

Opinions and Hypotheses

Postovulatory cell death: why eggs die via apoptosis in biological species with external fertilization

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Abstract. Spawned unfertilized eggs have been found to die by apoptosis in several species with external fertilization. However, there is no necessity for the externally laid eggs to degrade via this process, as apoptosis evolved as a mechanism to reduce the damaging effects of individual cell death on the whole organism. The recent observation of egg degradation in the genital tracts of some oviparous species provides a clue as to the physiological relevance of egg apoptosis in these animals. We hypothesize that egg apoptosis accompanies ovulation in species with external fertilization as a normal process to eliminate mature eggs retained in the genital tract after ovulation. Furthermore, apoptosis universally develops in ovulated eggs after spontaneous activation in the absence of fertilization. This paper provides an overview of egg apoptosis in several oviparous biological species, including frog, fish, sea urchin, and starfish.

Key words: Apoptosis, Egg ovulation, Meiotic exit, Spontaneous activation

(J. Reprod. Dev. 64: 1–6, 2018)

In oviparous species with external fertilization, ovulated eggs are deposited outside of the body, where they are fertilized. Fertilization causes transient elevation of the calcium concentration in eggs. The calcium signal represents the main early event of fertilization-induced egg activation observed in all species studied (reviewed in [1, 2]). On the other hand, unfertilized mature eggs undergo a time-dependent loss of quality, progressively deteriorate, and eventually die. Remarkably, eggs from different species have been found to die by apoptosis if they are not fertilized. It is conceivable that in viviparous species such as mammals with internal fertilization, apoptosis removes ovulated unfertilized eggs without a pronounced inflammatory response, thereby supporting optimal body function. Indeed, fragmentation of ovulated murine oocytes [3] and calcium-triggered degradation of rat eggs [4] are well known examples of classical caspase-mediated apoptotic cell

death. However, the occurrence of apoptosis in the eggs of oviparous species with external fertilization raises a question of its rationale. For externally deposited eggs, one might speculate that waiting for fertilization over an indefinitely long period would be a more efficient reproductive strategy than dying via apoptosis soon after ovulation.

In this work, we propose that egg apoptosis accompanies ovulation in species with external fertilization as a normal process to eliminate mature eggs retained in the genital tract after ovulation. Furthermore, apoptosis occurs in ovulated eggs after spontaneous activation in the absence of fertilization. Here, we give a brief overview of egg apoptosis as described recently in several oviparous species, such as frog, fish, sea urchin and starfish, and discuss further experiments that can support the proposed concept.

Common traits

Oocytes of most species grow in the ovaries while arrested at the diplotene stage of the first meiotic prophase. Fully-grown immature diplotene-arrested oocytes are not competent for fertilization. These oocytes are apoptosis resistant and can reside in the ovaries for many months (Fig. 1). At that time, the oocytes display low activity of the key meiotic regulators [5], maturation-promoting factor (MPF, a complex of cyclin B and Cdk1 kinase) and cytostatic factor (CSF, a multicomponent signaling system comprising the meiotic protein kinase Mos and the MAPK pathway). Importantly, Mos protein is present only during meiosis and disappears after fertilization [6, 7]. Hormonal stimuli release diplotene arrest and induce oocyte maturation when oocytes are still in the ovaries. During meiotic maturation, oocytes progress through the meiotic cell cycle and arrest again before fertilization (Fig. 1). Notably, the timing of the second meiotic arrest in the meiotic cycle varies, depending on the species [8]. In many species, such as mammals, frogs, and fishes, mature eggs arrest before fertilization at the metaphase of the second meiotic division. High activities of MPF and CSF are responsible for maintaining the metaphase II arrest. As a rule, meiotic

Received: July 12, 2017

Accepted: October 2, 2017

Published online in J-STAGE: October 27, 2017

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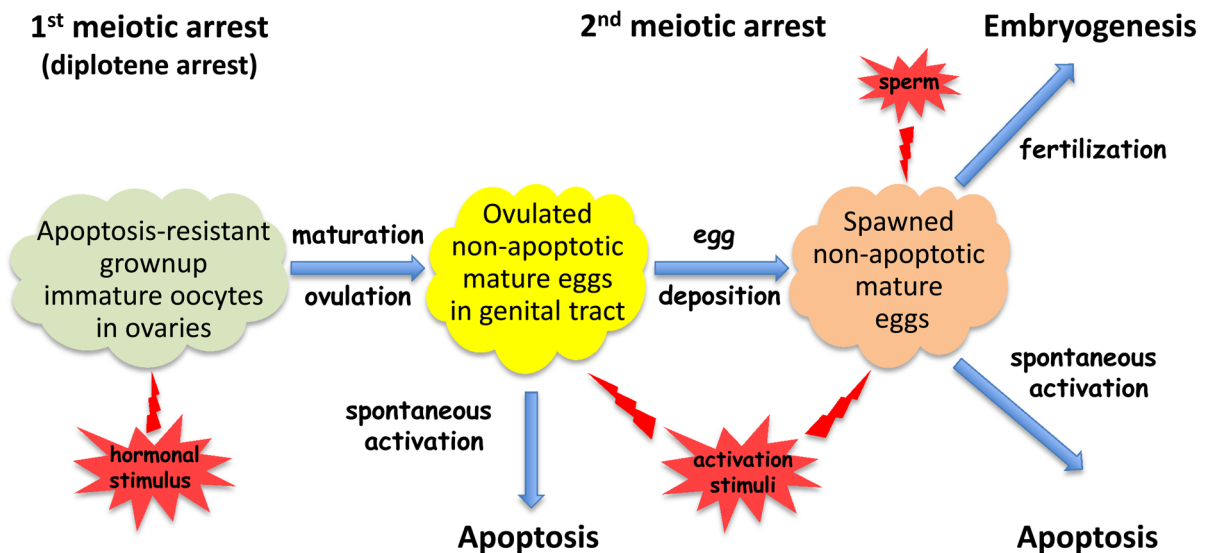


Fig. 1. Progression of oocytes and eggs in oviparous species with external fertilization.

maturation is accompanied or immediately followed by oocyte release from the ovaries, a process called ovulation. In the species with external fertilization, ovulated matured oocytes, also called eggs, are deposited outside of the female body, where they await fertilization. If the eggs are not fertilized within several hours to days (depending on species) of ovulation, they lose the capacity to be fertilized, deteriorate, and die. Although the spontaneous activation of eggs has been implicated in the loss of their fertilization capacity (Fig. 2), the mechanism of this process remains unclear. Eggs from several different species with external fertilization have been found to die by apoptosis as discussed below.

The case of frog

Fully-grown oocytes of the African clawed frog *Xenopus laevis* are naturally arrested in the first meiotic prophase. At ovulation, the steroid hormone progesterone, secreted from surrounding follicle cells, releases diplotene arrest and induces meiotic oocyte maturation. The maturing eggs advance from the ovaries into the open coelomic body cavity, pass through the oviduct, and accumulate in the uterus before being released out into the water where fertilization occurs. Before fertilization, the deposited eggs are arrested at metaphase II. During the reproductive season, egg deposition and fertilization may occur within a few hours. Unfertilized spawned

eggs die by a well-defined apoptotic process within 48 to 72 h after ovulation [9, 10]. The hallmark features of classical apoptosis, such as cytochrome c release, caspase activation, apoptotic nuclear morphology, increase of the ADP/ATP ratio, ATP depletion, etc., have been observed in apoptotic frog eggs. It has been demonstrated that exit from metaphase II arrest precedes apoptosis [9]. Apoptotic events were also observed in the cell-free extracts of *Xenopus* eggs [11]. Notably, only interphase extracts, but not extracts arrested in metaphase, are susceptible to apoptosis [12]. It was further found that a number of mature metaphase II-arrested eggs are retained in the genital tract of *Xenopus* frogs several days following hormone-induced ovulation [10, 13]. Falling temperatures were reported to cause retention of mature eggs in the uterus for several days [14]. Importantly, these retained eggs also displayed apoptotic features. All of the apoptotic events observed in unfertilized spawned eggs were also observed in the eggs retained in the genital tract, suggesting that the same apoptotic program unfolds in water-deposited and body-retained eggs [13]. Thus, apoptosis of post-meiotic eggs, both deposited and retained, accompanies ovulation in frogs. The observation of egg apoptosis in the frog genital tract points to its physiological relevance. Although it has little significance in the case of water-deposited eggs, it may be very important for elimination of the mature, overripe eggs retained in the

frog body after ovulation.

The case of fish

Most teleost fishes are oviparous species with external fertilization. During growth and vitellogenesis, when oocytes accumulate the nutritional reserves necessary for embryo development, fish oocytes reside in the ovaries arrested at the diplotene stage (Fig. 1). During the breeding season, environmental cues induce an acute increase in plasma luteinizing hormone levels. This hormone initiates production of maturation-inducing steroids (MIS), species-specific derivatives of progesterone, such as 17 α , 20 β -dihydroxy-4pregnen-3-one and 17 α , 20 β , 21-trihydroxy-4pregnen-3-one, by the follicle cells surrounding the oocytes. The process is accompanied by acquisition of maturational competence due to increase in MIS receptors on the oocyte cell membrane [15]. Engagement of these receptors triggers MPF activation, germinal vesicle breakdown (GVBD), and meiotic transition. Similar to frogs and mammals, fish oocytes arrest before fertilization in metaphase II after the completion of meiosis. In general, meiotic maturation is rapidly followed by ovulation, the release of mature oocytes (i.e., eggs) from their follicles into the ovarian cavity or into the abdominal cavity, depending on the species. Of note, several studies have suggested that eggs released during ovulation might not

have full developmental competence. For instance, the eggs of salmonids sampled 4–5 days after ovulation were reported to have significantly higher developmental capacity than freshly ovulated eggs. Thus, salmonids can hold the eggs in the abdominal cavity for several days after ovulation [15, 16]. Ovulated egg retained in the ovarian cavity for a prolonged period of time eventually become overripe, lose fertilization capacity, and degenerate [17]. At present, it is unclear whether apoptosis plays a role in this degenerative process. In addition, non-ovulated mature eggs and follicles break down in the ovaries by a degenerative process known as follicle atresia. Several studies indicated that apoptosis is involved in ovarian follicle atresia and postovulatory regression in teleosts [18–21]. It was reported that follicle cells display high phagocytic activity with digestive vacuoles at the advanced stage of follicular atresia [20]. Evidence was also presented showing that apoptosis occurs in ovulated fish eggs. It was found that the females of the sea bream *Sparus aurata* ovulate both floating and non-floating eggs, however only the floating eggs can be successfully fertilized [22]. The non-floating eggs present morphological and biochemical features of apoptosis, such as cell shrinkage, swollen mitochondria, decreased intracellular ATP content, high activity of PARP, and DNA fragmentation [22, 23]. It was further shown that the apoptotic pathway in these eggs involves the FAS/FAS-L signaling system [22].

The case of sea urchin

Sea urchin eggs complete meiosis and arrest in the haploid state at G1 phase of the first mitotic cell cycle before they are spawned and fertilized externally [8]. Unfertilized sea urchin eggs can stay arrested at this stage for weeks before being fertilized. The factors that stimulate gametogenesis and trigger sea urchin egg maturation, ovulation, and spawning are thought to be seasonal variations of seawater temperature and photoperiod. Under laboratory conditions, spawning of fertilization-competent eggs can be induced by KCl injection into females caught during the breeding season. Mature ovulated sea urchin eggs are arrested before S-phase of the first mitotic cell cycle with low activity of MPF due to the lack of a cyclin subunit and the inhibitory tyrosine phosphorylation

of Cdk1 [8, 24]. High activity of MAPK is essential for maintaining cell cycle arrest in the unfertilized eggs [25, 26], and abortive initiation of the mitotic cell cycle in the absence of fertilization was shown to cause cell death. Progressive loss of MAPK activity in aging unfertilized eggs was suggested to trigger apoptosis [27, 28]. The existence of the necessary apoptotic machinery in these cells was experimentally demonstrated using staurosporine, a nonspecific protein kinase inhibitor that is widely employed to induce apoptosis in various cell types [29]. Apoptotic progression in sea urchin eggs is characterized by a distinctive phenotype of blebbing and fragmentation, chromatin degradation, caspase activation, etc. [29]. A recent study demonstrated that unfertilized sea urchin eggs can execute apoptosis using different mechanisms involving changes in MAPK activity, intracellular calcium, pH, and acidity of vesicular organelles [28]. Importantly, residual reproductive cells are found in the female gonads after spawning; however, it is unknown whether apoptosis also occurs in these eggs. It was noted that nutritive phagocytes, the cells in sea urchin gonads that store the nutrients necessary for oocyte development, have the ability to absorb unused oocytes retained in the gonads after spawning [30].

The case of starfish

Apoptosis of unfertilized spawned eggs has been studied in great detail in starfish. Meiotic maturation of starfish oocytes is induced in the gonads in response to an unknown environmental signal by 1-methyladenine released by the follicle cells surrounding the oocytes. The hormone initiates MPF activation and re-entry into the meiotic cell cycle. The same hormone causes separation of the follicle cells from the oocytes, leading to ovulation and egg spawning. Uniquely, starfish eggs do not stop at the second meiotic arrest before fertilization; however, they do arrest after the completion of the meiotic cell cycle before apoptosis. Starfish eggs can be fertilized at any time after GVBD, which happens within 30 min of hormonal stimulation, with the optimum period being between GVBD and the end of meiosis I [31]. The MAPK pathway is activated in starfish eggs after GVBD by newly synthesized Mos, and fertilization causes Mos

degradation followed by inactivation of the MAPK pathway [32, 33]. Unfertilized starfish eggs complete meiosis and arrest in post-meiotic interphase. It was reported that unfertilized post-meiotic starfish eggs die rapidly and synchronously within 24 h of hormonal induction, while immature oocytes can live more than one week in seawater [31, 33]. Caspase activation, DNA degradation, membrane blebbing, and egg fragmentation occur during the cell death, indicating that mature starfish eggs die by apoptosis. It was shown that fertilization blocks apoptosis through a calcium-dependent mechanism via inactivation of the MAPK pathway [31, 33]. MAPK activity is maintained high in the mature starfish eggs by the newly synthesized Mos even after meiotic divisions. MAPK was found to be spontaneously inactivated just before caspase activation and blebbing [33]. This event is reminiscent of MAPK inactivation that occurs after fertilization and leads to mitotic divisions and embryogenesis. However, spontaneous inactivation of the MAPK pathway in unfertilized starfish eggs is followed by apoptosis. It was further demonstrated that, among the members of the MAPK family, ERK must stay activated in the mature starfish eggs for a period of about eight hours, in order for the eggs to develop the competence to apoptosis [34]. Following ERK inactivation at the onset of apoptosis, p38MAPK and JNK are activated and remain active during apoptosis. This sequential activation of MAPK family kinases seems to be necessary for apoptotic execution [34, 35]. It was suggested, after observing mature eggs and egg fragments resembling apoptotic bodies in the starfish ovaries at the end of the breeding season, that apoptosis may also occur in the ovary when oocytes reinitiate meiosis without spawning [33]. However, this hypothesis has not been proved experimentally. Interestingly, some starfishes are viviparous species that were suggested to have arisen from a free-spawning ancestor. The evolution of viviparity puts forward an additional demand for elimination of mature unfertilized eggs in the starfish gonads.

Spontaneous activation as a probable trigger of egg apoptosis

Although egg quality generally decreases gradually after ovulation (fish eggs may still

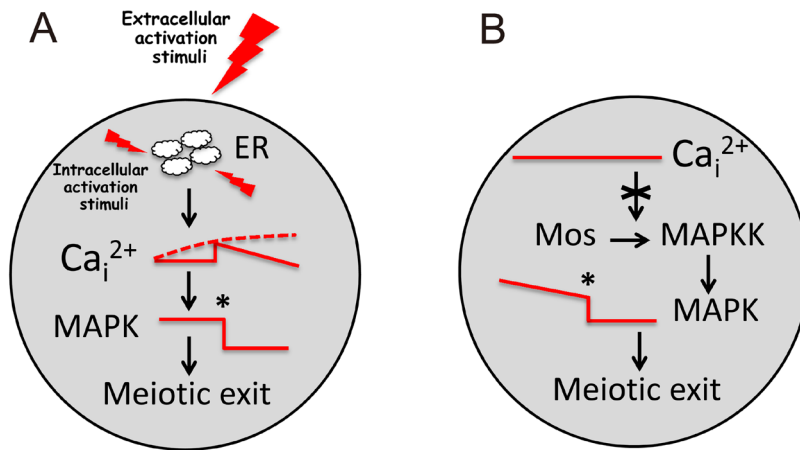


Fig. 2. Calcium-dependent and -independent spontaneous egg activation. Mature ovulated eggs at the second meiotic arrest can be spontaneously activated by calcium-dependent (A) or -independent (B) mechanisms. (A) Egg activation by extracellular or intracellular stimuli evokes calcium release from intracellular stores of the endoplasmic reticulum (ER), leading to MAPK inactivation and exit from meiotic arrest. A gradual increase in intracellular calcium, which occurs due to disruption of calcium homeostasis in aging eggs (dotted line), may eventually elicit the same effect. (B) Alternatively, a gradual decrease in MAPK pathway and/or CSF activity, below the threshold level necessary to maintain meiotic arrest, can cause meiotic exit in the absence of a calcium signal. The asterisk denotes the time of exit from the second meiotic arrest.

require some time after ovulation to develop full developmental competence, as discussed above in “The case of fish”), there exists a point of no return, after which successful fertilization becomes entirely impossible. Spontaneous activation and exit from the second meiotic arrest render eggs completely unfertilizable. Indeed, spontaneous egg activation has been implicated as a major factor responsible for the loss of fertilization capacity of ovulated eggs in many species. For instance, spontaneous activation of zebrafish eggs was shown to give rise to a fertilization membrane that prevents normal fertilization [36]; thus, in general, fish eggs entering aquatic environments remain fertilizable for a relatively short time [37]. Moreover, it seems that, at least in some oviparous species, egg apoptosis is preceded by spontaneous activation and exit from the second meiotic arrest. In starfish eggs, MAPK becomes completely inactive before caspase activation and blebbing [33]. Furthermore, the fact that apoptotic features cannot be observed in the metaphase II-arrested frog eggs, which maintain high activity of MPF and CSF, strongly suggests that egg activation may be a prerequisite for apoptosis [9].

Presently, little is known about the molecular mechanisms of spontaneous eggs

activation in the studied oviparous species. It was shown that this process might involve calcium-dependent mechanisms in mammalian eggs [38–40]. Indeed, calcium signal represents a main early event of fertilization-induced egg activation, triggering a plethora of calcium-dependent intracellular events [2]. It was demonstrated that spontaneous activation of fish eggs is associated with an increase of intracellular calcium [36], triggering cortical granule exocytosis and formation of the fertilization envelope. In addition, parthenogenetic activation of frog eggs by electric shock or H_2O_2 is accompanied by an elevation of intracellular calcium [41, 42]. The initial increase in cytosolic calcium in activated eggs relies mainly on its release from intracellular calcium stores, such as the endoplasmic reticulum [2]; (Fig. 2A). However, spontaneous activation of aging eggs may not necessarily involve a calcium-dependent mechanism (Fig. 2B). For instance, it has been suggested that the apoptotic pathway is triggered by gradual inactivation of MAPK in aging unfertilized sea urchin eggs [19]. In this case, an age-dependent decrease in the content and activity of the meiotic protein kinase Mos or MAPK, below a threshold level, may be involved in the shutdown of the MAPK pathway (Figs. 2B, 3). It was

demonstrated that aged mammalian eggs have decreased levels of MAPK and MPF [43, 44]. At present, it is unclear whether spontaneous activation of frog eggs is initiated by a calcium signal. Although the exit from metaphase II occurs in spontaneously activated unfertilized frog eggs [9], it is not known whether other calcium-dependent events take place in these eggs.

The MAPK pathway appears to play the major role in maintaining CSF activity during the second meiotic arrest in eggs of the analyzed oviparous species. Spontaneous egg activation leads to MAPK inactivation and disappearance of CSF (Fig. 2). These events are reminiscent of the transition that occurs after fertilization and leads to exit from meiotic arrest, mitotic entry, and embryogenesis. However, spontaneous egg activation in the absence of sperm DNA induces apoptosis. At present, the differences between sperm-induced and spontaneous egg activation are not clearly understood. An insufficient increase of cytosolic calcium was linked to postovulatory, aging-induced spontaneous activation of rat eggs [45], providing a probable clue to the different fates of fertilized and spontaneously activated eggs. Next, we discuss the molecular mechanisms underlying meiotic exit in frog eggs.

Meiotic exit in activated eggs

The mechanisms of the meiotic exit have been more fully investigated in parthenogenetically activated and fertilized eggs of the African clawed frog *Xenopus laevis*. They are briefly summarized in Fig. 3. Before activation, mature *Xenopus* eggs display high activities of MPF and CSF, maintaining meiotic metaphase II arrest. The calcium/calmodulin-dependent protein kinase, CaMKII, and the calcium/calmodulin-dependent serine/threonine protein phosphatase calcineurin, PP2B, were found to mediate calcium-induced exit from meiotic metaphase II arrest and cell cycle resumption in these eggs. The calcium signal independently activates CaMKII and calcineurin (Fig. 3). CaMKII directly phosphorylates Emi2 to promote the formation of a phosphorylation-dependent degradation signal [46, 47], which is recognized by the SCF1 (SKP2-cullin1-F-box protein)-E3 ubiquitin ligase complex targeting Emi2 for 26S proteasome-mediated degradation. Degradation of Emi2 leads to activation of

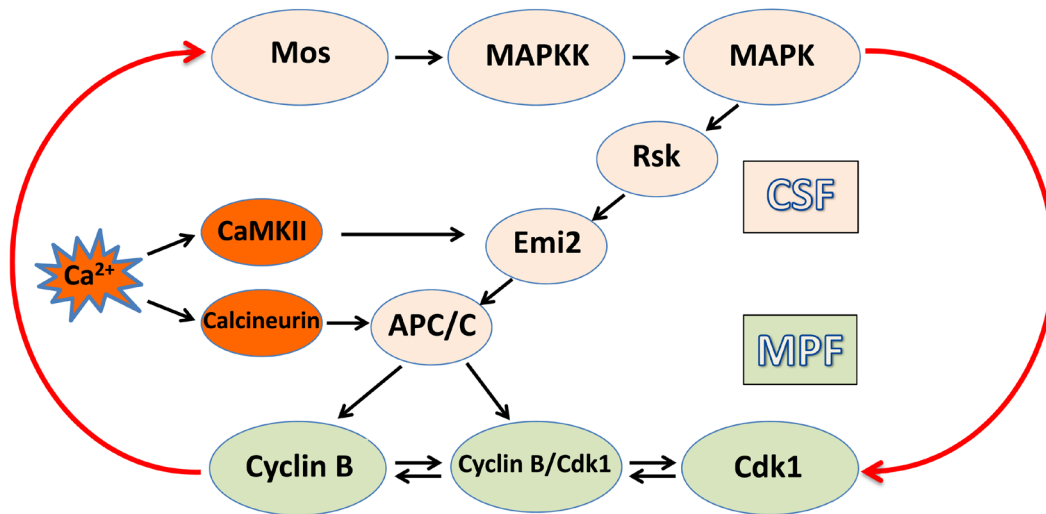


Fig. 3. Calcium-dependent meiotic exit in activated *Xenopus* eggs. Molecular components of CSF and MPF are colored pink and green, respectively. The factors involved in egg activation are shown in orange. CSF and MPF are embedded in a positive feedback loop (red arrows). Calcium-mediated egg activation signals stimulate APC/C, resulting in consecutive degradation of cyclin B, inactivation of MPF, destabilization of Mos protein, loss of CSF activity, and exit from meiotic metaphase II arrest.

the APC/C ubiquitin ligase, ubiquitination of cyclin B and its degradation by the 26S proteasome [reviewed in 48–50]. Similarly, calcineurin dephosphorylates the APC/C activator Cdc20 and the core APC/C component Apc3, supporting APC/C activation [51]. Thus, proteasome-dependent degradation of cyclin B by the two independent mechanisms inhibits Cdk1 activity in fertilized *Xenopus* eggs and inactivates MPF. Cdk1 inactivation triggers the disruption of the positive feedback loop between MPF and CSF [52] (Fig. 3). Mos protein is dephosphorylated at Ser3, a site of direct phosphorylation by Cdk1, and degraded by the N-terminal Pro2-dependent ubiquitin pathway [53]. Mos degradation leads to shutdown of the MAPK cascade, CSF inactivation, and meiotic exit. The factors involved in regulation of meiotic metaphase II arrest, including polo-like protein kinases, Myt1 and Wee1 kinases, Cdc25C phosphatase, Greatwall kinase, etc., have been described in greater detail elsewhere [2]. Of note, the involvement of CaMKII and calcineurin in egg activation is not evolutionary conserved. Mature eggs of many invertebrate species do not encode the CaMKII target protein Erp1 in their genomes and presumably use the “phosphatase-only” mechanism [54]. In mammalian eggs, however, exit from meiotic metaphase arrest and cell cycle resumption was found to rely solely on CaMKII activity [55, 56]. Investigations of egg activation

and meiotic exit in other animal species are necessary to delineate these findings from an evolutionary perspective.

Conclusion

Thus, it is evident that the functional apoptotic machinery is universally present in the eggs of different species with external fertilization, and apoptosis of mature unfertilized eggs is quite common in these species. Apoptosis is known to be the major mechanism involved in follicular atresia, a process that eliminates oocyte- and egg-containing follicles in the ovaries. Recently, unfertilized spawned eggs of several oviparous species with external fertilization were shown to die by apoptosis. It also appears that ovulated mature eggs retained in the genital tract of these animals after ovulation are degraded by apoptosis. However, the presence of apoptotic eggs in the genital tract has currently been confirmed only in frog and fish species, demanding validation of this phenomenon in other oviparous species with external fertilization. Additionally, the involvement of spontaneous activation and calcium signaling in the initiation of egg apoptosis requires further investigation. It is still unclear why and how the spontaneous activation and meiotic exit occur in mature unfertilized eggs. The differences between sperm-induced and spontaneous egg activation are not understood. Moreover, the natural

extracellular and intracellular inducers of spontaneous activation are largely unknown. At present, apoptosis of ovulated unfertilized eggs has been observed and studied only in a limited group of oviparous species, including frog, fish, sea urchin, and starfish. It is still impossible to generalize these findings to the mechanisms of meiotic exit and apoptosis in the unfertilized eggs of other species in the light of conservation and evolution. Investigation of egg apoptosis in many more animal species is necessary to reveal evolutionary trends.

Acknowledgements

This work was supported by the Grants-in-Aid for Scientific Research from the Ministry of Education, Culture, Sports, Science, and Technology of Japan (25440023 to AAT and 15K07083 to K-IS) and by the Collaboration Research Grant 281027 from the Kobe University, Japan (to AAT).

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