



Essential Role of Sperm-Specific PLC-Zeta in Egg Activation and Male Factor Infertility: An Update

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Sperm-specific phospholipase C zeta (PLC ζ) is widely considered to be the physiological stimulus responsible for generating calcium (Ca²⁺) oscillations that induce egg activation and early embryonic development during mammalian fertilization. In the mammalian testis, PLC ζ expression is detected at spermiogenesis following elongated spermatid differentiation. Sperm-delivered PLC ζ induces Ca²⁺ release via the inositol 1,4,5-trisphosphate (InsP₃) signaling pathway. PLC ζ is the smallest known mammalian PLC isoform identified to date, with the simplest domain organization. However, the distinctive biochemical properties of PLC ζ compared with other PLC isoforms contribute to its unique potency in stimulating cytosolic Ca²⁺ oscillations within mammalian eggs. Moreover, studies describing PLC ζ “knockout” mouse phenotypes confirm the supreme importance of PLC ζ at egg activation and monospermic fertilization in mice. Importantly, a number of clinical reports have highlighted the crucial importance of PLC ζ in human fertilization by associating PLC ζ deficiencies with certain forms of male factor infertility. Herein, we give an update on recent advances that have refined our understanding of how sperm PLC ζ triggers Ca²⁺ oscillations and egg activation in mammals, while also discussing the nature of a potential “alternative” sperm factor. We summarise PLC ζ localization in mammalian sperm, and the direct links observed between defective PLC ζ protein in sperm and documented cases of male infertility. Finally, we postulate how this sperm protein can be used as a potential diagnostic marker, and also as a powerful therapeutic agent for treatment of certain types of male infertility due to egg activation failure or even in more general cases of male subfertility.

Keywords: sperm, phospholipase C zeta, PLC zeta, egg activation, fertilization

SPERM PLC ζ IS THE PRIMARY STIMULUS FOR EGG ACTIVATION AND EARLY EMBRYONIC DEVELOPMENT

In mammalian fertilization, the fertilizing spermatozoon stimulates egg activation, a fundamental event that initiates embryonic development (Nomikos et al., 2017a). It is well established that the most crucial event of egg activation is an acute increase in cytosolic free Ca²⁺ concentrations, which in mammals occurs in the form of long-lasting Ca²⁺ oscillations that commence at or directly following gamete fusion, and persist for several hours beyond meiotic completion (Stricker, 1999; Malcuit et al., 2006; Kashir et al., 2013a). This Ca²⁺ signaling paradigm is essential

for the completion of the multiple events of egg activation. However, previous archetypes of our understanding regarding the distinct events of egg activation and the events controlling them are continuously being unraveled and questioned, with specifics still being investigated. It is, however, clear that Ca^{2+} -release is an integral component of egg activation in all species studied to date (Cran et al., 1988; Swann and Ozil, 1994; Jones, 1998; Nomikos et al., 2012; Limatola et al., 2019b). Over the last few decades, a number of sperm-derived molecules had been proposed as potential soluble sperm factors responsible for the generation of Ca^{2+} oscillations during mammalian fertilization (for more information see Nomikos et al., 2012, 2013a, 2017a). The fact that sperm-induced Ca^{2+} oscillations are caused by activation of the inositol 1,4,5-trisphosphate (InsP_3) signaling pathway (Miyazaki et al., 1992) suggested that the sperm factor might itself be a phospholipase C (PLC) isoform (Jones et al., 1998).

In 2002, a novel testis-specific PLC, termed PLC zeta ($\text{PLC}\zeta$), was discovered (Saunders et al., 2002) and abundant experimental evidence has accumulated over the years suggesting that $\text{PLC}\zeta$ fulfills all prerequisite criteria of the soluble sperm factor responsible for the generation of Ca^{2+} oscillations at mammalian fertilization (Cox et al., 2002; Saunders et al., 2002, 2007; Knott et al., 2005; Kouchi et al., 2005; Nomikos et al., 2005, 2013b, 2017a; Swann et al., 2006; Yu et al., 2008; Kashir et al., 2012a). Upon sperm-egg fusion, $\text{PLC}\zeta$ is proposed to be delivered by the fertilizing sperm into the ooplasm, triggering the Ca^{2+} oscillations via the InsP_3 signaling pathway, through the hydrolysis of its membrane-bound phospholipid substrate, PIP_2 (Saunders et al., 2002; Nomikos, 2015). The importance of this sperm specific protein in mammalian fertilization has been further highlighted by numerous clinical studies directly linking defects or deficiencies in human $\text{PLC}\zeta$ with documented cases of male factor infertility (Yoon et al., 2008; Heytens et al., 2009; Nomikos et al., 2011a, 2017b; Kashir et al., 2012b,c; Escoffier et al., 2016; Torra-Massana et al., 2019).

Intriguingly, two recent independent studies described the phenotype of a $\text{PLC}\zeta$ “knockout” mouse (Hachem et al., 2017; Nozawa et al., 2018). By using multiple transgenic models of $\text{PLC}\zeta$ “knockout” mice generated by CRISPR/Cas methodology, both studies reported that males can produce offspring, albeit with significantly reduced litter numbers (~25%). Interestingly, both studies showed that sperm lacking functional $\text{PLC}\zeta$ protein failed to induce Ca^{2+} release when microinjected into mouse eggs by ICSI. However, *in vitro* fertilisation (IVF) with such sperm, produced atypical and delayed patterns of Ca^{2+} oscillations (lower in number and frequency) with a high degree of polyspermy and activation failure, compared to the robust, physiological pattern triggered by physiological $\text{PLC}\zeta$ -induced egg activation (Nozawa et al., 2018; Satouh and Ikawa, 2018).

Perhaps the atypical and delayed pattern of Ca^{2+} release, observed alongside the low number of embryos and offspring, could be spontaneous activation, unrelated to Ca^{2+} release,

which is common in some strains of mice (Cheng et al., 2012), alongside with the introduction of $\text{PLC}\zeta$ knockout sperm. Indeed, eggs that had been fertilized by knockout sperm also displayed multiple pronuclei, consistent with the inability of a sufficient polyspermy block (Nozawa et al., 2018). Critically, however, eggs fertilized with $\text{PLC}\zeta$ knockout sperm exhibited a total of 3–4 oscillations in total, initiating following a 1-h delay. This was in contrast to normal fertilization where 3–4 oscillations were observed per hour over 3–4 h (Nozawa et al., 2018; Satouh and Ikawa, 2018). Such observations perhaps suggest that sperm containing a second molecule with Ca^{2+} releasing activity, albeit weaker than $\text{PLC}\zeta$ (Jones, 2018). From such results, one could potentially posit that perhaps $\text{PLC}\zeta$ is not an absolute requirement for natural fertilization, and that perhaps an alternative “primitive” or “cryptic” sperm factor may also be involved in leading to egg activation (Nozawa et al., 2018; Satouh and Ikawa, 2018).

It is possible that such a factor could be one of the previously proposed unsuccessful candidates for the “sperm factor,” including tr-kit (Sette et al., 2002), citrate synthase (Harada et al., 2007), or PAWP (Aarabi et al., 2010), which while not contributing to the majority of Ca^{2+} release at oocyte activation, may have a contributory function, especially, in the absence of $\text{PLC}\zeta$. However, it is worth noting that none of the aforementioned proteins is able to elicit Ca^{2+} release in the specific manner required for oocyte activation at physiological levels within sperm (Kashir et al., 2014; Nomikos et al., 2014; Satouh et al., 2015), while none of the alternatively proposed sperm factors (apart from $\text{PLC}\zeta$) has been shown to be directly involved in IP_3 -mediated Ca^{2+} release (Kashir et al., 2014). Furthermore, we cannot exclude the possibility that another sperm-associated enzyme, which might be only able to achieve critical levels due to absence of $\text{PLC}\zeta$ in the sperm of $\text{PLC}\zeta$ knockout mice, might play the role of the “cryptic” factor triggering embryogenesis by a distinct mechanism.

Theories regarding RNA involvement are also questionable since the total amount of $\text{PLC}\zeta$ RNA present within sperm may not be enough to elicit any Ca^{2+} release. On the other hand, this may have been altered as part of genetic compensation.

Intriguingly, starfish eggs pre-injected with heparin (which also blocks InsP_3 receptor function) to disrupt cytoskeletal arrangement were unable to exhibit a rapid Ca^{2+} wave response upon interaction with sperm, instead exhibiting a much more delayed pattern of release, and failed to prevent polyspermy. Furthermore, the amplitude of subsequent Ca^{2+} peaks were reduced, exhibiting an effect similar to observations made with sperm from $\text{PLC}\zeta$ -null mice. In starfish, it was suggested that heparin- or age-induced hyperpolymerization of the cortical actin disrupted actin cytoskeleton dynamics at fertilization influenced Ca^{2+} release (Puppo et al., 2008; Santella et al., 2015; Limatola et al., 2019a), potentially also impacting upon subsequent events in egg activation such as cortical granule exocytosis. It is thus possible that due to the lack of a sufficient response at fertilization due to deficient/absent $\text{PLC}\zeta$, a similar effect was observed in the $\text{PLC}\zeta$ -null mice, where similar symptoms of insufficient Ca^{2+} release and increased polyspermy were also observed. Indeed, it may be the case that the low number and frequency

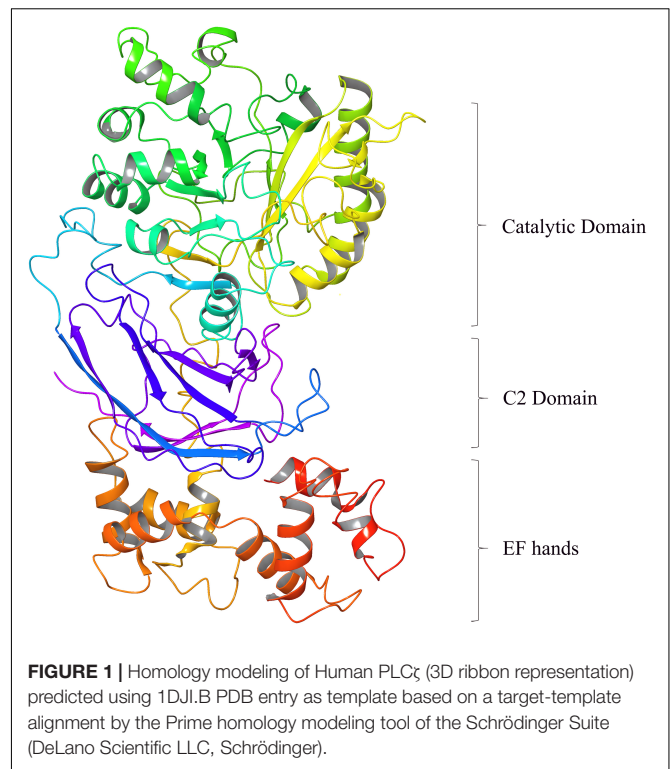
Abbreviations: ART, assisted reproductive technology; Ca^{2+} , calcium; ICSI, intracytoplasmic sperm injection; InsP_3 , inositol 1,4,5-trisphosphate; $\text{PI}(3)\text{P}$, phosphatidylinositol 3-phosphate; $\text{PI}(5\text{P})$, phosphatidylinositol 5-phosphate; PIP_2 , phosphatidylinositol 4,5-bisphosphate; $\text{PLC}\zeta$, phospholipase C zeta.

of Ca^{2+} observed could be due to events surrounding actin polymerization or associated InsP_3 -independent events of Ca^{2+} release (such as influx). However, these mechanisms are poorly understood in mammals, and require further investigation to fully ascertain.

It is clear that both Hachem et al. (2017) and Nozawa et al. (2018) represent keystone studies that support the notion that PLC ζ is the primary physiological stimulus that triggers the required specific pattern of Ca^{2+} oscillations, ensuring monospermy and eventually successful egg activation and early embryonic development (Hachem et al., 2017; Nozawa et al., 2018; Swann, 2018). Moreover, the presence of an alternative factor in other species and especially in humans is still questionable, particularly taking into consideration all the documented cases of male factor infertility due to PLC ζ deficiencies. However, this is an intriguing area of investigation that ongoing studies are now aiming to address. It would be interesting to examine how the increasing body of invertebrate animal work will direct the mammalian side of the coin in the future, particularly with relation to the early influence exerted by the egg actin cytoskeleton upon patterns of Ca^{2+} release and fertilization as is being unraveled in starfish. Furthermore, integral studies are required in particularly livestock mammalian models to demonstrate whether PLC ζ -loss resembles the mouse and/or human scenarios. Perhaps of particular interest should be attempts to generate transgenic knockout models of PLC ζ in porcine or bovine systems. While this would of course be considerably harder to perform than in the mouse, such data would undoubtedly assist in ascertaining the validity of “cryptic factor” theories. Further experiments that would be prudent would be to examine the specific timing of the reduced profiles of Ca^{2+} release in relation to PLC ζ knockout sperm-egg fusion and fertilization. Are such reduced frequency and amplitude oscillations due to fusion of a single sperm, or the cumulative effect of multiple sperm-egg fusion events? It is necessary that such experiments are performed to ascertain fully the conflicting data generated from knockout studies thus far.

SPERM PLC ζ STRUCTURE AND DOMAIN ORGANIZATION

Phospholipase C zeta is currently the smallest known mammalian PLC isoform (~70–75 kDa in size) with the most elementary domain organization (Cox et al., 2002; Saunders et al., 2002; Nomikos et al., 2013a, 2017a). Despite this, PLC ζ exhibits uniquely supreme potency in triggering Ca^{2+} oscillations within the fertilizing egg compared to other somatic PLCs. This is attributed to its novel biochemical characteristics, arising from the essential role of its domains that contribute to the unique biological function and mode of regulation of this distinctive PLC isozyme (Nomikos, 2015; Nomikos et al., 2017a). PLC ζ domain structure consists of four tandem EF hand domains at the N-terminus, the catalytic X and Y domain in the center of the molecule, followed by a single C2 domain at the C-terminus (Figure 1; Nomikos et al., 2017a). All these domains are common to other PLC isoforms. The X and Y catalytic domains are



separated by a short segment, the XY-linker, which through its net positive charge plays an important role in targeting PLC ζ to intracellular membranes by direct electrostatic interactions with its negatively charged substrate, PIP_2 (Nomikos et al., 2007, 2011b). The XY-linker region differs considerably between PLC isozymes (Nomikos et al., 2013a). By contrast, the XY catalytic domain between PLC isoforms is the most highly conserved region (Nomikos, 2015).

The XY domain of PLC ζ shares ~60% sequence similarity to that of all PLCs and is responsible for PIP_2 hydrolysis (Nomikos et al., 2005). The EF hands are Ca^{2+} -binding motifs and in PLC ζ these domains play a vital role in its high Ca^{2+} sensitivity compared with the other somatic PLCs, allowing PLC ζ to be active at resting Ca^{2+} levels within the egg cytosol, when PLC ζ enters after sperm-egg fusion (Nomikos et al., 2005). Additionally, we have demonstrated that the first EF-hand domain of PLC ζ , which contains a cluster of basic amino acid residues, plays an essential role together with the XY-linker region, in the interaction of PLC ζ with the PIP_2 -containing membranes (Nomikos et al., 2015b). The C-terminal C2 domain of PLC ζ , comprising ~120 amino acid residues is essential for PLC ζ function, as targeted deletion or replacement of this domain by the corresponding domain from PLC $\delta 1$ abolishes the Ca^{2+} -oscillation-inducing activity of PLC ζ in eggs, without altering its enzymatic activity or Ca^{2+} sensitivity (Nomikos et al., 2005; Theodoridou et al., 2013). We have provided biochemical evidence that this domain directly interacts with the membrane phospholipids, $\text{PI}(3)\text{P}$, and $\text{PI}(5)\text{P}$ and have suggested that C2 association with these phospholipids may facilitate in

the membrane targeting of PLC ζ (Theodoridou et al., 2013; Nomikos, 2015).

PLC ζ IN MAMMALIAN SPERM

Phospholipase C zeta mRNA has been identified during both early and late stages of spermatogenesis in mice and pigs (Yoneda et al., 2006; Young et al., 2009; Bedford-Guaus et al., 2011; Kaewmala et al., 2012). Specific localization patterns, however, remain elusive in the literature throughout the various spermatogenic cells within the testes (Kashir et al., 2018). Aarabi et al. (2012) indicated that PLC ζ is integrated as part of the acrosome during the Golgi phase of human and mouse spermiogenesis (Aarabi et al., 2012), suggesting that observable PLC ζ levels are diminished gradually throughout spermatid elongation (Kashir et al., 2018). PLC ζ was originally identified in mouse sperm extract fractions that were able to induce Ca²⁺ release. Subsequent immunofluorescence analysis indicated a post-acrosomal localization for PLC ζ ; a component of the post-acrosomal sheath (Saunders et al., 2002; Fujimoto et al., 2004; Young et al., 2009). Nevertheless, PLC ζ has been identified in the sperm of various mammalian species, and usually tends to be found within the sperm head in distinct subcellular regions, postulating differential functional roles for each population (Amdani et al., 2013; Kashir et al., 2014, 2018).

While in mouse and porcine sperm, PLC ζ has been observed mainly at acrosomal and post-acrosomal regions (Fujimoto et al., 2004; Young et al., 2009; Nakai et al., 2011; Kaewmala et al., 2012), in equine sperm, PLC ζ was recorded at the acrosome, equatorial segment, and head mid-piece, as well as the principle piece of the flagellum (Bedford-Guaus et al., 2011; Kashir et al., 2018). Several PLC ζ populations were observed in humans in multiple studies including the acrosomal, equatorial and post-acrosomal regions of the sperm head, with a potential tail localization (Grasa et al., 2008; Yoon et al., 2008; Young et al., 2009; Kashir et al., 2013b, 2018; Escoffier et al., 2015; Yelumalai et al., 2015; Yeste et al., 2016). While there is consensus regarding PLC ζ localization in mouse sperm, the veracity of the multiple populations identified in other mammalian sperm (particularly in humans) remains debated (Nomikos et al., 2017a; Kashir et al., 2018). In human sperm, this variation in PLC ζ localization is not only limited to observations between different studies but substantial variability in the PLC ζ localization pattern was found even within the same study (Kashir et al., 2013b).

Despite numerous efforts to examine PLC ζ localization within mammalian sperm, significant concern surrounds the specificity of the majority of antibodies used to date. More specifically, most antibodies used in the literature are unable to demonstrate a consistent motif of recognizing a single band following immunoblotting of human sperm, often detecting multiple protein bands other than, or in addition to, that of the expected size for native PLC ζ protein. Compounded by this non-specificity, multiple groups have identified varying populations between mouse and human sperm, even using the same antibodies, suggesting that varying protocols and the use of different antibodies are the main source of inconsistent results

between studies (Grasa et al., 2008; Yoon et al., 2008; Heytens et al., 2009; Kashir et al., 2011a,b, 2013b; Aarabi et al., 2012).

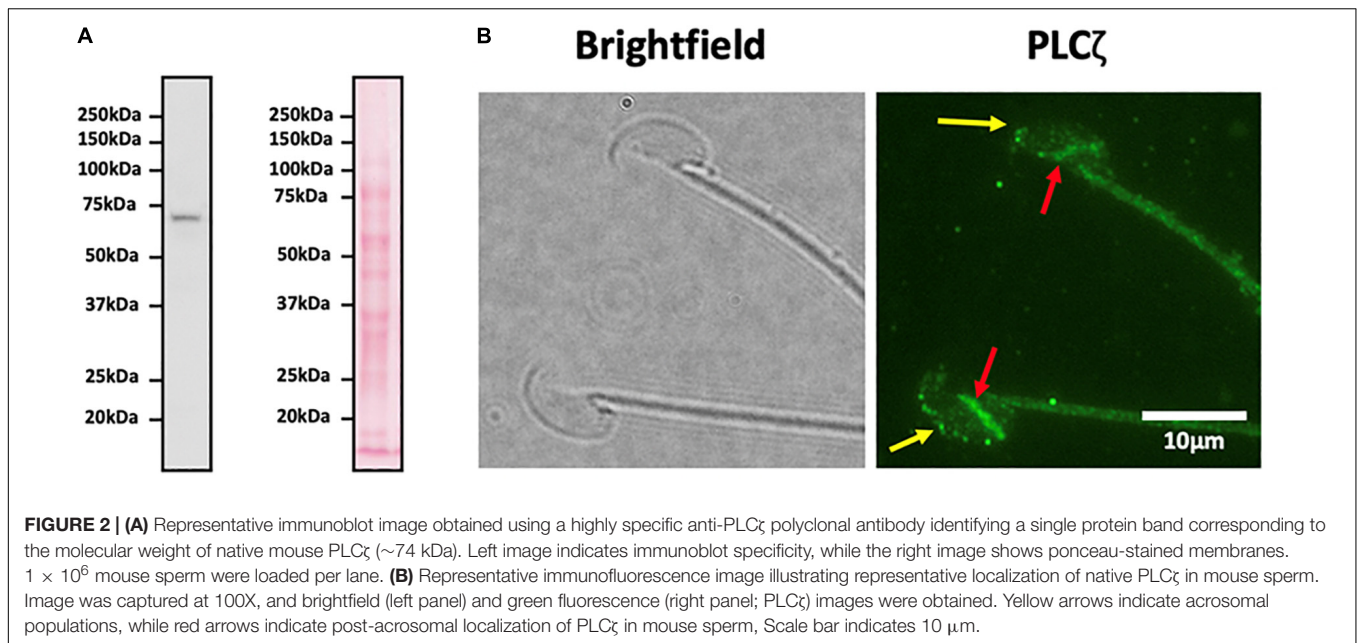
Addressing such concerns, we recently generated highly epitope-specific PLC ζ polyclonal antibodies against human, mouse, and porcine PLC ζ , that exhibit high consistency throughout numerous studies for both recombinant and native PLC ζ (Nomikos et al., 2013b, 2014, 2015a; Theodoridou et al., 2013). Furthermore, we have also developed specific antigen unmasking/retrieval protocols, which we previously demonstrated are essential to enhance the visualization efficacy of PLC ζ in mammalian sperm (Kashir et al., 2017). Using these enhanced protocols and materials, we have identified PLC ζ in the acrosomal and post-acrosomal, acrosomal and equatorial, and post-acrosomal and equatorial compartments of mouse, human, and porcine sperm, respectively. Furthermore, we have also consistently observed potential tail localization in all species (Kashir et al., 2017). **Figure 2** demonstrates the expression and distribution of PLC ζ in mouse sperm using our specific polyclonal antibodies and our recently developed and reported protocols (Kashir et al., 2017). It is now imperative that these specific antibodies and protocols are applied in a systematic manner to examine whether particular localization patterns or profiles of PLC ζ exhibit any relationships between male fertility parameters, or indeed between fertility treatment outcomes.

Another intriguing question is how PLC ζ , despite its high Ca²⁺-sensitivity and its potent enzymatic activity, is kept in an inactive state within the sperm, especially when it is likely to be present in much higher concentrations in a single spermatozoon than within the fertilizing egg. Indeed, our previous work where it was shown that PLC ζ is inactive in somatic cells even at levels over 1000 times that at which it is active in eggs (Phillips et al., 2011), suggests that either PLC ζ has an essential binding-partner within the egg, or that other factors within sperm and somatic cells may inhibit its catalytic activity.

REDUCED EXPRESSION LEVELS AND ABNORMAL FORMS OF SPERM PLC ζ LEAD TO MALE INFERTILITY

Infertility is estimated to affect ~15% of couples, with male infertility affecting ~7% of men worldwide (Kashir et al., 2010). While genetic causes of male infertility are estimated to underlie ~30% of such cases (Harton and Tempest, 2012; Jungwirth et al., 2012; Hotaling, 2014), ~50% of cases of male infertility remain unexplained (Kashir et al., 2018). While most forms of infertility can now be treated via a collection of laboratory techniques collectively termed ART, a number of conditions such as severe male infertility (19–57% of cases) cannot yet be treated (Botezatu et al., 2014). Despite the fact that powerful ART methods such as IVF or ICSI can successfully treat some infertility cases, it is concerning that this is achieved only after several fertility treatment cycles. A significant causative factor may be recurrent implantation failure, which even after fertility treatment leads to infertility (Polanski et al., 2014; Kashir et al., 2018).

Considering the indispensable contribution of PLC ζ to fertilization, defects in either egg activation, or in PLC ζ



protein itself, may underlie conditions of male infertility where fertilization failure occurs. The first evidence came from studies that reported sperm of infertile men, which consistently failed to fertilize eggs following routine IVF or ICSI, and were either unable to induce Ca^{2+} release upon microinjection into mouse eggs, or produced highly abnormal Ca^{2+} transients which were reduced in frequency and amplitude (Yoon et al., 2008; Heytens et al., 2009). Furthermore, such sperm also exhibited reduced or absent levels, as well as abnormal localization patterns, of PLC ζ within the sperm head (Heytens et al., 2009; Kashir et al., 2011a,b, 2013b), suggesting that deficiencies in PLC ζ protein may underlie currently unknown cases of male factor infertility. In a clinical scenario, in contrast to other causes, complete fertilization failure is attributed to egg activation failure in a species-specific manner (Kashir et al., 2010, 2018).

Moreover, PLC ζ gene abrogation in patients diagnosed with egg activation deficiency is now increasingly being reported within the scientific literature. The first two PLC ζ mutations were identified in the gene of an infertile male, whose sperm was unable to trigger the normal pattern of Ca^{2+} oscillations, leading to egg activation failure and potentially to his infertility (Heytens et al., 2009; Kashir et al., 2011b, 2012b,c). Both mutations were reported within the active catalytic site domains of PLC ζ (X and Y), disrupting local protein structural folding to cause reduction of enzymatic activity, subsequently leading to highly abnormal Ca^{2+} transients unable to initiate egg activation (Kashir et al., 2011b). Both mutations were reported to be heterozygous, with one mutation being inherited from the patient's father and the other from the patient's mother, indicating for the first time that such maternally inherited loss-of-activity mutations can lead to male infertility (Kashir et al., 2012b,c; Nomikos et al., 2017a). Subsequently, a further mutation in homozygosis was later reported by Escoffier et al. (2016) from two infertile brothers. This mutation is located within the

C2 domain of PLC ζ (Escoffier et al., 2016). Intriguingly, this PLC ζ mutant displayed similar enzymatic activity to wild type PLC ζ , but displayed a dramatically reduced relative binding-affinity to PI(3)P and PI(5)P-containing liposomes (Nomikos et al., 2017a). More importantly, this genetic report by Escoffier et al. (2016) and the identification of this novel missense homozygous PLC ζ mutation in these infertile brothers after whole exomic sequencing, strongly indicates that absence or defects in PLC ζ protein alone is sufficient to prevent human egg activation by the sperm, suggesting that PLC ζ is essential for human egg activation and thus human fertilization.

Furthermore, single nucleotide polymorphisms (SNPs) have also been reported by Yoon et al. (2008) and more recently by Ferrer-Vaquero et al. (2016), either within the PLC ζ coding sequence or its associated bi-directional promoter in human patients (Nomikos et al., 2017a).

Interestingly, Torra-Massana et al. (2019) very recently reported six new PLC ζ mutations after screening an egg activation deficiency group, one of which was previously described (Kashir et al., 2012b,c), in addition to four novel single-nucleotide missense mutations, located in the EF-hands, the X catalytic and C2 domains; while the sixth mutation identified was a frameshift variant, which was predicted to generate a truncated protein at the X-Y linker region (Torra-Massana et al., 2019). While further analysis indicated a potential deleterious effect of some of these mutations upon PLC ζ activity within eggs, further biochemical analysis is required to ascertain whether such variants of PLC ζ are deleterious as claimed at physiological levels, needing their accurate quantification in sperm and eggs. However, it is now clear that deleterious mutations in PLC ζ may be more widespread than previously thought, appearing not only in the catalytic active site, but also in other vital regulatory regions of this essential sperm protein, afflicting its membrane and/or substrate binding, its Ca^{2+} sensitivity, as well as its enzymatic activity.

Despite the relatively high success rate of ICSI in overcoming cases of failed fertilization after IVF, ~30% of such cases still repeatedly fail ICSI (Flaherty et al., 1998; van der Westerlaken et al., 2005). Low global pregnancy success rates following ART have been attributed to poor embryogenesis following fertility treatment (Fauque et al., 2007; Pelinck et al., 2010). Importantly, such poor embryonic competency can be directly linked to PLC ζ and competency of egg activation (Nomikos et al., 2017a; Kashir et al., 2018). Injection of increasing levels of PLC ζ in human eggs results in increasing frequencies and amplitudes of Ca²⁺ oscillations (Yamaguchi et al., 2017), which in turn affects subsequent gene expression (Ducibella et al., 2002, 2006). Furthermore, the frequency and amplitude of Ca²⁺ oscillations has been shown to play an important role in compaction, and blastocyst formation (Swann and Ozil, 1994; Miyazaki and Ito, 2006). Finally, taking into consideration that the rate of progression to the 2- and 4-cell stages of human eggs following fertilization has been suggested as an indicator of normal embryogenesis (Wong et al., 2010), PLC ζ -driven Ca²⁺ oscillations may not only be required for egg activation, but can also be equally important for subsequent embryogenesis. Thus, abnormalities in sperm PLC ζ levels may underlie not only infertility through fertilization failure, but also cases of male subfertility, whereby enough PLC ζ may be delivered into the eggs to trigger activation, but prove insufficient for embryonic competence.

CLINICAL APPLICATIONS OF PLC ζ AND FUTURE DIRECTIONS

Currently, cases of defective egg activation are clinically resolved using assisted egg activation (AOA), involving artificially mediated Ca²⁺ release (Santella and Dale, 2015; Kashir et al., 2018). Ca²⁺ ionophores like ionomycin, calcimycin are currently used to overcome unexplained fertilization failure in couples, who repeatedly fail ICSI cycles (Fawzy et al., 2018; Norozi-Hafshejani et al., 2018). Recently, a meta-analysis indicated that use of Ca²⁺ ionophores significantly improved fertilization and implantation rates in ICSI (Murugesu et al., 2017). However, Ca²⁺ ionophores induce a single Ca²⁺ transient, unlike the endogenous specific physiological pattern of Ca²⁺ oscillations observed during normal fertilization (Rinaudo et al., 1997). In fact, microinjection of human recombinant PLC ζ yielded higher blastocyst development rates than Ca²⁺ ionophore treatment (Sanusi et al., 2015). Thus, PLC ζ has long represented a physiologically endogenous alternative method to clinically treat cases of egg activation failure/deficiency, which would involve the *in vitro* production of active, purified versions of human recombinant PLC ζ protein. Indeed, following initial difficulties, multiple studies made advancements in the production of such a desired product (Kashir et al., 2011b; Yoon et al., 2012), culminating in efforts by Nomikos et al. (2013b) who made a significant breakthrough by generating purified, highly active recombinant PLC ζ , capable of inducing physiological patterns of Ca²⁺ oscillations following microinjection into mouse and human eggs (Nomikos et al., 2013b). More importantly,

recombinant PLC ζ was able to effectively rescue failed egg activation in a prototype of male infertility (Nomikos et al., 2013b). Further attempts to develop the use of recombinant PLC ζ protein as a therapeutic agent in a clinical setting are currently in progress, in order to eliminate any potential cytotoxic effects during embryonic development and confirm the overall safety of exogenous PLC ζ on the subsequent offspring.

Finally, PLC ζ not only represents a promising clinical therapeutic agent, but also a potentially powerful diagnostic biomarker, which may help in determining the criteria and requirements of the fertility treatment of male patients, significantly decreasing the number of cycles needed for a successful pregnancy to occur. Taking into consideration that PLC ζ analysis might be beneficial to identify not only cases of ICSI-failure but also cases of male subfertility, a simple immunocytological approach to routinely examine PLC ζ protein in sperm is currently widely regarded as a cost-effective approach, which could be easily applied by the majority of IVF clinics worldwide. However, it is essential that studies focus on the reliable investigation of PLC ζ parameters in relation to sperm health, using robust protocols and ultra-specific antibodies, before such clinical promise can be achieved. Indeed, significant issues remain regarding such analyses, particularly in humans, where PLC ζ antibodies used recognize multiple protein bands in addition to the full-length PLC ζ protein. The same antibodies have also been used to identify different immunoblotting profiles (for detailed review see Kashir et al., 2018). Predictably, such shortcomings have led to conflicting results between the association of specific PLC ζ localization patterns and quantification levels.

ETHICS STATEMENT

Use of mouse sperm cells was carried out in accordance with the principles of the Basel Declaration and recommendations of the Animal Care and Use Committee (ACUC) at the Office of Research Affairs (ORA) at the King Faisal Specialist Hospital and Research Center, Riyadh, Saudi Arabia. The protocols utilized for the relevant study (RAC-216004) were approved by the ACUC.

AUTHOR CONTRIBUTIONS

AS, JK, AT, and MN prepared the first draft of the manuscript, which was revised and approved by all authors.

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Conflict of Interest: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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