Birth of Normal Offspring from Mouse Eggs Activated by a Phospholipase Cζ Protein Lacking Three EF-hand Domains

Tomoko NAKANISHI1), Naoko ISHIBASHI1), Haruka KUBOTA1), Kimiko INOUE2), Narumi OGONUKI2), Atsuo OGURA2), Shin-ichi KASHIWABARA3) and Tadashi BABA1)

1)Graduate School of Life and Environmental Sciences, University of Tsukuba, Tsukuba 305-8572 and 2)Institute of Physical and Chemical Research (RIKEN), Bioresource Center, Tsukuba 305-0074, Japan
3)Present: Department of Molecular Biology, School of Life Science, Faculty of Medicine, Tottori University, Tottori 683-0853, Japan

Abstract. Sperm-specific phospholipase C, PLCζ, is a candidate for the Ca2+ oscillation-inducing factor that is introduced into the ooplasm upon sperm-egg fusion. In addition to the 647-residue full-length PLCζ, s-PLCζ lacking the N-terminal 110 amino acids is known to be present in the mouse testis. In this study, we attempted to obtain full-term offspring from s-PLCζ-activated eggs by round spermatid injection. Metaphase II-arrested eggs injected with a high RNA concentration of s-PLCζ RNA normally developed to blastocysts. When the round spermatid nucleus was injected into telophase II-stage eggs previously activated by s-PLCζ RNA, three live offspring were successfully obtained by transfer of the developed 4-cell embryos to pseudopregnant mice. These three offspring all grew to be normal adults and reproduced healthy second-generation mice.

Keywords: Ca2+ oscillations, Egg activation, Mouse, Round spermatid injection (ROSI), Sperm-specific phospholipase C (PLCζ)

In addition to intracytoplasmic sperm injection as a therapeutic intervention for male sterility [1, 2], round spermatid injection (ROSI) [3] is developing as another treatment for patients who have defects in spermatogenesis. In the mouse, the nuclei of round spermatids (1n haploid), secondary spermatocytes (2n haploid) and primary spermatocytes (4n haploid) all possess the ability to achieve fertilization and subsequent development of embryos into normal offspring [4, 5]. However, spermatogenic cells alone are incapable of activating eggs due to the lack of repetitive transient rises in intracellular Ca2+ concentration (Ca2+ oscillations) [6, 7], which is a prerequisite for fertilization. Various methods have been employed for artificial induction of Ca2+ oscillations in metaphase II-arrested eggs in vitro. An electrical pulse and strontium are typically used to induce single and multiple Ca2+ elevations, respectively [8–10]. Injection of the spermatogenic cell nucleus into eggs is recommended at the telophase II stage, no later than 1 h after the activating treatment, in order to obtain high developmental efficiency [11, 12]. A nonmetabolizable agonist of the inositol 1,4,5-triphosphate receptor, adenophostin, and a partially purified preparation of sperm extracts are also known to be useful for embryonic development of ROSI eggs [6, 10, 13]. Although these may be credible methods for inducing Ca2+ oscillations, the success rate for offspring production following ROSI is significantly lower than that provided by sperm.

Twelve isoforms (β1–4, γ1–2, δ1–4, ε and ζ) of phospholipase C, PLC, have been identified to date. Of these isoforms, 8 proteins, including PLCζ, are present in mouse sperm [14–16]. PLCζ has been identified as a candidate for the sperm factor in mouse, human and cynomolgus monkey, and has been shown to induce Ca2+ oscillations similar to those at fertilization and to develop diploid parthenotes to blastocysts [16–21]. The Ca2+ oscillation-inducing activity is lost by immunodepletion of PLCζ in sperm extracts [16], and transgenic RNA interference of PLCζ results in a low rate of egg activation [22]. Moreover, PLCζ containing the consensus nuclear localization signal between the X and Y domains accumulates in the pronuclei [23–26], which is consistent with the Ca2+ oscillation-inducing activity derived from sperm being localized in the pronuclei of fertilized eggs [7, 27, 28]. It is thus likely that PLCζ acts as the sperm factor that triggers the Ca2+ oscillations required for egg activation. However, there is a paucity of research that has investigated whether the Ca2+ oscillations induced by PLCζ result in egg activation leading to normal embryonic development and offspring.

Several isoforms of PLCζ, including s-PLCζ lacking the three EF-hand domains at the N-terminus, have been reported to be expressed in the mouse testis, presumably owing to alternative RNA splicing [26, 29–31]. Recombinant s-PLCζ exhibits phosphatidylinositol 4, 5-bisphosphate-hydrolyzing activity, but the Ca2+ oscillation-inducing activity is extremely weak, implying that the EF-hand domains of PLCζ are important for induction of Ca2+ oscillations [30, 32].

Artificially recreating the events of egg activation has become an important component of assisted reproduction technology. In this study, we reexamined whether PLCζ or s-PLCζ is applicable to activation of metaphase II-arrested mouse eggs and supports subsequent embryo development. Taking advantage of the delayed chromosome segregation in eggs activated by s-PLCζ, the nucleus of round spermatids was injected into telophase II-stage...
eggs. Three healthy offspring were born by transfer of 4-cell embryos developed from the ROSI eggs into pseudopregnant mice. Thus, s-PLCζ RNA injection is useful for embryonic development of ROSI eggs.

**Materials and Methods**

**Polymerase chain reaction (PCR)**

DNA fragments encoding the entire and N-terminal 231-residue protein-coding regions of mouse PLCζ were amplified by PCR from a ddY mouse testis cDNA library using PLCZ4/PLCZ3 and PLCZ4/PLCZ10 oligonucleotide primer sets (Fig. 1A), respectively: 5’-CGAAATTCGCCACCATGGAAAGCCAACCTTAC-TGAG-3′ for PLCZ4, 5’-GGTCTAGACTCTTAGGAATGCAC-CAAAACAT-3′ for PLCZ3 and 5’-AGAGAAGCTTGCTGGTG-GTAAT-3′ for PLCZ10. Reactions were performed in a 50-μl mixture containing 10 mM Tris/HCl, pH 8.8; 50 mM KCl; 1.5 mM MgCl2; 0.1% Triton X-100; 0.2 mM each of dATP, dCTP, dGTP and dTTP; 1 μM of each primer; template cDNA and 5 units of Taq DNA polymerase (Nippon Gene, Toyama, Japan). The reaction program consisted of 35 cycles at 94°C for 60 sec, 60°C for 60 sec and 72°C for 120 sec. The PCR products were then introdced into pcDNA3.1/Myc-His A (Invitrogen, Carlsbad, CA, USA) or pBlue-script II SK vectors. Nucleotide sequencing was carried out using an ABI Prism 310 genetic analyzer.

**Antibody preparation**

A DNA fragment encoding a 149-residue sequence of PLCζ at positions 271–419 was PCR-amplified, introduced into a pET23d vector (Novagen, Madison, WI, USA), and expressed in *Escherichia coli* BL21 (DE3). The recombinant protein containing a His tag at the C-terminus was purified on an Ni-NTA His column at the C-terminus was purified on an Ni-NTA His column. The resulting protein-coding regions of mouse PLCζ were amplified by cutting with PmeI. RNA was synthesized by T7 polymerase using a RiboMAX Large Scale RNA Production Systems-T7 kit (Promega, Madison, WI, USA), treated with phenol-chloroform, precipitated with ethanol and dissolved in water.

**Egg collection and microinjection**

Metaphase II-arrested eggs were collected from the oviducts of 8-week-old ICR or B6D2F1 mice (Japan SLC) that had been super-ovulated by pregnant mare’s serum gonadotropin (5 units, Teikoku Pharmaceutical, Tokyo, Japan) followed by treatment with human chorionic gonadotropin (5 units, Teikoku Pharmaceutical) 48 h later. The eggs were placed in FHM medium [35], treated briefly with 0.01% bovine hyaluronidase (Sigma-Aldrich, St. Louis, MO, USA), microinjected with RNAs (approximately 10 pl) using a Piezo-driven micromanipulator (PrimeTech, Ibaraki, Japan) and then incubated in drops of KSOM medium [35] covered with mineral oil (Sigma-Aldrich) at 37°C under 5% CO2 in air. All animal experiments were carried out according to the Guide for the Care and Use of Laboratory Animals at the University of Tsukuba.

**Immunohistochemistry**

Eggs were fixed in phosphate-buffered saline (PBS) containing 2% parafomaldehyde for 30 min and then in PBS containing 2% formaldehyde for 1 h. After treatment with 1% Triton X-100 for 15 min, the eggs were blocked in PBS containing 3% goat serum and 0.05% Tween-20 overnight. The eggs were incubated with anti-PLCζ antibody in the blocking solution for 3 h, washed with PBS and reacted with secondary antibody conjugated with Alexa Fluor 488 (Molecular Probes, Eugene, OR, USA), as described previously [33]. The pronuclei were stained with Hoechst 33342. The eggs were washed with PBS containing 0.05% Tween-20 and observed under an IX-70 inverted microscope (Olympus, Tokyo, Japan) equipped with a SPOT RT SE18 camera (Diagnostic Instruments, Sterling Heights, MI, USA). The 16-bit digital images were acquired and processed using the Metamorph software (Universal Imaging, Downingtown, PA, USA).

**Chromosome analysis**

Metaphase II-arrested eggs were activated by strontium [9] or RNA injection, fixed in 0.1 M cacodylate buffer, pH 7.4, containing 2.5% glutaraldehyde for 5 min, washed with water and dehydrated in ethanol. The chromosomes were stained with 1% aceto-orcein [36] and observed under an Olympus BX50 microscope.

**ROSI**

Spermatogenic cells were prepared from the seminiferous tubules of 8-week-old B6D2F1 mice [37]. The cell suspension was washed by centrifugation and placed in Dulbecco’s PBS containing 0.5% bovine serum albumin at 4°C. Using a Piezo-driven micro-manipulator [8], the round spermatid nucleus was injected into
pseudopregnant ICR mice. 48 h, the 4-cell embryos were transferred into the oviducts of day-1
in the induction of Ca2+ oscillations [26, 30].

Fig. 1. PLCζ isoforms in mouse testes and epididymal sperm. A: PLCζ is a single-chain protein of 647 amino acids containing EF, X, Y and C2 domains. Two isoforms of PLCζ, s-PLCζ and PLCζCs, lack the N-terminal 110 and C-terminal 594 amino acids, respectively. DNA fragments encoding the entire and N-terminal 231-residue protein-coding regions were amplified by PCR from a ddY mouse testis cDNA library using two sets of PLCZ4 and PLCZ3 or PLCZ10 primers. Anti-PLCζ antibody was raised against a 149-residue recombinant polypeptide at positions 271–419. B: At least three forms of PLCζ mRNA are synthesized by alternative splicing of PLCζ mRNA between exons 3 and 4. Parts of the nucleotide sequences of exon 3, exon 4 and intron 3 are indicated. Splicing variants AK005949 and AK006672 contained additional 3- and 30-nucleotide sequences (open boxes), identical to the third intron sequence at the 3’-end, between exons 3 and 4. The in-frame TAG stop codons included in the additional sequences are indicated by asterisks. Underlines: splicing acceptor sites. C: Immunoblot analysis of recombinant proteins produced by in vitro translation (rPLCζ and rs-PLCζ) and proteins in epididymal sperm extracts (5 μg) was carried out using affinity-purified anti-PLCζ antibody.

B6D2F1 telophase II-stage eggs that had previously been activated by s-PLCζ RNA (1 mg/ml). After incubation in kSOM medium for 48 h, the 4-cell embryos were transferred into the oviducts of day-1 pseudopregnant ICR mice.

Results

Isoforms of PLCζ

Several forms of PLCζ mRNA have been reported to be synthesized in the mouse testis by alternative RNA splicing from a single PLCζ gene [26, 29, 30]. s-PLCζ (GenBank/EMBL accession number AK006672) is characterized as a PLCζ isoform that lacks the N-terminal 110-residue sequence carrying the EF1, EF2, and EF3 domains (Fig. 1A), and exhibits a very weak but significant activity in the induction of Ca2+ oscillations [26, 30].

To ascertain the presence of PLCζ isoforms, a DNA fragment encoding a 231-residue sequence of PLCζ was amplified from a mouse testis cDNA library by PCR using a set of PLCZ4 and PLCZ10 primers (Fig. 1A), and subcloned into a pBluescript II SK vector. Sequence analysis of randomly selected clones indicated that 9 out of the 25 clones code for PLCζ. The other 16 clones contained an additional 3-nucleotide sequence, TAG, between exons 3 and 4 (Fig. 1B), consistent with the sequence of GenBank/EMBL accession number AK005949. Although we found no clone carrying a 30-nucleotide insertion already reported (GenBank/EMBL accession number AK006672), the 3- and 30-nucleotide sequences containing the in-frame TAG stop codon were identical to the third intron sequence at the 3’-end. Because the first ATG codon at the 5’-end of eukaryotic mRNA generally acts as the translation initiator [38], at least two PLCζ isoforms (termed PLCζCs), in addition to the 647-residue full-length PLCζ and 537-residue length s-PLCζ, are presumably synthesized as the 53- and 61-residue polypeptides in the mouse testis.

We next carried out immunoblot analysis of mouse sperm extracts using an antibody raised against a 149-residue recombinant polypeptide of PLCζ at positions 271–419 (Fig. 1A). Recombinant PLCζ and s-PLCζ proteins of 70 and 58 kDa, respectively, were immunoreactive with the affinity-purified anti-PLCζ antibody (Fig. 1C). However, the antibody was immunoreactive only with the 70-kDa protein in the sperm extracts. These results demonstrate that s-PLCζ is absent or exists at an undetectable level in mouse epididymal sperm. Since the antibody does not recognize PLCζCs, it is uncertain at present whether sperm indeed do contain PLCζCs.
Egg activation

The effects of PLCζ and s-PLCζ on egg activation were examined by injecting corresponding RNAs into metaphase II-arrested mouse eggs. In this study, most eggs (78%) previously injected with PLCζ RNA at a concentration of 1 mg/ml formed a female pronucleus within 5 h after RNA injection (Fig. 2A). However, all activated eggs (parthenotes) had degenerated by 24 h after injection, possibly owing to a high frequency of Ca^2+ oscillations [16]. Thus, we examined the optimal conditions for pronuclear formation and embryonic development using various concentrations (0.002–1 mg/ml) of synthetic RNAs.

When metaphase II-arrested eggs were injected with 0.002 mg/ml PLCζ RNA, most eggs (96%) formed a pronucleus 5 h after injection. In addition, the PLCζ RNA (0.002 mg/ml)-injected eggs developed into 2-cell embryos at a high rate (77%). Pronuclear formation and subsequent development into a 2-cell embryo in the eggs injected with s-PLCζ RNA required a high RNA concentration (Fig. 2A), despite PLCζ and s-PLCζ RNAs being equally translated in the eggs (Fig. 2B). PLCζ produced in the eggs was localized in the pronucleus, whereas a cytoplasmic distribution was observed for s-PLCζ (Fig. 2C). These data are consistent with the fact that s-PLCζ lacking three EF-hand domains has weak activity for inducing Ca^2+ oscillations and is barely accumulated in the pronucleus [25, 26, 30].

Development of haploid parthenotes

To examine whether PLCζ and s-PLCζ support the development of haploid parthenotes, metaphase II-arrested eggs were activated by injection of the corresponding RNAs. Because of the slow development of haploid parthenotes, the eggs were incubated for 120 h [39]. All or most eggs activated by PLCζ RNA (0.002 mg/ml), s-PLCζ RNA (1 mg/ml) and strontium (98, 100 and 93%, respectively) formed a female pronucleus under the experimental conditions employed. These parthenotes developed to 4-cell to 8-cell embryos, morulae and blastocysts 48, 96 and 120 h after pronuclear formation, respectively (Fig. 3). There was no significant difference in embryonic development among the eggs treated with strontium and injected with PLCζ or s-PLCζ RNAs.

Timing of transition to telophase II

The overall goal of this study was to obtain full-term offspring from PLCζ- or s-PLCζ-activated eggs by ROSI. Injection of round spermatid nuclei into telophase II-stage eggs is an effective technique for obtaining offspring [11, 12]. Thus, we examined the timing of chromosome segregation in the activated eggs (Fig. 4A). In eggs activated with the strontium treatment, chromosome segregation proceeded immediately after treatment and was complete within 2 h (Fig. 4B). The chromosomes of the PLCζ RNA (0.002 mg/ml)-injected eggs started to segregate 1.5 h after RNA injection, and chromosome segregation had finished in most eggs (92%) by 3.5 h. The s-PLCζ RNA (1 mg/ml)-injected eggs showed a significant delay in chromosome segregation; it started to occur 4 h after RNA injection. These data indicate that eggs 1.5–3.5 h and 4–6 h after injection of PLCζ and s-PLCζ RNAs, respectively, are suitable for ROSI.

Embryonic development of ROSI eggs

We speculated that the delayed egg activation caused by s-PLCζ RNA injection (Fig. 4) would enable effective production of offspring by ROSI because the egg plasma membrane may require a
certain time to repair after the RNA injection. Thus, in this study, s-PLCζ RNA was employed for induction of egg activation before ROSI. When ROSI was carried out using s-PLCζ-activated eggs at the telophase-II stage (4–6 h after the RNA injection), 66% of the activated eggs survived the ROSI treatment (Table 1). Following incubation of the ROSI-surviving eggs for 40 h, 87% of the eggs developed to the 4-cell embryo stage in vitro. Moreover, transfer of the 55 4-cell embryos to five pseudopregnant mice resulted in the birth of three live offspring (Table 2). No physical abnormalities were observed in these three newborn mice, all of which grew up to normal adults (Fig. 5A) that reproduced healthy second-generation mice (Fig. 5B).

### Discussion

The present study has demonstrated that s-PLCζ, which has Ca²⁺ oscillation-inducing activity, serves as a compensatory factor in ROSI for fertilization and embryo development in the mouse. To select an appropriate method for egg activation and subsequent embryo development, we reexamined the ability of PLCζ and s-PLCζ to induce egg activation. It has been reported that an RNA concentration of at least 0.5 mg/ml is required for s-PLCζ to induce Ca²⁺ oscillations, whereas an RNA concentration of 0.001–0.002 mg/ml is enough for PLCζ to induce Ca²⁺ oscillations [16, 26]. In the case of s-PLCζ, small Ca²⁺ oscillations begin later than when using PLCζ and they are followed by discrete small Ca²⁺ spikes [24, 26]. Coinciding with these reports, we found that resumption of meiosis as well as pronuclear formation was delayed in s-PLCζ RNA (1 mg/ml)-injected eggs compared with eggs injected with PLCζ RNA (0.002 mg/ml; Fig. 4). In spite of the weak Ca²⁺ oscillation-inducing activity of s-PLCζ, the present study describes that synthetic s-PLCζ RNA is effective for assisting the full-term development of ROSI-derived mouse embryos. However, the birth rate of offspring from the s-PLCζ RNA-activated eggs was very low (7%; Table 2). The low birth rate may be due to technical problem(s) in repeated injections of s-PLCζ RNA and the round spermatid nucleus. Further studies are required to improve the full-term development of embryos produced by ROSI coupled with RNA injection.

To ascertain the presence of PLCζ isoforms, including the s-PLCζ utilized in this study, two mRNA variants (GenBank/EMBL accession numbers AK005949 and AK006672) were analyzed in detail. They have both been proposed to derive from alternative splicing of the third intron of PLCζ mRNA (Fig. 1B). However, AK006672, reported by Yoda et al. [26], was not found under our assay conditions. The variant we identified was AK005949, which contained an additional in-frame stop codon between exons 3 and 4 of PLCζ mRNA. Our study also indicates that s-PLCζ is absent or exists at undetectable levels in mouse epididymal sperm (Fig. 2B). This result is inconsistent with previous reports [26, 31]; however, it suggests that translation of PLCζΔC may occur from the splicing variant because the first ATG codon at the 5'-end of eukaryotic mRNA generally acts as the translation initiator [38]. The different patterns of splicing variants and protein composition between various studies may depend on the mouse strain utilized to produce the testis cDNA library and the sperm extract.

### Table 1. In vitro development of ROSI embryos derived from s-PLCζ RNA-activated eggs

<table>
<thead>
<tr>
<th>No. of eggs injected</th>
<th>No. (% of eggs that survived)</th>
<th>No. (% of embryos that survived)</th>
</tr>
</thead>
<tbody>
<tr>
<td>ROSI</td>
<td>96</td>
<td>63 (66) 1 (2) 7 (11) 55 (87)</td>
</tr>
</tbody>
</table>

### Table 2. Birth of offspring from s-PLCζ RNA-activated eggs by ROSI

<table>
<thead>
<tr>
<th>Recipient</th>
<th>No. of 4-cell embryos transferred</th>
<th>No. (%) of offspring born</th>
<th>Sex of offspring</th>
</tr>
</thead>
<tbody>
<tr>
<td>A</td>
<td>11</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>B</td>
<td>11</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>C</td>
<td>11</td>
<td>1*</td>
<td>1?</td>
</tr>
<tr>
<td>D</td>
<td>11</td>
<td>1</td>
<td>1M</td>
</tr>
<tr>
<td>E</td>
<td>11</td>
<td>2</td>
<td>2F</td>
</tr>
<tr>
<td>Total</td>
<td>55</td>
<td>4 (7)</td>
<td>2F, 1M, 1?</td>
</tr>
</tbody>
</table>

* A pup died soon after birth, and its sex was not determined.

Fig. 5. Full-term development of ROSI embryos derived from eggs activated by s-PLCζ. Three offspring were born from the s-PLCζ-activated eggs, and grew to adulthood (A). These three mice (#1 and #2 are females, and #3 is a male) were all fertile. Female mouse #2 is shown with her pups in B.
In addition to the consensus nuclear localization signal between the X and Y domains, the region including Trp\(^{13}\), Phe\(^{14}\) and Val\(^{18}\) in the N terminus of the EF1 domain is necessary for PLC\(_{\zeta}\) to make the appropriate conformation for pronuclear translocation [24]. Thus, s-PLC\(_{\zeta}\) lacking the three EF-hand domains is localized in the cytoplasm (Fig. 2C). However, the aberrant localization of s-PLC\(_{\zeta}\) after egg activation did not affect the ratio of pronuclear formation or subsequent development in vitro (Figs. 2A and 3). This indicates that the nuclear localization of PLC\(_{\zeta}\) may not be essential for embryo development in vitro.

Moreover, full-term embryo development via combination of egg activation utilizing s-PLC\(_{\zeta}\) and ROSI means the process toward the activation is slow but normal. This prolonged period of egg activation utilizing s-PLC\(_{\zeta}\) catalysts that the nuclear localization of PLC\(_{\zeta}\) after egg activation did not affect the ratio of pronuclear formation and offspring from mouse eggs injected with round spermatids combined with Ca\(^{2+}\). Fertilization, embryonic development, and offspring from mouse eggs injected with round spermatids leading to full-term embryo development via combination of nuclear targeting of PLC\(_{\zeta}\) and egg activation in mammals is a novel phospholipase C-PLC\(_{\zeta}\) Reproduction 2004; 127: 431–439.


