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New Insights into the Molecular Events of Mammalian Fertilization

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Abstract

Currently, infertility affects ~16% of couples worldwide. The causes are reported to involve both male and female factors, including fertilization failure between mature spermatozoa and eggs. However, the molecular mechanisms involved in each step of mammalian fertilization are yet to be fully elucidated. Although some of these steps can be rescued with assisted reproductive technologies, it is important to clarify the molecular mechanisms involved for the treatment and diagnosis of infertile couples. This review illustrates recent findings in mammalian fertilization discovered by combining gene modification techniques with other new approaches, and aims to show how these findings will guide future research in mammalian fertilization.

Keywords

Genetically modified animals; CRISPR/Cas9 system; mammalian fertilization; sperm–egg fusion; egg activation

Main Text

Processes of Mammalian Fertilization

All eutherian mammal species utilize internal fertilization [1, 2]. This comprises many processes including sperm migration through the female reproductive tract, sperm capacitation and the sperm acrosome reaction, sperm–egg interaction and fusion, and subsequent initiation of embryonic development through egg activation. In more detail, ejaculated spermatozoa migrate from the uterus into the isthmus of the oviduct through the uterotubal junction, and fertilize eggs in the ampulla of the oviduct. During their migration in the female reproductive tract, spermatozoa change their physiological and morphological characteristics, such as drastic changes in their flagellar waveform (hyperactivation) and acquiring competency for the acrosome reaction. The acrosome reaction is an exocytotic event of a large enzyme-containing organelle in the sperm head and is a prerequisite for

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penetration of the egg's extracellular matrices, the zona pellucida (ZP), and for sperm–egg fusion. Thus, mammalian fertilization proceeds in a cascade-like manner, and the various steps are inseparable.

Identification of the essential factors, genes or proteins, for the processes is very important not only for understanding the mechanisms underlying the above processes at molecular levels, but also for presumptive diagnosis and studying treatments for human infertility. Gene knockout (KO) animals have most effectively contributed to identifying the essential factors in these processes [3, 4]. CRISPR/Cas9 genome editing approaches have now accelerated the *in vivo* screening of essential genes for reproduction [5, 6]. More importantly, genome–editing approaches allow us to dissect the important regions of gene products at the amino-acid level. In addition, human patient-derived mutations can also be examined in live animal models.

This review highlights the last two fertilization processes, sperm–egg fusion and egg activation (**Figure 1; Key Figure**). Striking reports have now been published about these two processes, effectively combining analysis using animal models and other approaches. These articles not only describe the precise functions of the essential factors in the fertilization steps, but suggest the involvement of new factors and concepts for understanding the nature of mammalian fertilization.

Sperm-Egg Fusion and IZUMO1-JUNO Conjugation

Ejaculated eutherian spermatozoa migrate through the female reproductive tract and encounter ovulated eggs in the ampulla of the oviduct [1]. During this process in the mouse, the numbers of spermatozoa decrease dramatically from 10^6 – 10^7 to 10–100. After reaching the ampulla, only spermatozoa that are fully capacitated with sufficient motility can penetrate the surrounding material of eggs, the hyaluronic acid-filled cumulus cell layer and the ZP, and finally fuse with the egg (Figure 1). Thus, sperm–egg fusion is the final goal of spermatozoon's long journey in the female reproductive tract, and is the process that determines which one contributes to the next generation.

IZUMO1 is the only sperm protein proven to be essential for sperm–egg fusion. IZUMO1 was isolated as the antigen of the anti-sperm antibody, OBF13, which inhibits sperm–egg fusion in mice. This type I transmembrane protein is expressed in a testis-specific manner and belongs to the immunoglobulin superfamily. *Izumo1* KO male mice are completely infertile, and their spermatozoa accumulate in the space between the ZP and the egg (perivitelline space; PVS) due to successful penetration of ZP and failure of sperm–egg fusion [7]. Therefore, this protein was named IZUMO after the Japanese Shinto shrine dedicated to marriage. Time-lapse observation of spermatozoa carrying fluorescently tagged IZUMO1 further indicated that IZUMO1 localizes to both the inner and outer acrosomal membranes in fresh spermatozoa and migrates out to the cellular surface between the hybrid outer acrosomal–plasma membrane vesicles formed by the acrosome reaction [8]. This surface exposure of IZUMO1 is consistent with the fact that only acrosome-reacted spermatozoa are capable of fusing with eggs [2].

On the other hand, the receptor for IZUMO1 on the egg plasma membrane was identified by the combination of polymerized IZUMO1 and an iterative expression cloning approach using a mouse egg complementary DNA library [9]. The receptor is a glycosylphosphatidylinositol-anchored protein originally known as folate receptor-like protein 4 (FOLR4) to be a surface marker of regulatory T-cell, but no function in fertilization has been reported. Because receptor gene KO females show complete infertility because of failure of sperm–egg fusion, Bianchi *et al.* renamed the receptor JUNO after the ancient Roman goddess (the official name of the receptor has been changed to IZUMO1R, but we use JUNO in this manuscript to avoid confusion). Interestingly, JUNO disappears from the egg plasma membrane within 40 min after fertilization, suggesting its involvement in the establishment of the block to polyspermy. Moreover, its recognition is suggested to be involved in determining the species specificity of gamete interaction [9, 10].

Both IZUMO1 and JUNO are conserved in eutherian mammals including humans. Despite the suggested 1:1 interaction of IZUMO1 and JUNO [9], domains significant for their interaction have yet to be identified.

Structures of IZUMO1 and JUNO

In 2016, five reports were published as for the crystal structures of IZUMO1 and JUNO [11–15]. Ohto *et al.* and Aydin *et al.* clarified the tertiary structures of human IZUMO1, JUNO, and the IZUMO1–JUNO complex at atomic level resolution (2.0–3.2 Å). At the same time, our group and Han *et al.* solved the crystal structure of mouse JUNO, and Nishimura *et al.* reported the structure of mouse IZUMO1 [12]. Here, we summarize different and common results among these studies, and discuss issues of sperm–egg interactions that need to be resolved.

In previous studies, IZUMO1 was predicted to be a rod-shaped protein consisting of α -helices, an immunoglobulin-like (Ig-like) domain, a transmembrane domain, and a short cytoplasmic tail (N- to C-termini). The N-terminus of the extracellular region, called the IZUMO domain, was suggested to be involved in sperm–egg adhesion by a peptide competition assay [16, 17]. X-ray crystallography of the extracellular part of human IZUMO1 was carried out by two groups [11, 15]. They found that the IZUMO domain consists of a bundle of four α -helices (α -1–4: termed the 4HB domain), whereas the Ig-like domain consists of seven stranded β -sheets (β -3–9). They found a pair of antiparallel β sheets (β -1 and -2) linking the two domains above, termed the hinge region (Figure 2A).

Interestingly, Aydin *et al.*, reported that IZUMO1 has a boomerang-like shape bend near the hinge region as a monomer and a conformational change was observed in the 4HB and hinge regions when bound to JUNO. The 4HB region shifts ~20 Å towards JUNO upon binding, whereas the hinge regions shift by ~8 Å, resulting in a change from a boomerang-like shape to a right-angled or upright conformation. In contrast, Ohto *et al.* reported no conformational change of IZUMO1 upon JUNO binding and the structure remained upright (Figure 2B). This difference in the putative free IZUMO1 structure might have been caused by a difference in pH, as observed for other molecules [18]. Free human IZUMO1 with a boomerang-like conformation was crystallized under a low pH condition (pH 4.6; [15]), whereas free mouse or human IZUMO1 molecules with more upright conformations were

crystallized at pH 5.6–6.6 [12, 14]. This flexibility of the IZUMO1 structure might facilitate IZUMO1–JUNO interaction and play important roles during subsequent fusion events.

The structure of the human/mouse JUNO ectodomain is reported to be globular with no conformational change upon IZUMO1 binding [11, 13–15]. Within JUNO, the cavity corresponding to the folate binding pocket was confirmed to be smaller than for other folate receptors, which is consistent with a report that JUNO cannot bind folate [9].

Interactions between IZUMO1 and JUNO

Structural analysis of the IZUMO1/JUNO complex provides very useful information in terms of their interaction. For IZUMO1 and JUNO, amino acid residues residing at the interacting surface and their interspecies conservation were reported. In IZUMO1, the interactive surface spans all three regions (4HB, hinge, and Ig-like) with the hinge region comprising the largest parts (Figure 2A). In JUNO, the interactive surface consists of a relatively flexible region with hydrophobic residues, which differs from the folate binding pocket of other folate receptors. Specific surface amino acid residues were also reported to be involved in van der Waals interactions and hydrogen bonding between IZUMO1 and JUNO, and these results were mostly consistent for the two reports [11, 15].

The same groups further analyzed amino acid residues affecting the interaction between these molecules to elucidate their significance. These approaches are one of the most beneficial aspects of structural analysis. As a result of their measurement of binding affinities between recombinant IZUMO1 and JUNO carrying specific mutations E71, W148, H157, and R160 of IZUMO1 and E45, W62, L81, and K163 of JUNO were shown to be important for IZUMO1–JUNO interaction. In particular, the mutations in W148 of IZUMO1 or L81 of JUNO were shown to interfere strongly compared with other mutations (Figure 2C) [11, 15].

Ohto *et al.* further expressed various mutant IZUMO1 in Cos-7 cells, coincubated them with mouse eggs, and found that mutations in the hinge region (including W148A, H157A, and R160A) significantly decreased the numbers of cells bound to eggs [11]. These cell-based assays allow assessment of the effect of mutations on cell–cell interactions; however, these assays are heterologous, and no cellular fusion was detected. In contrast, we generated *Juno*-deficient mice and injected various mutant JUNO mRNAs into the KO eggs to assay if these mutant JUNOs rescue the fusion ability of eggs with wild-type (WT) mouse spermatozoa. *Juno* mutants were designed by predicting IZUMO1-binding sites including the central pocket corresponding to the folate-binding pockets of other folate receptors. W62 of JUNO was successfully revealed to be necessary for sperm–egg fusion, indicating that the central pocket is not included in the interactive surface to IZUMO1 [13].

The mRNA complementation assay described above also allows us to assess whether the misrecognition of IZUMO1–JUNO might contribute to human infertility. As human JUNO-complemented *Juno* KO mouse eggs could be fertilized by mouse spermatozoa [13], the impact of ~120 mutations found in human *JUNO* (ExAc; <http://exac.broadinstitute.org/>) can be evaluated in living mouse eggs. Similarly, comparative studies using JUNOs from various species, including the golden hamster whose eggs are capable of fusing with sperm from a

variety of species [19], would allow us to study whether the IZUMO1–JUNO interaction is species-specific and whether misrecognition might establish interspecific reproductive isolation.

Other Factors in sperm-egg Fusion

The molecular mechanisms underlying cell–cell fusion in general are yet to be fully elucidated; however, fusion of two independent cellular membranes is mediated by proteins called fusogens that overcome an energy barrier between two opposing lipid bilayers [20]. Fusogens function bilaterally (e.g., soluble N-ethylmaleimide-sensitive factor attachment protein receptor) or unilaterally (e.g., hemagglutinin HA2 subunit from the influenza virus) by forming homo- or heteroprotein complexes bridging the two membranes. Despite the differences in their functions, fusogens share fundamental properties: (1) their core proteins possess a transmembrane region; and (2) they exhibit conformational transitions between pre- and postfusional events such as conversion of trans- to cisbinding, or a protein bending at $\sim 180^\circ$. These properties are presumably required for them to integrate into plasma membranes and generate a force to pull two membranes close enough to enable fusion [20, 21].

The IZUMO1–JUNO complex does not seem to satisfy the fundamental requirements of fusogens. JUNO is not a transmembrane protein, and the conformational changes of IZUMO1 upon binding to JUNO (Figure 2B) appear insufficient compared with those of known fusogens [21]. IZUMO1 is a transmembrane protein: dimerization of IZUMO1 through its cytoplasmic tail was proposed and the involvement of such dimerization in IZUMO1's conformational changes was suggested [22]. However, removal of the IZUMO1 cytoplasmic tail by CRISPR/Cas9 genome editing revealed that only the ectodomain of IZUMO1 is sufficient for sperm fusion competency [23]. Importantly, Cos-7 cells expressing IZUMO1 did not fuse with eggs [11, 17] and WT spermatozoa did not fuse with HEK293 cells expressing JUNO [13], suggesting that the IZUMO1–JUNO complex function in adhesion rather than in fusion per se. This is consistent with the observation that JUNO molecules are dislocated from the interactive site of eggs and IZUMO1-expressing cells, whereas IZUMO1 remained on the site even after the binding event [22].

These findings raise questions of whether IZUMO1 participates in a fusogenic complex, and if so, what fusogens interact with IZUMO1 on the sperm/egg membranes? Spaca6, another immunoglobulin superfamily protein, was reported from a screening of male-infertile transgenic mouse strains that display fusion failure phenotype [24], but no further studies have been reported. The participation of Spaca6 in sperm–egg fusion and the relationship with IZUMO1 need to be examined in further detailed analysis.

Egg Activation and sperm PLC ζ 1 as a SOAF

In eutherian mammal fertilization, stimulation by a fused spermatozoon triggers the egg to undergo changes in egg cytosolic Ca^{2+} concentrations with repetitive increases and decreases that last for hours, referred to as Ca^{2+} oscillations [25]. During these oscillations, the egg establishes block systems against excess sperm entry (block to polyspermy) and the egg starts to form the male and female pronuclei. In addition, changes in ion levels, large-

scale degradation of maternal mRNAs and proteins, changes in protein phosphorylation, and new protein synthesis commence [26, 27]. These processes are termed collectively egg activation (Figure 1). The number of Ca^{2+} spikes is shown to affect the developmental quality of embryos, as suggested by the correlation of attenuated sperm or egg ability to induce Ca^{2+} oscillations and human infertility [28, 29].

The successful activation of eggs by the sperm head after intracytoplasmic sperm injection (ICSI), and the failure of Ca^{2+} oscillations by the inhibitors for inositol 1,4,5-triphosphate (IP_3) receptor channels implied that a sperm borne oocyte activation factor (SOAF) in the sperm head induces the Ca^{2+} oscillations by increasing IP_3 levels. Although various SOAF candidates have been proposed, they were not shown to be responsible for the oscillations [30–32]. However, Phospholipase C zeta 1 ($\text{PLC}\zeta 1$) was suggested by a finding that injection of *Plcz1* mRNA induced Ca^{2+} oscillations equivalent to that caused by sperm entry [33]. PLC enzymes digest phosphatidylinositol-4,5-bisphosphate into inositol 1,4,5-triphosphate (IP_3) and diacylglycerol, but $\text{PLC}\zeta 1$ is the only one that exhibits activity under Ca^{2+} concentration as low as those found in the egg cytosol (~100 nM) [38]. Moreover, $\text{PLC}\zeta 1$ is a testis-specific protein that localizes to the sperm head cytoplasm where an ideal SOAF candidate could diffuse into the ooplasm after sperm–egg fusion. The reports of *PLCZ1* mutations in infertile patients with egg activation failure support its significance in mammalian egg activation [35, 36].

The *Plcz1* KO mouse was first described to exhibit spermatogenesis failure in a preliminary report at a conference. In contrast, two groups including ours succeeded in generating *Plcz1*deficient or mutant mice using the CRISPR/Cas9 system without affecting spermatogenesis. Hachem *et al.* introduced frameshift-producing small indels in the exons of *Plcz1* [37], while all the exons coding *Plcz1* were removed in our study [38]. The common phenotypes between the two lines include the following: (1) testicular morphology, spermatogenesis, sperm motility, the acrosome reaction, and sperm fusion ability are similar to those of WT mice; (2) the *Plcz1*-deficient spermatozoa fail to induce Ca^{2+} oscillations following ICSI; but, surprisingly, (3) they are capable of activating eggs following *in vivo* fertilization or *in vitro* fertilization (IVF), but with a high incidence of polyspermy and activation failure, which results in subfertility, not infertility.

The discrepancy between the common phenotype (listed as 1 above) and the previous preliminary report is likely caused by artifacts such as truncated *PLCz1* gene expression and/or silencing of neighboring genes in the initial study. *Plcz1* genes in mammalian genome share a bidirectional promoter with the actin filament capping muscle Z-line alpha 3 (*Capza3*) gene that is required for spermatogenesis [39], and point mutations in their promoter region were reported to cause expression failure of both genes [40]. The CRISPR/Cas9 system applied in recent studies allowed us to introduce fine-tuned mutations and/or large deletions to circumvent these problems. Importantly, the common phenotype (listed as 2 above) reveals that $\text{PLC}\zeta 1$ is the long-sought SOAF responsible for inducing the Ca^{2+} oscillations and egg activation following ICSI. However, the common phenotype (listed as 3 above) clearly indicates that $\text{PLC}\zeta 1$ is not the only molecule that can activate mammalian eggs (Figure 3).

PLC ζ 1-independent Activation

Using a low-invasive imaging system for mammalian embryos [41], we visualized the correlation of Ca²⁺ oscillations with the number of fused spermatozoa for each of the eggs fertilized by PLC ζ 1-deficient spermatozoa. Atypical Ca²⁺ oscillations with delayed onset and decreased numbers of spikes were observed for all fertilized eggs, and an incremental relationship between the numbers of fused sperm and that of Ca²⁺ spikes was found with 2.75 ± 0.64 for one spermatozoon vs. 5.00 ± 2.94 for two spermatozoa [38]. A significant difference was also discovered between the numbers of Ca²⁺ spikes in eggs that failed to form pronuclei, and that of the eggs with pronuclei (2.30 ± 0.48 vs. 2.96 ± 0.56), implying that the mouse egg requires more than three spikes to be activated [38]. On the other hand, Hachem *et al.* reported that none of the eggs subjected to IVF with *Plcz1* mutant sperm showed Ca²⁺ oscillations, while 1/40 eggs showed a single Ca²⁺ transient [37]. This apparent discrepancy between the two studies might be explained by the use of different imaging techniques, as well as by the fact that they frozen spermatozoa and B6 strain mouse eggs were used for IVF analysis. It has been suggested that B6 mouse eggs exhibit spontaneous activation at a higher frequency [42] and freezing of spermatozoa might cause the loss of PLC ζ 1-independent egg activation ability.

The newly found PLC ζ 1-independent egg activation mechanism seems to require sperm-egg interaction. It reminds us of the multiple hypotheses for mammalian egg activation proposed before the SOAF theory [43]. In the Ca²⁺ bomb hypothesis, sperm bring a high concentration of Ca²⁺ to cause Ca²⁺-induced Ca²⁺ release in the egg. In the Ca²⁺ conduit model, ion channels in the sperm membrane facilitate extracellular Ca²⁺ uptake after integration of the sperm and egg plasma membranes. In the sperm receptor hypothesis, the binding of sperm surface molecules to specific egg receptors activates membrane-bound egg PLC to produce IP₃ [44]. Species-specific differences should be considered carefully when applying the findings about PLC ζ 1 deficiency to the other mammals, especially to human infertility. However, interestingly, a sperm transient receptor potential-3 channel was recently identified as being important for the sperm Ca²⁺ conduit model in *C. elegans* [45], and a sperm receptor model acting through Src activity has also been reported in *Xenopus* egg activation [46, 47]. The mouse orthologues of these genes and their involvement in PLC ζ 1-independent egg activation can now be examined using *Plcz1* KO mice. The increase in activation efficiency afforded by the numbers of fused spermatozoa is also reminiscent of physiological polyspermy found in reptiles, urodeles, anurans, monotremes, and marsupials [48]. This mechanism may be masked by the presence of PLC ζ 1 in mice, but is consistent with evolutionary aspects of both egg activation mechanisms and establishment of the block to polyspermy.

Another major cause of subfertility of PLC ζ 1-deficient male mice is polyspermy. Thus, the two major mechanisms of blocks to polyspermy, the zona pellucida block (ZPBP) and the plasma membrane block (PMBP), were analyzed. After the establishment of these blocking systems, the ZP or the PM loses the ability to bind and/or fuse with spermatozoa. Both mechanisms need Ca²⁺; the ZPBP in particular is known to be established by the digestion of ZP protein by astacin-like metalloendopeptidase (Astl) and release of the ASTL protein was triggered by the first a few Ca²⁺ spikes [41, 49], but the molecular mechanisms for

establishment of the PMBP have not been elucidated. The onsets of both ZPBP and PMBP were delayed in eggs fertilized with *Plc ζ 1*-deficient spermatozoa [38], and assays using *AstI* KO eggs, which completely lose their ZPBP [49], clearly showed that loss of this mechanism does not cause polyspermy during *in vivo* fertilization in mice [38]. These results indicate that the main cause of polyspermy in *Plcz1* KO sperm is the delay in establishing the PMBP. Simultaneously, a fundamental discovery that the PMBP, rather than the ZPBP, plays critical roles in monospermic fertilization *in vivo* was shown through this study [38]. The significance of ZPBP in IVF was clearly shown by gene KO analysis [49], but this is the first study of the significance of these two mechanisms for fertilization *in vivo*.

Concluding Remarks

As described in this review, the combined use of live imaging with gene-manipulated animals has provided a robust approach to solve unanswered questions in the field of fertilization. The behavior of mCherry-tagged IZUMO1 during the acrosome reaction could be observed in transgenic mouse spermatozoa [8], and the developments of recent low-invasive imaging systems not only enabled the visualization of normal physiological responses of activated eggs [38] but also allowed us to obtain healthy pups after these observations [41]. Furthermore, although it has long been proposed that spermatozoa undergo the acrosome reaction when they interact with the ZP, imaging of fertilizing spermatozoa with fluorescent midpieces and acrosomes [50] showed that most fertilizing spermatozoa undergo the acrosome reaction before this [51]. Recent developments in optical coherence tomography techniques have also enabled us to visualize sperm behavior in the mouse oviduct without introducing any transgene [52].

The development of CRISPR/Cas9 gene-editing tools has broadened the avenues for fertilization research [53]. One can easily generate tens of knockout mouse lines to screen for essential genes involved in fertilization [5]. Further, targeted mutagenesis can also be achieved efficiently by introducing CRISPR/Cas9 as well as template DNA into zygotes (e.g., reporter gene knockin for imaging, nucleotide substitution to assess human mutations, and tag sequence insertion for protein pulldown assay). These genome-edited animals will enable mechanistic insights into how the various factors play roles in fertilization and provide us with animal models for human infertility. Because the CRISPR/Cas9 system can be applied for a wide variety of species, this opens the way for investigating species-specific fertilization mechanisms.

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Highlights

- Recent discoveries of the IZUMO1–JUNO complex structure suggest that its intermolecular bridge is required for sperm–oocyte binding prior to fusion. Juno complementation in oocytes mediated by mRNA provides novel strategies to study the origin of species-specific fertilization mechanisms.
- Gene KO studies in the mouse have revealed that PLC ζ 1 is the long-sought SOAF. A PLC ζ 1-independent mechanism has been elucidated but was inefficient in activating oocytes as well as in establishing blocks to polyspermy. Thus, PLC ζ 1 ensures monospermic fertilization in mice.
- Considering the difficulties in generating fully functional human gametes in vitro, CRISPR/Cas9 genome-edited mouse strains will provide essential platforms to study fertilization mechanisms and develop treatments for human infertility.

Outstanding Questions Box

Are the fusogenic components involved in sperm-egg fusion specific for gametes or ubiquitously used in other cellular functions?

How are the fusogenic components recruited or regulated at the site of sperm-egg fusion on gamete cells?

Is it possible to distinguish IZUMO / JUNO-dependent intercellular junction from other (nonspecific) bindings, even under the condition in which sperms are motile?

In the PLC ζ 1-independent fertilization, although both PMBP and ZPBP delayed as well as the onset of Ca²⁺ spike, does the delayed Ca²⁺ spike directly trigger both block systems?

Since it is known that PMBP is not established on ICSI fertilized egg whereas ZPBP is established, the essential factor for PMBP establishment seems to be activated or introduced through sperm-egg membranous fusion, but does the factor participate in the PLC ζ 1-independent egg activation?

Figure 1. (Key Figure) Mammalian sperm–egg fusion and egg activation processes. Gene deletion analysis revealed that both IZUMO1 on the spermatozoa and JUNO on the egg are required for sperm–egg fusion. PLCζ1 is a sperm-borne oocyte activation factor that induces Ca²⁺ oscillations, subsequent cell cycle resumption, and other postfusion processes such as the blocks to polyspermy.

Figure 2. Interaction between IZUMO1 and JUNO. (A) complex are shown as a ribbon diagram (Protein Data Bank code 5F4E; [15]). Alpha helices or β -sheets in the HB domain, hinge region, and Ig-like domain of IZUMO1 are colored in orange, green, and blue, respectively. Residues shown in (C) are colored in red. (B) Conformational changes in IZUMO1 upon interaction with JUNO. In Ohto *et al.* reported no conformational change in IZUMO1 [11]; on the other hand, Aydin *et al.* reported that IZUMO1 changes its structure from a boomerang-like shape to an upright or right-angled conformation [15]. (C) Significant residues in the interactive surface between IZUMO1 and JUNO suggested from *in vitro* affinity assays using recombinant proteins.

Figure 3. Egg activation mechanisms with or without PLC ζ 1

Without sperm-borne PLC ζ 1 (upper), eggs exhibit atypical Ca²⁺ oscillations (delayed onset and low Ca²⁺ spike frequency) leading to higher incidences of polyspermy and activation failure compared with PLC ζ 1-dependent and robust egg activation (lower). The PLC ζ 1-independent egg activation is observed only following natural fertilization (*in vivo* or *in vitro*) during sperm–egg interactions, but not after ICSI.