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SPERM FACTORS AND EGG ACTIVATION

The phenotype of PLCZ1-deficient mice

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Abstract

In 2002, a report suggested that oocyte activation is induced by *Plcz1* in mouse oocytes, which prompted great interest in exploring the role of sperm PLCZ1. Thus, PLCZ1 loss-of-function experiments became a crucial tool for addressing this subject. Although the only option to completely delete a target protein in fully functional spermatozoa is to use gene-deficient animals, *Plcz1*-deficient mice were not reported until 2017. Challenges to obtain suitable *in vivo* models have been related to altered expression of *Capza3*, a neighbor gene to *Plcz1* locus in mammalian genomes that is required for spermatogenesis. With the advancement of genome-editing technologies, two groups independently and simultaneously produced *Plcz1* mutant mouse lines, which were the first animal models to be artificially and reliably deficient for sperm PLCZ1. All *Plcz1* mutant mouse lines display normal spermatogenesis and, surprisingly, subfertility rather than complete infertility. Moreover, analysis of oocyte Ca^{2+} dynamics indicates that mouse PLCζ1 is an essential sperm-derived oocyte activation factor via intracytoplasmic sperm injection, as PLCZ1 deficiency causes a complete lack of Ca^{2+} oscillations. This seemingly contradictory phenotype can be explained by atypical Ca^{2+} oscillations that are provoked slowly and less frequently in the case of fertilization accompanied by physiological sperm–egg fusion. These findings not only raise new questions concerning the sperm basic biology, by clearly demonstrating the existence of a PLCZ1-independent oocyte activation mechanism in mice, but also have implications for the treatment and phenotypic interpretation of patients presenting oocyte activation failure.
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Introduction

Mammalian oocytes arrested at metaphase II stage while they are spawned into the oviduct and wait for the spermatozoon to resume their cell cycle. This resumption, as part of oocyte activation processes, is triggered by serial increases (and decreases) of cytosolic-free calcium ion, named Ca^{2+} oscillations (Sanders & Swann 2016, Swann & Lai 2016). The success of intracytoplasmic sperm injection (ICSI) accompanied with the Ca^{2+} oscillations in mammalian oocytes suggests the existence of a sperm-borne oocyte activation factor (SOAF) in the sperm head (Kurokawa & Fissore 2003). Moreover, SOAF identification as a proteinaceous factor has been explored by researchers in this field (Perry *et al.* 1999, 2000). Parrington *et al.* also suggested the involvement of glucosamine-6-phosphate isomerase in this process in mice, also called Oscillin in hamsters (Parrington *et al.* 1996); however, Wolny *et al.* denied the involvement of Oscillin in mammalian oocyte activation, as determined by the expression of a recombinant Oscillin homolog (Wolny *et al.* 1999). Moreover, the postacrosomal sheath WW domain-binding protein (PAWP), which is a WBP2 N-terminal-like protein that localizes in the cytosolic portion of the sperm head, has also been suggested to be involved in the mammalian oocyte activation (Wu *et al.* 2007). Nevertheless, PAWP was shown to be dispensable for oocyte activation in mice, as PAWP-null sperm is still able to trigger Ca^{2+} oscillations identical to those of WT sperm and *PAWP* knockout male mice can originate healthy litters (Satouh *et al.* 2015).

As demonstrated in the PAWP case, analyzing cells lacking the gene that encodes the factor of interest can greatly help in understanding how important the factor is. Although significant technological innovations have been made to assess spermatogenesis *in vitro*, no strategies are currently available to produce fully functional and swimming spermatozoa *in vitro* and allow an accurate assessment of sperm proteins (Sato *et al.* 2011, Komeya *et al.* 2016). Therefore, genetically modified animals are currently necessary for investigating the molecular and cellular features of spermatozoa.

In 2002, Saunders *et al.* reported the generation of an almost physiological pattern of oocyte Ca^{2+} oscillations upon *Plcz1* cRNA microinjection, which opened new knowledge avenues regarding the oocyte-activation mechanism by spermatozoon and paved the way for

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the hypothesis that PLCZ1 is a SOAF (Saunders *et al.* 2002). Therefore, obtaining *Plcz1*-deficient animals has been regarded as a key for answering the question, 'do sperm from *Plcz1* deficient animals lose the ability to activate oocytes?' However, for a long time after the seminal report on PLCZ1 role on Ca^{2+} oscillation induction, only one model of *Plcz1*-deficient mice was described in conference proceedings (Ito 2010), and no peer-reviewed report on the subject was published. Nevertheless, the lack of sperm PLCZ1 was observed in mutant animals, such as wobbler mice that exhibit malformed sperm and infertility (Heytens *et al.* 2010). Moreover, almost complete loss of human PLCZ1 was observed in the spermatozoa of infertile men (Yoon *et al.* 2008, Heytens *et al.* 2009, Taylor *et al.* 2010, Chithiwala *et al.* 2015, Yelumalai *et al.* 2015), and changes in the coding region of *PLCZ1*, such as loss-of-function H398P and less functional H233L mutations, were also reported in human patients (Kashir *et al.* 2011, 2012).

Although these experimental approaches strongly suggest that PLCZ1 is essential for oocyte activation, they do not supplant the use of a targeted gene deletion model due to the following reasons. First, given the nature of spermatozoa, heterogeneity in cellular quality may cause a discrepancy between experimental assays and the real phenotype, as minor populations of spermatozoa with superior fertilizing ability may determine the assay outcome. In particular, human spermatozoa have higher heterogeneity than those of murine origin (Sousa *et al.* 2011, Yaniz *et al.* 2016). Secondly, if the type of genetic change is a point or small indel mutation, it is often difficult to guarantee the complete loss of gene expression, and a small amount of residual or truncated protein expression cannot be disregarded. Finally, if *Plcz1* is not specifically targeted, it is difficult to accurately discuss its importance, since unspecific loss of proteins other than PLCZ1 cannot be prevented. It should be noted that what is expected for *Plcz1*-deficient mice is to obtain spermatozoa that were absolutely null for PLCZ1.

Plcz1 locus and spermatogenesis failure

The spermatogenesis phenotype derived from target gene deletion affects significantly the subsequent experimental procedures. If the gene deletion causes complete failure of spermatogenesis, so that no elongated sperm is obtained, the options to analyze the target protein function in mature sperm are largely limited. Moreover, the more the target gene deletion affects the sperm morphology and/or motility the more difficult it becomes to accurately interpret the experimental outcomes using a population of mature live spermatozoa; for example, *in vitro* fertilization (IVF). The common phenotype among the *Plcz1*-edited mouse lines (Hachem *et al.* 2017, Nozawa *et al.* 2018), in which *Plcz1* depletion itself does not cause significant problems on spermatogenesis, allows to analyze PLCZ1-deficient mature spermatozoa through various types of assays.

Before the establishment of the CRISPR/Cas9 technology to produce gene-deficient models (Cong *et al.* 2013, Wang *et al.* 2013), the homologous recombination method using embryonic stem cells was the mainstream method to achieve *in vivo* models of the targeted gene deletion. Noteworthy, a preliminary report in 2010 indicated that *Plcz1* knockout causes spermatogenesis failure (Ito 2010), which is consistent with the previous findings in patients and the wobbler mice phenotype (Heytens *et al.* 2009, 2010, Taylor *et al.* 2010, Chithiwala *et al.* 2015, Yelumalai *et al.* 2015). However, a mutant mouse line with whole exon deletion of *Plcz1* generated using the CRISPR/Cas9 system does not exhibit spermatogenesis failure. In addition, spermatogenesis failure was avoided when point mutations equivalent to those reported in human diseases were introduced (Nozawa *et al.* 2018). In agreement, small indel mutant mouse lines (17 or 22 bp deletions), which were generated by other groups using the CRISPR/Cas9 approach and were carefully examined for the loss of PLCZ1 expression, did not present spermatogenesis failure (Hachem *et al.* 2017). These discrepancies can be explained by the lower rate of unspecific effects on neighbor genes caused by the CRISPR/Cas9 technology.

In the conserved mammalian genomic region in the vicinity of *Plcz1* is located (in the opposite direction, and with a gap of 100 bp in the mouse genome and 19 bp in the human genome) the gene that encodes the capping actin protein of the muscle Z-line subunit alpha 3 (*Capza3*) (Fig. 1). CAPZA3 is specifically expressed in the testis and is located in the post-acrosomal region of the mouse sperm head (Tokuhiro *et al.* 2008). Moreover, it was reported that a single nucleotide missense mutation (T/A transversion changing an ATG (M) to an AAG (K) codon

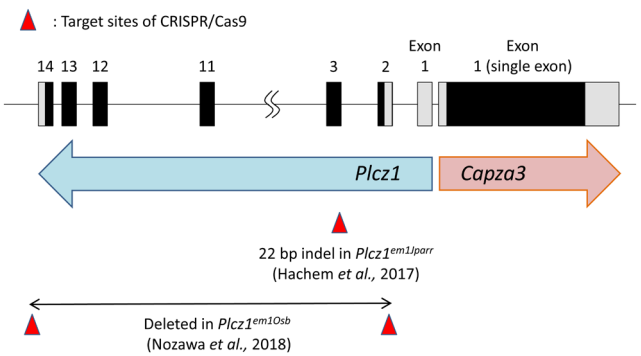

Figure 1 The *Plcz1* gene locus and *Capza3* gene. Red arrowheads indicate targeted sites for CRISPR/Cas9 genome editing. Black or gray solid box indicates coding or non-coding region in exons, respectively.

in the open reading frame) in *Capza3* leads to severe globozoospermia in mice (Geyer *et al.* 2009), contrary to *Plcz1* mutant mice generated with CRISPR/Cas9 technology (Hachem *et al.* 2017, Nozawa *et al.* 2018). Furthermore, *Capza3* expression was reported to be associated to that of *Plcz1* (Javadian-Elyaderani *et al.* 2016). The homologous recombination method often involves embedding vector components in the vicinity of the genomic region of the target gene; thus, it may lead to reduced or absent expression of *Capza3* when targeting *Plcz1*.

There are a number of reports regarding the localization of PLCZ1. In the spermatozoon of WT mouse, it is localized in a perinuclear cytosolic structure near the post-acrosomal sheath, which is close to where CAPZA3 is located. In humans, PLCZ1 was also reported to be localized in the cytosolic portion surrounding the acrosome and equatorial segment (Escoffier *et al.* 2015). Recently, it was reported that the actin-like protein 9 (ACTL9), another actin-related protein that interacts with ACTL7, is located in the vicinity of the post-acrosomal sheath and that its attenuation causes damage to the perinuclear theca, resulting in decreased levels of PLCZ1 (Dai *et al.* 2021). Although the direct interaction of PLCZ1 with CAPZA3 or ACTL9 remains unclear, these observations suggest that PLCZ1 localization in the head cytoplasmic fraction of mature sperm is associated with its actin-based structure, which may contribute to PLCZ1 persistence in mature mammalian spermatozoa. It has also been reported that the development of globozoospermia is correlated with the loss or abnormal expression of *DPY19L2*, and that the severity of the sperm morphological abnormalities correlates with the degree of PLCZ1 loss or abnormal localization (Escoffier *et al.* 2015). Therefore, considering the process of spermatogenesis and the pattern of PLCZ1 localization, it is expected that PLCZ1 localizes close to actin filament structures in the head cytoplasmic fraction of mature sperm during the later stages of spermatogenesis (Pleuger *et al.* 2020). Hence, when sperm are malformed due to reasons other than *Plcz1* mutations, their abundance is reduced via destabilization of actin filament-dependent structures, suggesting that PLCZ1 can be a biomarker of sperm quality.

Importantly, the phenotype of *Plcz1*-deficient mice suggests that abnormalities in the genomic regions near *Plcz1* can cause sperm malformation, and, conversely, sperm malformation (from mutations in other than *Plcz1* locus) can occur first, resulting in PLCZ1 reduction or loss. These two phenomena may occur independently (Fig. 2), a duality that is suggested to underlie the challenges in interpreting the relationship between PLCZ1 abundance and sperm morphology. Moreover, these events may also lead to sperm functional heterogeneity, such as in the case of ICSI treatment of human patients with abnormal sperm morphology.

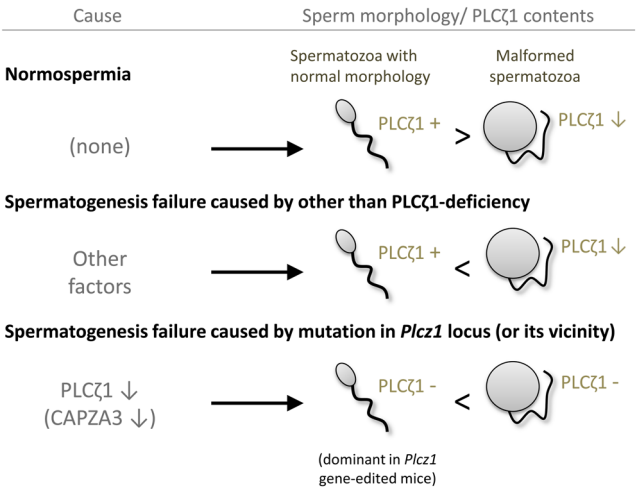

Figure 2 Relationship of spermatogenesis failure and PLCZ1 deficiency. Note that PLCZ1 has an aspect as a sperm quality marker, and that relatively higher heterogeneity exists in human spermatozoa.

Reproductive phenotype of *Plcz1* deleted mice

Two different research groups have generated several mutant mouse lines using the CRISPR/Cas9 system (Hachem *et al.* 2017, Nozawa *et al.* 2018). Among them, homozygous male mice without all the coding exons (exon 2–14) of *Plcz1* (named *Plcz1*^{em1Osb}) and mutant mice harboring a homozygous 22 bp deletion in exon 3 (named *Plcz1*^{em1Jparr}) showed complete loss of PLCZ1 expression (Fig. 1). These male mice showed no problems regarding the testis weight, spermatogenesis, and sperm swimming ability. Moreover, ICSI using their spermatozoon did not result in the formation of pronuclear (PN) in any of the mutants, which clearly demonstrated that PLCZ1 is the SOAF in ICSI. However, surprisingly, these mutant males showed subfertility in crosses with WT females – the detailed litter size was 2.3 ± 0.50 for *Plcz1*^{em1Osb} (heterozygous control: 8.9 ± 0.26) and 4.2 ± 0.6 for *Plcz1*^{em1Jparr} (WT control: 7.8 ± 0.8) (Hachem *et al.* 2017, Nozawa *et al.* 2018). When fertilized oocytes were collected from the oviducts after crossing with these mutant males, polyspermic fertilization was found in about 20% of the oocytes, at, which was a significantly higher rate than that obtained with WT males. In addition, the number of oocytes that exhibited 0PN with no PN but with fertilization cone(s), and 1PN oocytes with only one PN also increased. Although, 1PN is often considered to result from parthenogenetic activation, it was noted that swollen sperm chromosomes united with oocyte chromosomes in the oocyte cytoplasm after IVF using spermatozoa from *Plcz1*^{em1Osb} (unpublished observation by Satouh and Nozawa). IVF with spermatozoa from both *Plcz1*^{em1Osb} and *Plcz1*^{em1Jparr} male mice showed a higher rate of polyspermic fertilization than upon mating (Hachem *et al.* 2017, Nozawa *et al.* 2018), an effect retained even when sperm concentration in the IVF was lowered. The

intracellular Ca^{2+} pattern in ICSI was the same in both mutants, and no Ca^{2+} spike was observed. Noteworthy, no Ca^{2+} spike nor PN was observed upon injection of three sperm heads of *Plcz1*^{em1Osb} spermatozoa (at the same time) or whole sperm (Nozawa *et al.* 2018).

As described above, most of the results from the two mutant models are equivalent, confirming the solidity of the phenotypes of PLCZ1 deficiency in IVF, ICSI, and *in vivo* fertility. Nevertheless, different results regarding the occurrence of Ca^{2+} oscillations in IVF were reported. In particular, all oocytes fused with *Plcz1*^{em1Osb} sperm exhibited Ca^{2+} spikes delayed by several tens of minutes in their onset and were reduced in number compared with those fused with WT spermatozoon (2.75 ± 0.65 spikes generated vs 12.0 ± 5.68 spikes generated) (Fig. 3) (Nozawa *et al.* 2018). Moreover, Hachem *et al.* reported that almost all oocytes inseminated with *Plcz1*^{em1Jparr} spermatozoa fail to generate Ca^{2+} spikes (Hachem *et al.* 2017).

This apparent disparity was solved by the discovery of a positive correlation between the number of Ca^{2+} spikes detected in the atypical Ca^{2+} oscillations and PN formation rate (discussed in the following section), indicating that these atypical Ca^{2+} oscillations have physiological significance. Furthermore, in support of this, the atypical Ca^{2+} oscillation was also observed by Swann and colleagues (reviewed in Swann 2020). It is reasonable to assume that the different interpretations are results of the imaging system used, especially owing to its toxicity and sensitivity. Nozawa *et al.* employed the low-invasive directed system which supports healthy birth of mouse pups even after Ca^{2+} recording for 5 h by using protein-based indicator GECO (genetically-encoded Ca^{2+} indicators for optical imagings) series (Zhao *et al.* 2011), a spinning-disk confocal microscope, and a highly sensitive camera (Satouh *et al.* 2017, Nozawa *et al.* 2018). On the other hand, Hachem *et al.* employed the imaging system using Fura-2 acetoxymethyl ester, a chemical indicator excitable at 340 and 380 nm UV light (Hachem *et al.* 2017). Although it could be argued that differences in sperm condition can also contribute to give the difference between two groups (fresh in Nozawa *et al.* (2018) and cryopreserved in Hachem *et al.* (2017)), identical waveforms were observed using cryopreserved *Plcz1*^{em1Osb} spermatozoa (unpublished observation by Satouh and Nozawa). Therefore, the PLCζ1-independent atypical Ca^{2+} oscillation may be more sensitive to the observation method than the typical Ca^{2+} oscillations and easily disappear.

Findings by applied use of PLCZ1-deficient spermatozoa

The number of the atypical Ca^{2+} oscillations elicited by PLCZ1-deficient spermatozoa in IVF was approximately three and varied among oocytes. The number of Ca^{2+} spikes is suggested to affect the developmental ability of mammalian embryos, as indicated by the correlation between the attenuated number of Ca^{2+} spikes and infertility (Yoon *et al.* 2012, Yeste *et al.* 2016). Indeed, Hoechst staining of oocytes collected from the oviducts of female mice after mating with *Plcz1*-deficient males indicated that the success rate of oocyte activation varies even for oocytes that are fertilized by a single sperm (Nozawa *et al.* 2018). Therefore, it is possible that a single PLCZ1-deficient spermatozoon can elicit either an insufficient or sufficient number of Ca^{2+} spikes required for oocyte activation.

Using the abovementioned observation method that does not interfere with the activation process of embryos while simultaneously recording oocyte intracytoplasmic Ca^{2+}, it was possible to record and retrospectively match the number of Ca^{2+} spikes and fused sperm, as well as the rate and number of PN formed in individual eggs during IVF. Overall, a significant difference in the number of Ca^{2+} spikes was observed between monospermic-fertilized oocytes that were able to form PN and those that failed to do so, and that they were activated by three or more Ca^{2+} spikes (Nozawa *et al.* 2018). This finding resolves the long-debated question of 'how many Ca^{2+} spikes are minimally required to initiate the initial development?' Interestingly, Perry and colleagues

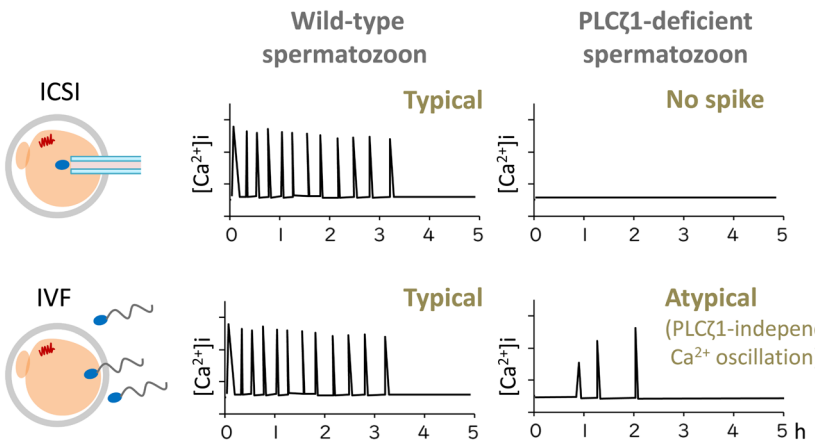

Figure 3 Calcium oscillation outcomes in different fertilization modes. Atypical, or PLCZ1-independent, Ca^{2+} oscillations are evoked only in the IVF and exhibit delayed onset and less number of spikes.

succeeded in achieving very efficient parthenogenesis of mouse oocytes by removing Zn^{2+} using a chelator (Suzuki *et al.* 2010). Zn^{2+} ions in eggs are stored in vesicles just below the plasma membrane (Que *et al.* 2015) and are released synchronously with the first one or two Ca^{2+} spikes when oocytes are artificially activated (Duncan *et al.* 2016). These suggest the importance of the first few spikes to activate the oocyte. However, with respect to the number of spikes that occur in the mouse, which is more than a dozen, Ducibella *et al.* suggested that the involved mechanisms are progressively turned on to ensure not only the resumption of the cell cycle but also the early development and birth of the litter (Ducibella & Fissore 2008); therefore, the importance of the remaining Ca^{2+} spikes (e.g. in the epigenetic state of the progeny) should be analyzed in the future.

Addressing why polyspermy occurs frequently in PLCZ1-independent atypical Ca^{2+} waveforms, focusing on the cases of atypical Ca^{2+} oscillations displaying delayed onset is also important. In particular, the relationship between the two polyspermy block systems (zona pellucida block to polyspermy (ZPBP) and plasma membrane block to polyspermy (PMBP)), and their correlations to Ca^{2+} waveform were explored. *In vitro* analyses suggest that PLCZ1-independent atypical Ca^{2+} waveforms induce retarded establishment of both ZPBP and PMBP, resulting in polyspermic fertilization (Nozawa *et al.* 2018). Using female mice lacking astacin-like metalloendopeptidase (ASTL), which completely lack ZPBP (Burkart *et al.* 2012), showed that all ASTL-deficient oocytes present 2PN as a result of 1:1 fertilization between the oocyte and WT spermatozoon, indicating that the PMBP system can solely ensure monospermy in mouse fertilization (Nozawa *et al.* 2018). Thus, raised frequency of polyspermy in PLCZ1-independent fertilization results from the delay of PMBP establishment, not ZPBP. This result suggests a superiority of PMBP in the establishment of the mammalian polyspermy block and may affect even on the comprehension of *in vivo* fertilization studies indicating that mouse fertilization occurs slowly and in a very small number of gametes (La Spina *et al.* 2016, Muro *et al.* 2016).

Human disease and mutant mice phenotypes

It may be difficult to relate the phenotype of complete loss of PLCZ1, which does not cause infertility in mice, to human symptoms, unless it is carefully ensured that PLCZ1 expression is fully suppressed in humans. Moreover, there are limited approaches to replicate human symptoms in mice. Generation of point mutant mice that harbor similar amino acid substitution to those identified in human patients may be a useful strategy.

Plcz1 mutant mouse lines harboring the H435P mutation, which corresponds to the human H398P variant (Heytens *et al.* 2009), and the D210R mutation, which leads to almost complete loss of *Plcz1* ability to induce Ca^{2+} oscillations (Saunders *et al.* 2002), were generated using the CRISPR/Cas9 system (Nozawa *et al.* 2018). In both point mutants, male mice were phenocopies of the $Plcz1^{em1Osb}$ model, with no spermatogenesis abnormalities. However, PLCZ1 expression was almost completely lost in spermatozoa of the D210R mutant, whereas a low molecular weight protein was detected in the spermatozoa of the H398P mutant, which may be derived from a truncated protein form. The spermatozoa from H398P, even with the truncated form, or that from the D210R mutant, showed complete loss of Ca^{2+} oscillations in ICSI, and atypical and slowly initiated oscillations were produced in IVF, similar to $Plcz1^{em1Osb}$ spermatozoa (Nozawa *et al.* 2018). However, detection of the truncated form by antibodies is an important point to note when testing protein levels using antibodies without considering molecular weight, such as ELISA or immunostaining.

Spermatozoa from all *Plcz1* mutants exhibits high incidence of polyspermic fertilization in IVF (up to approximately 80%) per fertilized oocytes. Thus, H435P and D210R mutant spermatozoa were examined regarding the ICSI outcome along with artificial oocyte activation by *Plcz1* mRNA injection to ensure both monospermic fertilization and reliable oocyte activation. The success of this approach was confirmed when healthy pups were obtained at identical birth rate to that of ICSI using WT oocyte and spermatozoa (Nozawa *et al.* 2018). As for the linkage with sperm malformation, since sperm heterogeneity in morphology is relatively high in humans (Sousa *et al.* 2011, Yaniz *et al.* 2016), it should be particularly considered that PLCZ1 behaves similarly to a sperm quality biomarker. Recently, several reports have examined the correlation between infertility, sperm malformation, and the presence of *PLCZ1* mutations in humans (Escoffier *et al.* 2016, Dai *et al.* 2020, Mu *et al.* 2020, Wang *et al.* 2020, Yan *et al.* 2020, Yuan *et al.* 2020*a*,*b*). Additional details on other *PLCZ1* mutations will be addressed in other sections herein. Nevertheless, the analysis of these pathologies with abnormal sperm morphologies may require attention to the presence of mutations in the neighbor of *PLCZ1*, and the combination of ICSI with artificial oocyte activation is suggested to be effective for investigating PLCZ1 deficiency.

Summary and future perspectives

The PLCZ1-independent, minor, and atypical Ca^{2+} oscillations could not be discovered without the analysis of animals in which PLCZ1 expression is completely prevented. Conversely, as PLCZ1-containing spermatozoon can ensure a sufficient number of spikes in a single sperm clearly highlight that PLCZ1 is an essential SOAF to ensure single sperm fertilization in mammalian fertilization. Noteworthily, investigations using PLCZ1-deficient mice proved that PLCZ1 contents

can be an absolute parameter to predict the success of oocyte activation, especially in the ICSI, which is the most applied treatment in artificial reproductive therapy. Furthermore, the phenotype of PLCZ1-deficient mice, as subfertile rather than fully infertile, also suggests the possibility that human patients with *PLCZ1* mutations are less likely to be detected as potentially infertile. Thus, diagnosis based on *PLCZ1* sequencing may be useful; however, considering that point mutations can exhibit different expression patterns (Nozawa *et al.* 2018), additional analysis strategies may be warranted, such as the use of recombinant proteins to determine the precise effect of each mutation on the oocyte-activating potency of the spermatozoon.

In the PLCZ1-independent mechanism, the number of Ca^{2+} spikes increases with the number of sperm to be fused, and consequently, the PN formation rate is higher when multiple spermatozoa are fused to one oocyte (Nozawa *et al.* 2018). These findings resemble those reported in birds, reptiles, and amphibians, called physiological polyspermy (Iwao 2012). Since PLCZ1-dependent full and typical Ca^{2+} oscillation is obtained in the ICSI using WT mouse sperm heads, at least some soluble content in sperm heads is transmitted during the ICSI. Perry *et al.* suggested that multiple SOAFs exist in the mouse sperm head, as heat-sensitive and -stable factors participating in oocyte activation (Perry *et al.* 2000). However, the Ca^{2+} waveform was completely lost in the ICSI of PLCZ1-deficient spermatozoa even when multiple sperm heads were injected.

In avian oocyte, litters were successfully obtained via ICSI by injecting not only PLCZ1 but also aconitate hydratase and citrate synthase, which may be of mitochondrial origin, implying a synergic effect between these proteins (Mizushima *et al.* 2014). Diffusion of organelle-contained soluble content into the egg cytoplasm by fertilization is expected to take longer than that of the soluble content in the sperm cytoplasm, considering the complexity of the membrane structure and the directionality of degradation. This is also in common with the delayed occurrence of PLCZ1-independent atypical Ca^{2+} waves. However, in mice, this contradicts the no Ca^{2+} spike nor PN formation phenotype obtained by the injection of the whole (with intact tail) PLCZ1-deficient spermatozoon by ICSI (Nozawa *et al.* 2018). Kang *et al.* recently reported that extramitochondrial citrate synthase (eCS) is the second SOAF in mouse spermatozoon (Kang *et al.* 2020). However, the phenotype of eCS-deficient spermatozoon elicits a series of Ca^{2+} oscillations with a similar pattern to that caused by PLCZ1-deficient spermatozoon, whereas eCS deficient spermatozoa retain PLCZ1 content.

Considering that ICSI, compared with IVF or *in vivo* fertilization, promotes the loss of the interaction and fusion between plasma membranes of spermatozoon and oocyte, it is possible that membranous activation factors from the sperm plasma membrane diffuse into the oocyte plasma membrane by sperm–egg fusion, as observed in *Caenorhabditis elegans* (Takayama & Onami 2016). In addition, the Ca^{2+} bomb theory, which was postulated before the SOAF theory dominated, may also be reconsidered (Machaty 2016). *PLCZ1*-deficient mouse spermatozoon introduced in this review is currently the only material that does not generate PLCZ1-dependent Ca^{2+} oscillation, preserving swimming and fusion abilities, and it would be an essential tool for analyzing PLCZ1-independent oocyte activation. Studies using *Plcz1*-deleted mice have addressed various questions in the field of basic biology and assisted reproduction, as well as have provided clues for further questions or enigmas. Elucidation of PLCZ1-related underlying mechanisms, including those that explain these multiple contradictions, remains to be addressed in the future.

Declaration of interest

The author declares that there is no conflict of interest that could be perceived as prejudicing the impartiality of this review.

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