meiosis, when COCs or intact follicles are cultured in vitro a MEK inhibitor significantly suppresses this process, presumably by blocking the activity or ERK1/2 in cumulus cells and granulosa cells [24, 52]. Moreover, EGF-like factors can induce oocytes to resume meiosis and reach metaphase II stage, indicating that the activated EGF receptor pathway in cumulus cells is required for the resumption of meiosis [53, 54]. Collectively, these results indicate that the EGF-like factor/EGFR/ERK1/2 pathway in cumulus cells is critical for oocyte maturation. This hypothesis has been supported recently by observation of a total block of oocyte maturation in mutant mice lacking ERK1/2 in somatic cumulus/granulosa cells but not in the oocyte [55].

One of the targets of ERK1/2 in cumulus cells is Cx-43. Cx-43 has numerous phosphorylated sites, and phosphorylation of these residues plays a key role in regulatory mechanisms governing the assembly of connexons into gap junctions in the plasma membrane, and gating the gap junction that is formed [56–58]. In particular, phosphorylation of serine on Cx-43 is stimulated by ERK1/2 in rat liver cells [57, 59]. In fact, the phosphorylation of Cx-43 is induced in an ERK1/2-dependent manner in mice and rat COCs in culture [60], which results in a reduction of Cx-43 and then the close of gap junctional communication among the cumulus cells [61]. In porcine COCs, at least three Cx-43-positive bands were detected by immunoblotting, and the upper bands disappeared after treatment with phosphatase, indicating that Cx-43 is phosphorylated in cumulus cells of porcine COCs [47]. Time-dependent changes were examined when porcine COCs were cultured with FSH up to 28 h. The staining intensities of the fast (43 kDa) and moderate (45 kDa) migrating bands were

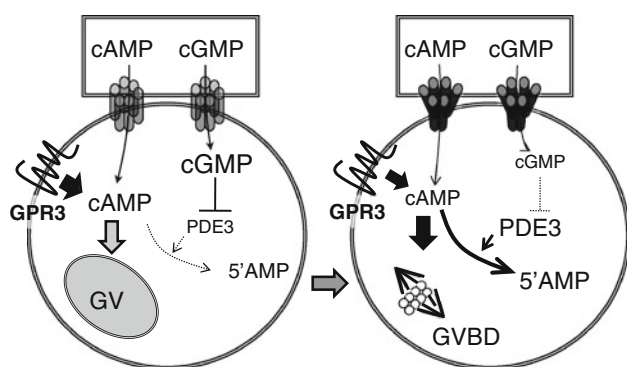


Fig. 3 A schematic diagram with regard to the induction of meiotic resumption of porcine COCs. The close of gap junctional communication reduces the amount of cAMP and cGMP transferred from cumulus cells to oocyte, which results in the induction of meiotic resumption of oocyte

significantly increased at the 8-h culture point as compared with those of cumulus cells separated from COCs immediately after collection [47]. However, after culture for 16 h and up to 28 h, significant reductions in the intensity of the 43-kDa of Cx-43 band were noted. In contrast, the intensity of the phosphorylated forms of 45- and 47-kDa Cx-43 were increased at 16 h and up to 28 h. Thus, within the first 4 h of culture, a large amount of Cx-43 was synthesized in all layers of the cumulus cells, while after 16 h of culture Cx-43 expression disappeared in cumulus cells, with increasing levels of phosphorylated Cx-43.

In conclusion, the transfer from cumulus cells to oocytes is shut down after ovulation stimuli via ERK1/2-induced phosphorylation of Cx-43, because gap junctional communication is closed by this phosphorylation. The reduction in cGMP induces the activation of PDE3 to degenerate cAMP produced in a GPR3-dependent manner and/or transferred from cumulus cells, and the pathways are essential for exhibiting meiotic resumption (Fig. 3).

How to activate the ERK1/2 pathway in cumulus cells

In mice, the EGFR-RAS pathway is a key signaling pathway to induce the phosphorylation of ERK1/2 in cumulus cells [62, 63]. The EGF receptor (EGFR, ERBB1) is a member of the EGF receptor superfamily that is expressed in cumulus cells but not in oocytes and, based on a specific receptor tyrosine kinase inhibitor, is known to affect oocyte maturation in LH-stimulated preovulatory follicle cultures [53, 64, 65]. Specifically, the EGF-like factors amphiregulin (*Areg*), betacellulin (*Btc*) and epiregulin (*Ereg*) are transiently expressed after LH stimulation in granulosa cells and act by binding to ERBB1 expressed on cumulus cells [53, 66, 67]. We also showed the expression of

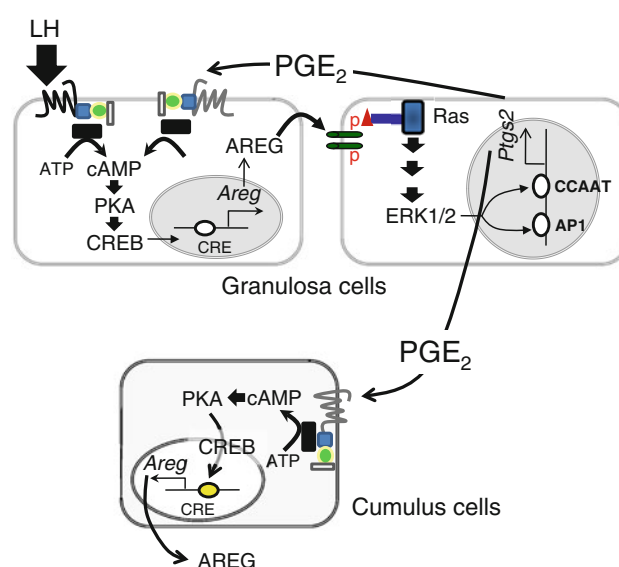


Fig. 4 Schematic showing potential paracrine and autocrine pathways by which expression of amphiregulin (*Areg*) is regulated in granulosa cells and cumulus cells of ovulating follicles. The proposed sequence is as follows: (1) LH binds to its cognate receptor localized to granulosa cells stimulating cAMP production to activate PKA–CREB pathway. (2) As a consequence, *Areg* mRNAs are induced rapidly providing ligand that (3) bind EGF-receptors on granulosa cells (autocrine) as well as cumulus cells (paracrine) leading to activation of ERK1/2 and induced expression of *Ptgs2* in both cell types. (4) Increased production of prostaglandins (PGs; PGE) then provides ligands that bind EP2 on granulosa cells and cumulus cells (paracrine and autocrine) that (like the FSH and LH receptors) activate cAMP–PKA–CREB pathway. (5) By the PGE/EP2 pathway, *Areg* mRNA can be induced in both cell types

EGF-like factors in cumulus cells during the ovulation process [67]. Because cumulus cells have low or undetectable levels of LH receptor [1], other stimulatory factors are required to induce the expression of EGF-like factors in cumulus cells. In the mouse *Areg* gene promoter region, a putative cAMP-responsive element (CRE) site is observed [68, 69]. Mutation of this region decreases the promoter activity in a luciferase promoter assay using primary culture of mouse granulosa cells, and the CRE sequence binds to phosphorylated CRE-binding protein (CREB) at 2 h after LH stimulation [69]. The expression of *Areg* is directly regulated by the cAMP–PKA–CREB cascade in granulosa cells and cumulus cells during the ovulation process. It has been well known that cumulus cells express the G protein-coupled receptor subtypes EP2 (PTGER2) or EP4 (PTGER4), which, when activated, stimulate adenylate cyclase to produce cAMP [70]. EP2 and EP4 are receptors for prostaglandin E2 (PGE2), which is converted from arachidonic acid by the rate-limiting enzyme PTGS2 [71–73]. Using an in vivo approach, we documented that *Areg* and *Ereg* expression levels were markedly reduced in COCs and granulosa cells of *Ptgs2*-null mice [67]. Thus,

the initial induction of EGF-like factor expression is directly induced by LH via the cAMP–PKA–CREB pathway, and expression is maintained in a PGE₂ production-dependent manner (Fig. 4).

Neuregulin is a novel inducer of oocyte developmental competence

In our microarray analysis of rat COCs, we found the expression of neuregulin (NRG1), another EGF-like factor that acted on ErbB2/3 hetero-dimers expressed in cumulus cells. Activation of the hetero-dimer was also observed in cumulus cells and granulosa cells during the oocyte maturation (ovulation) process [74]. The murine *Nrg1* gene has three promoter and transcriptional start sites [75], and the type I and type III sites are expressed in granulosa cells during ovulation [74]. Expression of type III *Nrg1* was dramatically increased in granulosa cells by hCG, whereas that of type I was not changed. The type III *Nrg1* promoter region has three putative C/EBP binding sites and a CRE site. Based on the transfection of specific promoter-reporter constructs in granulosa cells, the most distal C/EBP-binding site appears to play a critical role in the increase in promoter activity after stimulation. Because we have shown recently that the ERK1/2–C/EBPb pathway is essential for inducing cell fate decisions in granulosa cells and cumulus cells of preovulatory follicles [50], it is clear that *Nrg1* expression is also dependent on this pathway.

To clarify the roles of NRG1 in oocyte maturation, we carried out in vitro maturation of COCs with or without NRG1. When mouse COCs were cultured with NRG1, spontaneous meiotic resumption of oocytes was suppressed. The induction of meiosis initiated by AREG was also delayed by NRG1 in the presence of 4 mM hypoxanthine. The matured oocyte cultured with both NRG1 and AREG had significantly higher developmental competence as compared with oocytes cultured with AREG alone. Thus, the AREG–EGFR–ERK1/2 pathway accelerates meiotic resumption, but it controls timing via activation of the NRG1–ErbB2/3–PKB pathway that works as a brake, and both pathways are required to regulate the timing of meiotic progression that has an impact on oocyte developmental competence (Fig. 5).

Conclusion

In preovulatory follicles, oocytes are surrounded by numerous layers of cumulus cells, which form the cumulus cell–oocyte complex (COC). Although after stimulation of

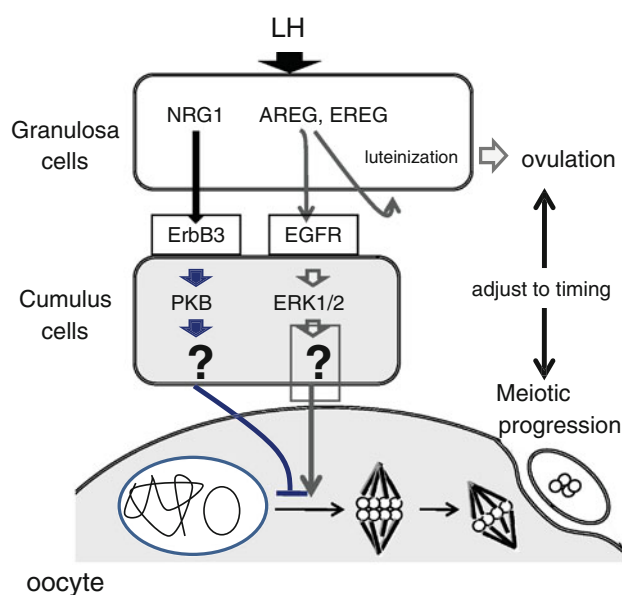


Fig. 5 Schematic showing the roles of AREG that acts on EGFR and NRG1 that binds to ErbB3 to increase ERK1/2 or PKB pathway, respectively, in the regulation of meiotic progression in mice ovulating follicles

ovulation by an LH surge the oocyte resumes meiosis by progressing to metaphase II, expression of LH receptors is not detected in the oocyte and is minimal (negligible) in cumulus cells as compared with granulosa cells. However, cumulus cells express members of the EGF receptor family (ErbB family) that respond to specific ligands, and members of the EGF-like factor family are secreted by granulosa cells during the ovulation process. Via these intermediary steps, cumulus cells mediate LH signaling from granulosa cells to induce oocyte meiotic resumption. One of the key signaling pathways in cumulus cells is the EGFR–ERK1/2 pathway that regulates gap-junctional communication among cumulus cells and between cumulus cells and the oocyte. Closing gap-junctional communication by ERK1/2 decreases the level of cGMP and/or cAMP to transfer into the oocyte, which requires activation of PDE3 in the oocyte. PDE3 brakes down cAMP to decrease PKA activity in the oocyte. Because the PKA pathway activates Wee1B and downregulates both Cdc25C activity and cyclin B synthesis to decrease CDK1 activity, the decrease in PKA activity caused by PDE3 induces activation of CDK1 to resume meiosis from the GV stage. Thus, control of the cAMP level in oocytes by cumulus cells is essential for oocyte meiotic resumption.

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