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Fertilization 2: Polyspermic fertilization

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Abstract

During fertilization in animals, a haploid egg nucleus fuses with a haploid sperm nucleus to restore the diploid genome. In most animals including mammals, echinoderms, and teleostei, the penetration of only one sperm into an egg is ensured at fertilization because the entry of two or more sperm is prevented by polyspermy block systems in these eggs. On the other hand, several animals such as birds, reptiles, and most urodele amphibians exhibit physiological polyspermy, in which the entry of several sperm into one egg is permitted. However, in these polyspermic eggs, only one sperm nucleus is involved in zygotic formation with a female nucleus, thereby avoiding syngamy with multiple sperm nuclei. In the chicken, 20-60 sperm are generally found within the egg cytoplasm at fertilization and this number is markedly higher than that of other polyspermic species; however, avian-specific events such as the degeneration and mitosis of supernumerary sperm nuclei during early embryo development allow a polyspermic egg to develop normally. This chapter describes current knowledge on polyspermy-related events in avian eggs during fertilization, and is characterized by a comparison to the fertilization modes of other vertebrates. The close relationship between sperm numbers and egg sizes, and the movement of supernumerary sperm nuclei towards the periphery of the egg cytoplasm and their degeneration are summarized. The molecular mechanisms by which polyspermy initiates egg activation to start embryo development are also discussed.

Key words: Diploid genome, Polyspermy block, Physiological polyspermy, Supernumerary sperm, Fertilization modes, Sperm numbers, Egg sizes, Egg activation

7.1

Polyspermy and embryo development

Fertilization is an indispensable event in the restoration of diploid genomes and the initiation of several reactions that lead to embryonic development in all animals. Most animals exhibit monospermy, in which a single sperm has the ability to activate the reaction important for early development (Swann 1996; Stricker 1999; Runft et al. 2002; Yanagimachi 2005). Conversely, the simultaneous fertilization of an egg by two or more sperm is a lethal condition leading to aneuploidy and developmental arrest. Polyspermy in humans mostly results in spontaneous abortion. Although the birth of triploid or tetraploid children has been reported, severe malformations and multiple abnormalities are associated with polyploidy births (Uchida and Freeman 1985; Sherard et al. 1986; Shiono et al. 1988; Roberts et al. 1996; Dean et al. 1997; Sun 2003). On the other hand, several sperm have been observed in porcine eggs at a high incidence under physiological conditions or *in vitro* fertilization (Sun and Nagai 2003); however, porcine egg cytoplasm has the ability to remove successive sperm (Xia et al. 2001), such that some polyspermic pig eggs may develop to term if successive sperm do not interrupt the diploid zygotic genome (Han et al. 1999). Polyspermic fertilization in this species is an extraordinary case, and, in most cases of monospermic fertilization in mammals, defenses against polyspermy are rapidly established after penetration of the first sperm (Galeati et al. 1991; Yanagimachi 1994).

The number of sperm reaching the egg surface is reduced during passage through the female reproductive tract and the egg extracellular matrix, zona pellucida, jelly layer, and vitelline envelope. In mice, only 100 or 200 out of the 50 million sperm ejaculated were previously shown to reach the ovulated egg (Wassarman 1994). Although the possibility of the penetration of multiple sperm into an egg decreases at fertilization, eggs still remain at risk of polyspermy. Therefore, most eggs have established a membrane block and zona pellucida block in order to prevent polyspermy (Yanagimachi 1994; Abbott and Ducibella 2001). Following sperm penetration, the rapid and transient depolarization of membrane potential at the level of the plasma membrane (membrane block) is elicited in order to prevent the fusion of additional sperm in the frog, *Xenopus laevis*, and several marine invertebrates (Jaffe and Gould 1985; Gould and Stephano 2003). This change in membrane potential is not observed in fertilized mouse, hamster, rabbit, or bony fish eggs, in which the membrane block employs a different mechanism to changes in membrane potential (Nuccitelli 1980; Miyazaki and Igusa 1981; Igusa et al. 1983; Jaffe et al. 1983; McCulloh et al. 1983). After a temporal fast block, the contents (e.g. ovastacin in human and mouse; Quesada et al. 2004; Liu 2011; Burkart et al. 2012) of cortical granules, a special organelle in eggs, are released into the perivitelline space via their exocytosis (a cortical reaction). Although the structure and molecules involved in the zona reaction differ among species, this reaction makes the zona pellucida refractory to the binding and fusion of a second sperm (the zona reaction), which is common to all vertebrate species (Wong and Wessel 2006). An egg must recognize the binding and fusion of the first fertilizing sperm and rapidly establish a polyspermy block. Therefore, the fertilizing sperm must immediately activate and propagate a signal cascade throughout the whole egg. Although the molecular mechanisms underlying the activation of a polyspermy block have not yet been elucidated in detail, fertilization-mediated Ca^{2+} release from intracellular stores and the activation of protein kinase C (PKC) are known to be involved in the establishment of two different polyspermy blocks (Sun 2003; Gardner et al. 2007).

The eggs of some species, including ctenophores, elasmobranchs, urodele amphibians, reptiles, and birds, physiologically permit the penetration of more than one sperm into the ooplasm at fertilization (Elinson 1986; Iwao 2000; Wong and Wessel 2006; Snock et al. 2011; Mizushima 2012). Unlike monospermic eggs, neither a membrane block nor intracellular organelles similar to cortical granules have been observed in these polyspermic eggs; therefore, there is no block against the entry of a second or more sperm after the first sperm is incorporated into the egg cytoplasm. Since only one sperm is eventually involved in zygotic formation with a female pronucleus, embryo development with a diploid configuration is ensured. During natural fertilization, the avian egg receives a markedly higher number of sperm than other polyspermic species (Fig.8.1). Although it is technically difficult to calculate the total number of sperm in the germinal disc, it appears likely that, at the lowest estimate, 20 sperm are typically incorporated into an egg (Harper 1904; Patterson 1910, Fofanova 1965; Nakanishi et al. 1990; Waddington et al. 1998). The maximum number of sperm found within the germinal disc was previously reported to be 25 for the pigeon and 62 for the chicken (Harper 1904; Nakanishi et al. 1990). Furthermore, more than 70 sperm were detected in the quail egg within 1 hour of fertilization (our unpublished data).

The reason why the avian ovum may accept the entry of numerous sperm remained unclear until recently. The findings of recent experiments on birds suggest that the number of sperm incorporated into an egg cytoplasm affects the fate of the egg. The rate of chicken and turkey embryos developing to the blastoderm stage was found to be approximately 50% when approximately 3 sperm penetrated the inner perivitelline layer over the germinal disc region (in this case, the number of sperm that penetrated the egg cytoplasm was estimated to be less than 3), while the probability of egg development was almost 100% when more than 6 sperm penetrated the region (Bramwell et al. 1995; Wishrt 1997). This finding suggests that the fertilization rate increases when the number of sperm incorporated into an egg is higher. In addition, the direct injection of sperm into the cytoplasm of a mature quail egg, namely, an intracytoplasmic sperm injection (ICSI), directly revealed that a single sperm was insufficient for a high rate of fertilization and subsequent blastoderm development (approximately 20%;

Hrabia et al. 2003; Takagi et al. 2007a; Mizushima 2008, 2009, 2012; Shimada et al. 2014; Kang et al. 2015). It has been proposed that the purpose of polyspermy is to increase the opportunity of a sperm nucleus migrating to the center of the germinal disc and making contact with the female nucleus because the surface area of the avian germinal disc in relation to that of sperm is very large. However, the ICSI technique revealed that the microinjection of a single sperm together with soluble protein equivalent to multiple sperm induced fertilization and subsequent development up to the blastoderm stage in 70% of eggs tested (Mizushima et al. 2014). Therefore, instead of the frequency of migrating sperm nuclei, an alternative hypothesis in which many spermatozoa are necessary to provide sufficient amounts of sperm-derived egg-activating proteins to ensure the successful initiation of egg development in birds is currently considered the most likely theory (see Sect. 8.2 for more details). On the other hand, there may be a physiological limitation to excessive polyspermy in the avian egg. For example, an increase in the number of sperm that make contact with the ovum by intramagnal insemination, which is greater than the normal numbers of sperm, induces an increase in the frequency of early blastoderm developmental arrest (Van Krey et al. 1966). More critically, a recent study showed that cytoplasmic segmentation occurs at a high rate in the absence of nuclear divisions in the germinal discs of quail and chicken ova inseminated *in vitro* using 1–2 x 10^4 semen, and eggs underwent development to the early pseudo-blastoderm stage (Olszanska et al. 2002; Batellier et al. 2003), indicating that an excessive number of supernumerary sperm interferes with normal fertilization and subsequent embryo development (Fechheimer 1981; Olszanska et al. 2002; Mizushima et al. 2009). A novel structure coated with calcium carbonate in the infundibulum part of the female reproductive tract in which fertilization occurs and sperm are stored until fertilization, termed the sperm-associated body (SB), was identified in domestic birds, and SB-accompanied sperm only were found to pass through the vitteline membrane (Sultana et al. 2004; Rabbani et al. 2006, 2007). This SB may contribute physiologically to reducing the number of excessive fertilizing sperm.

7.2

Egg activation

7.2.1

Ca^{2+} increase during fertilization

Physiological polyspermy occurs in oviparous species that exhibit internal fertilization such as cartilaginous fishes, urodele amphibians, reptiles, and birds (Elinson 1986; Iwao 2000;

Wong and Wessel 2006; Snook et al. 2011). Fertilizing sperm provide a signal to trigger the initiation of egg development (egg activation) as well as the nucleus because the development of animal eggs is arrested at the species-specific phase of meiosis until fertilization, e.g. at metaphase of second meiosis (metaphase II) in most vertebrate eggs (Stricker 1999). In all nomospermic and polyspermic animals studied to date, an increase in intracellular Ca^{2+} concentrations ($[Ca^{2+}]i$) has been observed immediately after sperm-egg binding or fusion during fertilization (Stricker 1999; Runft et al. 2002), and this increase in $[Ca^{2+}]i$ plays a pivotal role in restarting cell cycle events in the egg, which comprise the resumption of meiosis and extrusion of the second polar body (Ducibella et al. 2002; Miyazaki 2006). Furthermore, in mammalian species, the establishment of polyspermy prevention is also evoked by an increase in $[Ca^{2+}]i$ (McAvey et al. 2002; Gardner et al. 2007). In spite of its universality, the spatiotemporal patterns of the Ca^{2+} signal associated with egg activation vary widely among species (Stricker 1999). In monospermic fishes and the frog, *Xenopus laevis*, a transient increase in $[Ca^{2+}]i$ induced by a single sperm was shown to propagate throughout the whole egg from the sperm-entering position as a Ca^{2+} wave (Ridgway et al. 1977; Busa and Nuccitelli 1985; Fluck et al. 1991; Abraham et al. 1993; Keating et al 1994; Creton et al. 1998). An increase in $[Ca^{2+}]i$ during the activation of mammalian eggs is known to occur periodically in the form of long-lasting oscillations, which are known as Ca^{2+} oscillations (Miyazaki et al. 1993; Jones et al. 1995), and continues to approximately the first interphase stage, similar to that in mouse eggs (Jones et al. 1995; Marangos et al. 2003), or to the first mitotic cell cycle, as observed in bovine eggs (Nakada et al. 1995).

In mice, artificial stimuli such as electrical pulses or exposure to ethanol have revealed that a single Ca^{2+} pulse induces second polar body extrusion, but causes only partial egg activation due to the incomplete inactivation of cytostatic factor, which is the cytosolic protein responsible for meiotic arrest at metaphase II (Tatone et al. 1999; Ducibella et al. 2002; Jones 2005). In contrast, continuous stimuli for 24 hours fully activate eggs, which include an increase in cortical granule exocytosis and the formation of both polar bodies (Deguchi and Osanai 1995; Lawrence et al. 1998; Ozil 1998; Ducibella et al. 2002; Jones 2005, 2007). Accordingly, the mammalian egg needs to be exposed to a series of repetitive Ca^{2+} pulses in order to ensure it escapes meiosis because a number of hours are needed for a lapse between meiotic resumption and the interphase stage. Alternatively, unlike mammals, fertilization in fishes and *Xenopus laevis* triggers a rapid transition to pronuclear formation (Rugh 1951; Iwamatsu and Ohta 1978). The short post-fertilization phase in these zygotes may obviate the second or more Ca^{2+} oscillations (Jones 1998).

In physiological polyspermic eggs, a few sperm successively enter at different points on the egg surface, and increases in $[Ca^{2+}]i$ spread concentrically into the egg cytoplasm at each sperm entry site as small waves (Harada et al. 2011; Iwao 2012). However, unlike monospermic eggs such as those of fishes, *Xenopus laevis*, and mammals, each Ca^{2+} wave does not reach the opposite site of the egg. The $[Ca^{2+}]i$ intensity in the entire egg region of polyspermic eggs, as revealed by a Ca^{2+}-sensitive fluorescence dye, shows a slow increase that continues for approximately 40 min, with the peak level reached being markedly lower that than in *Xenopus laevis* eggs (Fontanilla and Nuccitelli 1998). In addition, a slow wave-like increase in $[Ca^{2+}]i$ is also caused by a single injection of sperm extract (SE) containing soluble proteins into the newt egg cytoplasm, which propagates concentrically from the injection site through the entire egg without a local small wave, but is also able to evoke meiotic resumption (Harada et al. 2007). In polyspermy of the frog, *Discoglossus*, an increase in $[Ca^{2+}]i$ lasts for 50 min after fertilization (Nuccitelli et al. 1988). However, several rapid spike-like depolarizations caused by sperm entry precede the major depolarization mediated by Ca^{2+}-activated Cl^- efflux (Talevi 1989), suggesting that a non-propagative small Ca^{2+} increase occurs at each sperm entry point in advance of the major Ca^{2+} wave (Iwao 2012).

In birds, a microinjection of SE into an ovulated egg retrieved from the infundibulum or upper part of the magnum revealed a very unique pattern for the increase in $[Ca^{2+}]i$ in the quail egg, which has not been observed in other species (Fig.8.2). Its Ca^{2+} signal pattern is classified as two different kinds of Ca^{2+} waves, namely, a transient, slow wave and multiple, spiral-like oscillations (Mizushima et al. 2014). The slow Ca^{2+} wave was observed immediately after the microinjection, and an increase in $[Ca^{2+}]i$ was initiated at the injection site of the germinal disc and its Ca^{2+} wave spread concentrically into the egg cytoplasm, but was restricted to the germinal disc. The increase in $[Ca^{2+}]i$ at the injection site continued for approximately 5 min, and then decreased gradually before returning to the basal level within 30 min of the microinjection. On the other hand, an initial spiral-like Ca^{2+} signal occurred at the injection site 10-15 min after the microinjection, which was prior to the restoration of increases in $[Ca^{2+}]i$ by the Ca^{2+} wave to the basal level. Second and further spiral-like Ca^{2+} waves originated successively before the disappearance of the previous Ca^{2+} spiral with a mean interspike interval of less than 1 min, and its oscillation lasted for at least 3 hours. Furthermore, a few Ca^{2+} spirals overlapping partially in one egg were found to have complicated waveforms, which markedly differed from the Ca^{2+} oscillations observed during mammalian egg activation. It is important to note that these two different $[Ca^{2+}]i$ patterns play different roles in the activation of the quail egg. A slow Ca^{2+} wave has the ability to evoke the resumption of meiosis and subsequent zygote formation (Mizushima et al. 2007, 2008, 2014), whereas spiral-like Ca^{2+} oscillations are not involved in these events. However, the induction of spiral-like Ca^{2+} oscillations in a slow Ca^{2+} wave-generated ICSI quail egg showing developmental arrest at the early embryo stage (Mizushima et al. 2008, 2010, 2014) activate full-term development to hatching, suggesting that spiral-like Ca^{2+} oscillations are necessary for cell cycle progression in the advanced zygote stage. More than 20 sperm generally enter chicken and quail eggs and then progressively form male pronuclei between 1 and 3 hours (Perry 1987; Nakanishi et al. 1990). Although the spatiotemporal changes that occur in $[Ca^{2+}]i$ in the avian egg following natural fertilization or *in vitro* insemination still remain unknown, multiple Ca^{2+} spirals appear to be necessary, in part, for a continuous stimulation to complete pronuclei formation besides the cell cycle stimulation (Fig.8.4).

7.2.2

Egg size and sperm number

The microinjection of a whole sperm into a mammalian egg (ICSI), which avoids any membrane contact between sperm and the egg, generates a Ca^{2+} oscillation similar to that observed during fertilization, and this Ca^{2+} oscillation is also triggered by a microinjection of SE corresponding to the content of a single sperm (Homa and Swann 1994; Wu et al. 1997, 1998; Dong et al. 2000; Parrington et al. 2000; Tang et al. 2000). Thus, the quantity of egg-activating proteins contained in a single sperm is necessary and sufficient for fully activating the eggs of monospermic species. It is important to note that avian SE equivalent to approximately 1 sperm or a whole avian sperm possess the ability to initiate Ca^{2+} oscillations and subsequent zygotic formation in a mouse egg (Dong et al. 2000; Takagi et al. 2007b), but have a negligible effect on the activation of an avian egg (Hrabia et al. 2003; Takagi et al. 2007a; Mizushima et al. 2009, 2014). These findings led to the hypothesis that a large number of sperm may be necessary in order to provide egg-activating factors not fulfilled by a single sperm due to the large size of an avian egg in comparison to that of a mammalian egg. Indeed, the volume of quail egg cytoplasm is more than 1-2 μL, whereas that of the mouse is approximately 200 pL.

Egg-activating proteins equivalent to 100-200 sperm are required for fertilization and full-term development to hatching in quails, which supports the strong correlation between egg size and polyspermy (Birkhead et al. 1994; Mizushima 2012, 2014; Shimada et al. 2014; Kang et al. 2015).

Unlike mammals in which a sperm-triggered Ca^{2+} wave propagates through the entire mammalian egg, the Ca^{2+} wave generated by a single sperm only propagates in one-eighth to one-quarter of the egg surface in physiologically polyspermic newts (Harada et al. 2011, Iwao 2012). Consistent with the egg size theory, several sperm must enter from different points in order to increase $[Ca^{2+}]i$ throughout the large eggs of newts. Similarly, in polyspermy in birds, the propagation of a Ca^{2+} signal by a single sperm may not reach the whole egg, and, as such, a larger number of sperm than that needed by newts is required for the activation of the whole egg because the size of an avian egg is markedly larger than those of newts (Fig.8.4). Although little is known about the discrepancy that a larger amount of SE (equivalent to 100-200 sperm) than that from the 2 to 60+ observed during natural fertilization is required for the full-term development of quail eggs following ICSI with a single sperm (Mizushima et al. 2014), these differences lie in the fact that 2-20 sperm typically penetrate newt eggs *in situ* (Iwao et al. 1985, 1993), whereas complete egg activation by a microinjection of newt SE requires a protein content equivalent to 330 sperm (Harada et al. 2011). Regarding egg activation, although limited information is available on the minimum number of sperm needed for the full activation of an avian egg, at least 20 sperm may be required to increase $[Ca^{2+}]i$ throughout the entire avian egg. Otherwise, egg size may influence where and when sperm enters, and, thus, potentially affect the timing of sperm aster formation and migration of potential microtubule organization centers (MTOCs), which are responsible for the movement of the pronucleus to karyogamy. The extra space in the egg cytoplasm of large eggs is one of the most important factors for eliminating supernumerary sperm nuclei in the same egg cytoplasm (Elinson 1986; Iwao 2012). Based on various aspects of internal fertilization, centrosome dynamics, evolutionary history, and egg cytological polyspermy blocks (see Sect. 8.3 for details), the close relationship between increased egg sizes and the number of sperm in the acquisition of polyspermy needs to be discussed in more detail (Snook et al. 2011). This evidence based on an

7.2.3

Molecular mechanisms underlying egg activation in physiological polyspermy

An increase in $[Ca^{2+}]i$ in the eggs of vertebrates is initiated by Ca^{2+} release via the inositol 1,4,5-trisphosphate (IP_3) receptor (IP_3R). A large number of studies on vertebrate eggs have demonstrated that an injection of IP_3 induces the release of Ca^{2+} from Ca^{2+} stores, mainly the endoplasmic reticulum, in the egg (Miyazaki 1988; Miyazaki et al. 1992; Fissore and Robl 1993; Swann and Ozil 1994; Wang et al. 1999; Amano et al. 2004; Lee et al. 2010; Mizushima et al. 2014). The repetitive increases observed in $[Ca^{2+}]i$ (Ca^{2+} oscillations) during mammalian fertilization are no exception. IP_3-induced Ca^{2+} release as well as Ca^{2+}-induced Ca^{2+} release, which operates by the sensitizing effects of Ca^{2+} on the IP_3R, contribute to the regenerative process of Ca^{2+} release (Miyazaki et al. 1993). Since IP_3 is generated by enzymes of the phospholipase C (PLC) family, which catalyze the hydrolysis of phosphatidylinositol 4,5-bisphosphate (PIP_2) into IP_3 and dyacylglycerol (Rhee 2001), two main molecular signaling models have been proposed for the production of IP_3 in the egg. The first suggestion proposes that sperm-egg fusion induces the activation of a receptor on the egg plasma membrane and couples to either a G-protein or tyrosine kinase (Src kinase), and this is followed by the activation of PLCβ or PLCγ, respectively (Runft et al. 2002). In *Xenopus laevis*, Src kinase is phosphorylated and then stimulates PLCγ at the time of sperm-egg contact (Sato et al. 1999, 2000, 2001, 2003). Sperm induces the transient phosphorylation of Uroplakin III (UPIII) on the egg membrane, which is a substrate of the egg cytoplasmic Src kinase, and Src (Sakakibara et al. 2005; Mahbub Hasan et al. 2014). In addition, a sperm-derived protease associated with a sperm surface glycoprotein (Nagai et al. 2009) serves as a target of the extracellular domain of UPIII (Mahbub Hasan et al. 2005; Sakakibara et al. 2005). Although the molecular signaling pathway for egg activation in the monospermy of the primitive jawless fish lamprey remains unknown, the involvement of a receptor on the egg membrane, and not sperm-egg fusion has been postulated because most eggs clamped at a positive potential (+20 to +40 mV) were found to undergo egg activation (Kobayashi et al. 1994).

An alternative hypothesis has been proposed for increases in $[Ca^{2+}]i$ in which the sperm itself contains soluble egg-activating factors that diffuse directly into the cytoplasm of the egg after fusion. Since fertilization-induced increases in $[Ca^{2+}]i$ are not prevented in the mouse egg in spite of the introduction of a G-protein-specific inhibitor or PLCγ (Moore et al. 1993; Mehlmann et al. 1998; Williams et al. 1998), this appears to be the case in most mammalian species (Swann 1996; Stricker 1999; Runft et al. 2002). The most likely candidate for the initiator of an increase in $[Ca^{2+}]i$ in mammalian eggs is a sperm-specific member of the PLC isozyme, PLCzeta (PLCζ; Saunders et al. 2002). Although other sperm-inducing factors have been reported (Sette et al. 2002; Perry et al. 1999, 2000), they have not yet been substantiated and important evidence now exists to show that PLCζ alone has the ability to generate Ca^{2+} oscillations in mouse eggs at an amount equivalent to the content in a single sperm (Saunders et al. 2002; Kouchi et al. 2004). Furthermore, a PLCζ orthologue has also been identified in humans, monkeys, rats, pigs, bovines, and horses as a common egg-activating factor in mammals (Cox 2002; Yoneda et al. 2006; Ito et al. 2008; Ross et al. 2008; Sato et al. 2013). The mouse PLCζ contains a nuclear localization signal that promotes its accumulation in the pronuclei (Larman et al. 2004; Yoda et al. 2004; Kuroda et al. 2006). The nuclear accumulation of PLCζ appears to terminate long-lasting Ca^{2+} oscillations. In addition, the sperm of the teleost fish, tilapia, contains an egg-activating factor for increasing Ca^{2+} in mouse eggs or sea urchin egg homogenates (Coward et al. 2003), and the medaka testes also contain PLCζ that initiates Ca^{2+} oscillations in mouse eggs (Coward et al. 2011). However, PLCζ in the pufferfish, Fugu, is expressed in the ovary and brain, but not in the testis, and its ovarian form does not have the ability to trigger Ca^{2+} oscillations in mouse eggs (Coward et al. 2011). Therefore, PLCζ does not appear to be involved in the sperm-induced activation of all fish eggs.

On the other hand, citrate synthase (CS) is the most likely candidate for an initiator of increases in $[Ca^{2+}]i$ in newt eggs (Harada et al. 2007). Although the molecular mechanisms underlying the initiation of an increase in $[Ca^{2+}]i$ by sperm CS have not yet been elucidated in detail, an experimental hypothesis was recently proposed in which CS released from sperm forms a complex with maternal microtubules and the ER with the IP_3R as well as PLCγ in the midpiece region of the sperm, and this complex acts on small Ca^{2+} wave propagation by the sequential activation of PLCγ in order to stimulate the IP_3R (Ueno et al. 2014). Or paternal CS cleaves citrate into acetyl-CoA and oxaloacetate in the egg cytoplasm, and the former and latter then trigger Ca^{2+} release from the ER and mitochondria, respectively (Harada et al. 2011). In somatic cells, CS produces citrate from acetyl-CoA and oxaloacetate in the mitochondrial tricarboxylic acid (TCA) cycle, but may inversely cleave citrate, which is abundant in the egg cytoplasm, to produce acetyl-CoA and oxaloacetate (Srere 1992; Iwao, 2012). Increases induced in acetyl-CoA in the egg cytoplasm by sperm CS may sensitize IP_3Rs on the ER (Missiaen et al. 1997). Oxaloacetate has been suggested to have the potential to induce the release of Ca^{2+} from mitochondria (Leikin et al. 1993). Therefore, paternal CS functions as an enzyme in the egg in order to produce acetyl-CoA and oxaloacetate and/or as a PLCγ and IP_3R stimulator to generate the release of Ca^{2+} from Ca^{2+} stores. Furthermore, since SE derived from newts cannot generate Ca^{2+} oscillations in the mouse egg, the mechanism responsible for egg activation in newts differs from PLCζ signaling (Harada et al. 2007). A PLCζ orthologue has not yet been identified in newts.

In contrast to PLCζ and CS alone being sufficient to evoke the release of Ca^{2+} in mammalian and newt eggs, at least 3 egg-activating factors, namely, PLCζ, CS, and aconitate hydratase (AH), are essential for the complete release of Ca^{2+} in order to activate the full-term development of avian eggs to hatching (Fig.8.3; Mizushima et al. 2014). Even though avian PLCζ has the ability to generate Ca^{2+} oscillations in the mouse egg (Coward et al. 2005), it is only involved in an initial slow Ca^{2+} wave in quail eggs (Mizushima et al. 2014). This difference in responses to PLCζ in these eggs may be associated with the desensitization of the IP_3R channel to IP_3 and IP_3-induced Ca^{2+} resulting from PLCζ activity, and not to any reductions in enzyme activity. The IP_3Rs in mammalian eggs are progressively desensitized by ubiquitination and subsequent proteasome activity, which corresponds to the termination of Ca^{2+} oscillations at the interphase stage (Zhu et al. 1999; Brind et al. 2000; Zhu and Wojcikiewicz 2000; Malcuit et al. 2005; Lee et al. 2010), suggesting that IP_3 binding to IP_3Rs initiates the down-regulation of IP_3Rs to terminate PLCζ-induced egg activation immediately after fertilization in birds. Although limited information is available for the down-regulation of maternal IP_3Rs during avian fertilization, the long-lasting spiral-like Ca^{2+} oscillation initiated 10-15 min after the PLCζ-induced Ca^{2+} wave slowly occurs irrespective of the absence of IP_3Rs because the introduction of an antagonist of IP_3R did not prevent Ca^{2+} oscillations in the quail (Mizushima et al. 2014). On the other hand, CS and AH are both responsible for the generation of spiral-like Ca^{2+} oscillations. This spiral-like Ca^{2+} oscillation is not induced by CS or AH alone. Egg-activating activity in SE was found to be abolished by a treatment with each antibody, which supports these findings. Spiral-like Ca^{2+} oscillations induced by CS and AH may be partly ascribed to the release of Ca^{2+} from ryanodine receptors on the ER because the microinjection of an agonist of ryanodine receptors into quail eggs was found to initiate repetitive Ca^{2+} spikes similar to CS- and AH-induced spike-like Ca^{2+} oscillations (Fig.8.3; Mizushima et al. 2014). Previous studies reported that ryanodine receptors participated in the Ca^{2+} waves observed in sea urchin eggs during egg activation (Galione et al. 1993; Lee et al. 1993; Miyazaki et al. 2006). The elucidation of variations in egg activation systems and particularly increases in Ca^{2+} at fertilization in vertebrates may contribute to a more detailed understanding of the evolutionary history of egg activation concomitant with the acquisition of polyspermy (Table 8.1).

7.2.4

Variations in and evolution of the sperm factor

The egg-activating factors responsible for increases in $[Ca^{2+}]i$ in eggs are characterized as sperm-specific molecular triggers in all vertebrates studied. Although AH and CS are originally mitochondrial genes involved in the TCA cycle, the molecular weights of proteins detected in sperm are slightly higher for CS and lower for AH than those in body cells (Mizushima et al. 2014). Since their predicted amino acid sequences are partially different from those in somatic cells, their sperm-specific isoforms appear to be diversified and specified by gene duplication as an egg-activating factor. The molecular weight of sperm-specific newt CS was also previously shown to be slightly higher than that of other tissues (Harada et al. 2007), and this difference has been attributed to the hyper-phosphorylation of sperm CS (Ueno et al. 2014). Unlike PLCζ in monospermic species, the acquisition of novel egg-activating factors in the polyspermic species, quails and newts, may have played a pivotal role in the evolution of slower activation in polyspermic eggs and may also have promoted the reproductive isolation necessary for speciation in vertebrates. However, a review of variations in sperm-specific CS and AH between quails and newts indicates divergent evolution in the molecular mechanisms underlying increases in $[Ca^{2+}]i$ concomitant with a species-specific transition in the mode of polyspermic fertilization. A previous study showed that a microinjection of quail CS alone into a homogeneous egg did not have the ability to generate Ca^{2+} waves (Mizushima et al. 2014).

On the other hand, PLCζ is solely expressed in the sperm of most animals such as mammals (Cox et al. 2002; Saunders et al. 2002; Yoneda et al. 2006, Young et al. 2009), birds (Coward et al. 2005; Mizushima et al. 2008, 2009), and medaka (Ito et al. 2008). This ensures the sperm-specific enrichment of egg-activating factors, consistent with a gamete-specific role. Genomic DNA analyses have revealed that the PLCζ gene is located back to back with another testis-specific gene, the actin-capping protein gene *CAPZA3* (Hurst et al. 1998; Yoshimura et al. 1999; Miyagawa et al. 2002), which is inserted in the 5'-region of PLCζ and shares a bidireactional promotor with PLCζ (Coward et al. 2005, 2011), suggesting that the expression of CAPZA3 and PLCζ is male germ cell- or testis-specifically transcripted in the same process. However, the PLCζ of puffer fish was expressed in the brain and ovaries, but not in the testes. More interestingly, the

CAPZA3 genes of puffer fish and medaka were not adjacent to the PLCζ genes (Coward et al. 2011). Although difficulties are associated with accounting for variations in the tissue-specific expression patterns of PLCζ in these fishes, during the rapid evolution of egg activation mechanisms, PLCζ seemed to be the first important differentiation factor for sperm function in the vertebrate species. Pufferfish PLCζ does not have the ability to activate the release of Ca^{2+} in mouse eggs; however, this does not exclude the possibility that the activation of PLCζ by a fertilizing sperm functions as an egg-derived Ca^{2+}-releasing factor and/or plays some other role in the egg. Further investigations will provide an insight into the functional role and genesis of PLCζ.

PLCζ has not yet been detected in intermediate species between fishes and higher eutherian mammals, such as primitive mammals including the *monotrematous* platypus and small marsupial mammals (Table 8.1). The platypus, *Ornithorhynchus anatinus*, lays large yolky eggs that exhibit polyspermy, and several sperm have been suggested to enter the egg cytoplasm (Gatenby and hill 1924; Hughes and Hall 1998). Furthermore, the eggs of the small marsupial mammal, *Sminthopsis crassicaudata*, are relatively small, but occasionally exhibit polyspermy (Breed and Leigh 1990). Although changes in $[Ca^{2+}]i$ at fertilization have not yet been examined in primitive mammals, egg activation in the ancestors of mammals may be achieved by polyspermy, with eggs showing primitive Ca^{2+} oscillations. Since the platypus, the most primitive mammal, shares common molecular, genetic, and morphological features with birds and reptiles (Rens et al. 2007) and are in a comparatively close cluster taxonomically (Warren et al. 2008), the spiral-like Ca^{2+} oscillations observed in avian egg activation may be closely related to an ancestor of mammalian Ca^{2+} oscillations. In this respect, these primitive mammalian species may possess a PLCζ orthologue or intermediate molecules similar to three egg-activating factors.

7.3

Syngamy and elimination of supernumerary sperm in the egg cytoplasm

In monospermic species, frog, and *Hynobium* salamander eggs, increases in Ca^{2+} open Ca^{2+}-dependent Cl^- channels on the egg plasma membrane in order to produce rapid depolarization, which prevents the penetration of additional fertilizing sperm (Cross and Elinson 1980; Iwao 1989; Iwao and Jaffe 1989). Previous studies demonstrated that rapid depolarization or a cortical reaction does not occur in polyspermic urodeles (Charbonneau et al. 1983; Iwao 2012). In polyspermic frog, *Discoglossus* eggs exhibit a fertilization-activated membrane potential due to the opening of Cl^- channels; however, it does not block additional sperm entry (Talevi 1989). On the other hand, hyperpolarization mediated by Na^+ channels in response to each sperm penetration has been indicated in polyspermic newt eggs (Iwao 1985), but does not prevent second sperm penetration (Iwao and Jaffe 1989). Another polyspermy block system, the cortical reaction described above, is well-developed in mammalian eggs (Yanagimachi 1994; Quesada et al. 2004; Wong and Wessel 2006; Liu 2011; Burkart et al. 2012), whereas the dynamic movement and exocytosis of cortical granules are not likely in newt eggs (Iwao 2000). Although electrical responses in avian eggs have not yet been examined, a review of findings obtained using several amphibian eggs suggests that there is neither electrical regulation on the surface of egg plasma membranes nor alterations in the extracellular matrix that prevent polyspermy in all polyspermic species.

Even though numerous sperm are incorporated into polyspermic eggs, the egg nucleus proceeds to karyogamy with a single sperm nucleus because of the presence of an ooplasmic block to escape polyploidy. In birds, a large number of sperm penetrate the egg cytoplasm, most of which undergo transformation into male pronuclei after swelling, a change from the elongated form to the spherical head, chromatin decondensation, and reconstitution of the nuclear envelope (Fofanova 1965; Okamura and Nishiyama 1978; Perry 1987; Nakanishi et al. 1990; Waddington et al. 1998). However, supernumerary sperm (accessory sperm) nuclei, except for the one (principal sperm) that unites with the female pronucleus to form a zygote, move towards the periphery of the germinal disc, it is not in the yolk, but in the vicinity of cytoplasm, and supernumerary sperm nuclei undergo one or two mitoses (Perry 1987). This dynamic movement of accessory sperm in avian eggs has not been observed in other polyspermic species. The mitosis of accessory sperm occurs in synchrony with the zygotic nucleus, and the resultant cell division at the peripheral region of the egg cytoplasm disappears during the early cleavage stage (Paterson 1910; Eyal-Gilidi and Kochav 1976). Such nuclear division without DNA synthesis may represent a deviation from periodic nuclear activation (Emanuelsson 1965; Gurdon and Woodland 1968). It is important to note that, in addition to this, since ICSI with a single sperm, without accessory sperm-activating temporal cleavage, has been shown to develop quail eggs to the late blastoderm stage (Hrabia et al. 2003; Takagi et al. 2007; Mizushima et al. 2007, 2008, 2012, 2014; Kang et al. 2015), accessory sperm nuclei are not essential for any cellular events related to zygotic development. Although the molecular mechanisms suppressing supernumerary sperm nuclei have not been elucidated fully, the involvement of maternal-derived deoxyribonucleases (DNase I and II) in birds, which are not expressed in monospermic mammalian eggs (Stepinska et al. 2001, 2003; Olszanska and Stepinska 2008), is under debate.

On the other hand, the mechanisms by which the movement of the male and female pronuclei forms the zygote nucleus remain obscure in birds. This assures that only one sperm is selected as the principal sperm by some unknown mechanisms and is subsequently paired with the female pronucleus in the center of the germinal disc 3-4 hours after fertilization (Fig.8.4; Perry 1987). Female metaphase II chromatin is localized in the superficial cytoplasm near the center of the germinal disc when the avian egg is ovulated (Perry 1987); however, even in the presence of numerous sperm, the female pronucleus still occupies a central position (Perry 1987), indicating that the selected principal sperm pronucleus appears to move toward the female pronucleus. In fertilized newt eggs, only one sperm nucleus, possibly that nearest to the female nucleus, forms a larger sperm aster than that of accessory sperm, and then makes contact with the female pronucleus in the center of the animal hemisphere (Iwao et al. 2002). The maternal γ-tubulin predominantly distributed in the animal hemisphere strongly accumulates in the centrosomes of the one principal sperm nucleus and subsequent zygote nucleus in order to promote microtubule polymerization, whereas only a small amount of γ-tubulin is associated with those of other sperm nuclei (Iwao et al. 2002; Morito et al. 2005). γ-tubulin is a well-known major component of the MTOC, and is involved in the movement of the male pronucleus to fuse with the female pronucleus (Haren et al. 2006; Eot-Houllier et al. 2010), suggesting that γ-tubulin is one of the key factors in the event related to the selection of the principal sperm nucleus in newts (Reinsch and Karsenti 1997; Iwao et al. 2002). Furthermore, since the principal sperm and female nuclei enter the DNA synthesis phase of the first cleavage earlier than the accessory sperm nuclei, zygotic nuclei can enter the mid-term of the first cleavage. This is because cyclin B that forms the MPF complex accumulates in and disappears from the zygotes earlier than accessory sperm cells (Iwao and Elinson 1990; Iwao et al. 2002). The failed progression of cyclic nuclear activities in accessory sperm may be due to insufficient exposure of the MPF complex (Iwao et al. 1993; Sakamoto et al. 1998). In addition to the possible participation of the motor proteins, dynein, dynactin, and kinesin in opposite movements between the principal and accessory sperm nuclei (Payne et al. 2003; Waitzman and Rice 2014), further investigations on the sequential distribution of avian cdc2 (Mori et al. 1991) and cyclin

B as well as γ-tubulin in the egg will provide insights into not only the selection of principal sperm nuclei, but also the cellular process of zygotic fusion.

7.4

Conclusion

Development of avian ICSI technique has brought us a new schematic diagram in avian fertilization (Fig.8.3). However, new finding such as novel sperm-derived egg-activating factors is only a part of mysterious events of polypsermic fertilization. Therefore, more information will be needed in order to understand the comprehensive molecular mechanism of avian fertilization. Experiments using gene-disrupted animals are very powerful tools for validating which factors are essential, which has also contributed to find novel genes in many species. In particular, in vitro fertilization study is one of the suitable research fields to use gene-manipulated animals. Fortunately, the TALEN and Crisper/Cas 9 systems have opened a new door for avian gene-disruption (see Sect.13 for more details). The combination of avian ICSI and gene-manipulation systems will make a significant progress in our understanding of avian fertilization system.

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Figure Captions

Fig.7.1. Scanning electron microscopic observation of two sperm and egg plasma membrane fusion in Japanese quail (photograph provided by Dr. Norio Yoshizaki, Gifu University). Usually, 2-60+ sperm penetrate egg cytoplasm in birds.

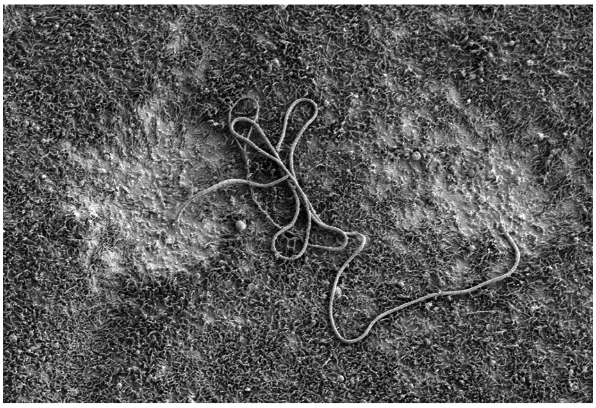
Fig.7.2. Ca^{2+} rise in quail egg cytoplasm after microinjection of sperm-derived egg activating factors. An intracellular Ca^{2+} increased immediately from injecting site and the Ca^{2+} signal propagated over the egg cytoplasm. The Ca^{2+} rise continues for about 5 minutes. Thereafter, long-lasting spiral-like Ca^{2+} oscillations are generated and continue every 1 min for over 1 hour. Small circle indicates injecting site.

Fig.7.3. Schematic signaling pathway in avian egg activation. Sperm-specific PLCζ introduced from each sperm induces a slow Ca^{2+} wave, while sperm-specific CS and AH induces a periodic spiral-like Ca^{2+} spike. Inositol 1,4,5-trisphosphate (IP_3) hydrolyzed from phosphatidylinositol 4,5-bisphosphate by PLCζ actvity evokes Ca^{2+} relrease from endoplasmic reticulum (ER) via IP_3 receptor. CS and AH may induce Ca^{2+} relrease from ER or mitochodria. In a part, another molecules and ryanodin receptor may be involved in Ca^{2+} release.

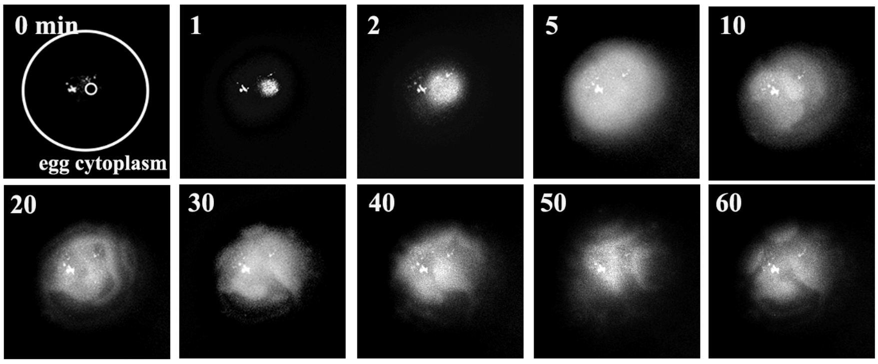
Fig.7.4. Ca^{2+} rise and zygote formation in avian egg cytoplasm. (A) At immediate after sperm penetration, Ca^{2+} waves spreads in the whole egg cytoplasm from each sperm entry site. (B) At 15 min, a spiral-like Ca^{2+} spikes from each sperm entry site are generated and it continues to pronuclear stage. (C) At 1.5 hours, the female chromatin is in metaphase II (MII) of the second meiosis and the incorporated sperm are starting to transform to pronuclei in order of entry into egg cytoplasm. (D) At 3 hours, female (FPN) and principal male pronucleus (PMPN) places in the center of egg cytoplasm, whilst supernumery sperm pronuclei (SPN) move towards the in the vicinity of cytoplasm. (E) At 4.0 hours, the zygotic nucleus and several supernumerary sperm nuclei undergo mitosis. (F) At 5 hours, a zygotic nucleus progresses towards 4-cell stages and supernumerary sperm are degraded by DNases. Modified from J Anat 150:99-109 (Perry 1987).

Table 8.1 Comparison of fertilization mode, pattern of Ca^{2+} rise in egg activation, and sperm-derived egg-activating factor among vertebrates

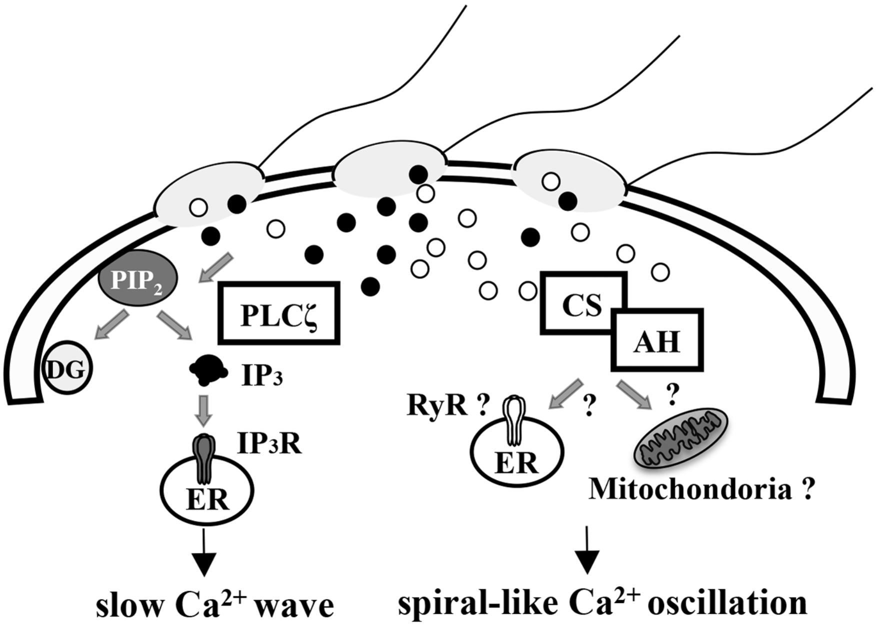
Species	**Fertilization mode**	**Ca^{2+} pattern**	**Ca^{2+} store**	**Sperm factor**
fish				
Fugi	monospermy	single	endoplasmic reticulum	?
medaka	monospermy	single	endoplasmic reticulum	PLCζ?
Discoglossus	polyspermy	multiple	endoplasmic reticulum	?
amhibian				
anuran (*Xenopus*)	monospermy	single	endoplasmic reticulum	receptor
anuran (*Discoglossus*)	polyspermy	?	?	?
urodele (*Cynopus*)	polyspermy	multiple	endoplasmic reticulum, mitochondria?	CS
bird				
Japanese quail	polyspermy	multiple	endoplasmic reticulum, mitochondria?	PLCζ, CS, AH
chicken	polyspermy	?	?	PLCζ?
mammal				
platypus	polyspermy	?	?	?
higher eutherians	monospermy	multiple	endoplasmic reticulum	PLCζ



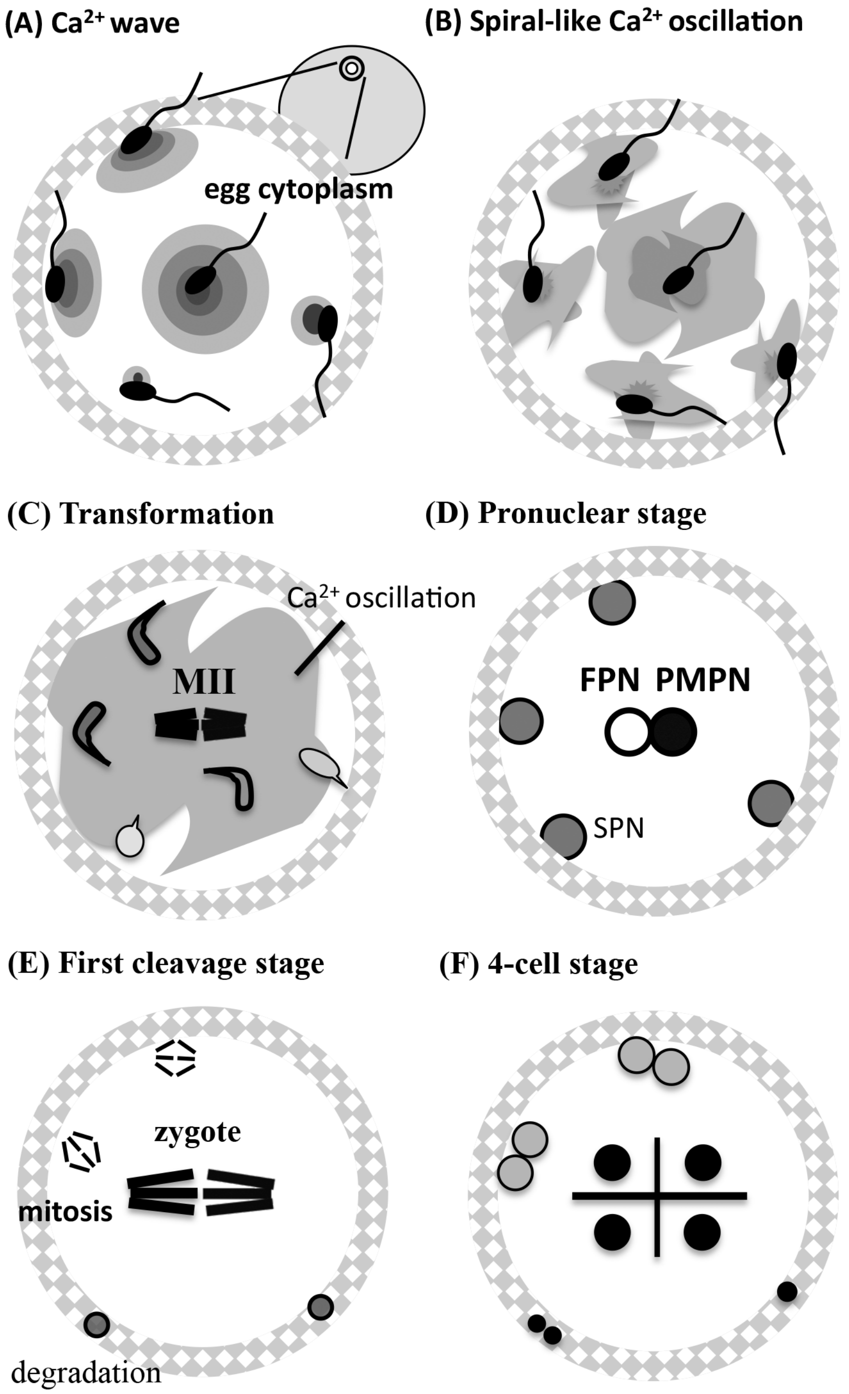
Mizushima Figure 7.1



mizushima Figure 7.2



Mizushima Figure 7.3



Mizushima Figure 7.4