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**Abstract**

Phospholipase C-zeta (PLCZ1), a strong candidate of egg-activating sperm factor, can induce Ca2+ oscillations and cause egg activation. For the application of PLCZ1 to clinical use, we examined the pattern of Ca2+ oscillations and developmental rate by comparing PLCZ1 RNA injection methods with the other current methods, such as cytosolic aspiration, electrical stimulation and ionomycin treatment in human oocytes. We found that the pattern of Ca2+ oscillations after PLCZ1 RNA injection exhibited similar characteristics to that after ICSI treatment. We also determined the optimal concentration of PLCZ1 RNA to activate the human oocytes. Our findings suggest that human PLCZ1 RNA is a better therapeutic agent to rescue human oocytes from failed activation, leading to normal and efficient development.



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| **Keywords** | human oocyte; egg activation; PLCZ1 RNA; Ca2+ oscillations; preimplantation development. |



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**The establishment of appropriate methods for egg-activation by PLCZ1 RNA injection into human oocyte**

Running title: human egg-activation by PLCZ1 RNA

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Keywords: human oocyte, egg activation, PLCZ1 RNA, $Ca^{2+}$ oscillations, preimplantation development.

Abbreviations

PLCZ1, Phospholipase C-zeta;

ICSI, intracytoplasmic sperm injection;

ER, endoplasmic reticulum;

InsP3R, inositol 1,4,5-trisphosphate receptor;

PN, pronucleus:

$[Ca^{2+}]_i$, intracellular calcium ion concentration;

ROSI, round spermatid injection

IVF, *in vitro* fertilizaition;

CaMKII, $Ca^{2+}$/CaM-dependent protein kinase II;

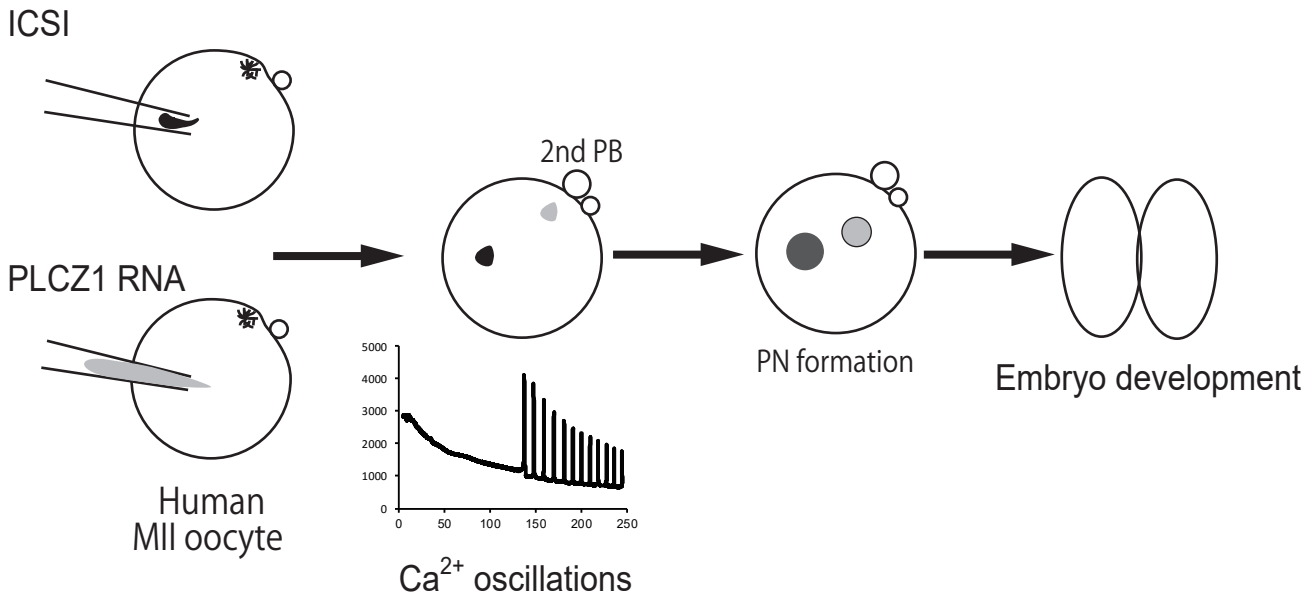
MAPK, mitogen activated protein kinase;

APC/C, anaphase-promoting complex/cyclosome;

iPS cells, induced pluripotent stem cells.

**Abstract**

Phospholipase C-zeta (PLCZ1), a strong candidate of egg-activating sperm factor, can induce $Ca^{2+}$ oscillations and cause egg activation. For the application of PLCZ1 to clinical use, we examined the pattern of $Ca^{2+}$ oscillations and developmental rate by comparing PLCZ1 RNA injection methods with the other current methods, such as cytosolic aspiration, electrical stimulation and ionomycin treatment in human oocytes. We found that the pattern of $Ca^{2+}$ oscillations after PLCZ1 RNA injection exhibited similar characteristics to that after ICSI treatment. We also determined the optimal concentration of PLCZ1 RNA to activate the human oocytes. Our findings suggest that human PLCZ1 RNA is a better therapeutic agent to rescue human oocytes from failed activation, leading to normal and efficient development.


ICSI
PLCZ1 RNA
Human
MII oocyte
2nd PB
5000
4000
3000
2000
1000
0
0
50
100
150
200
250
Ca2+ oscillations
PN formation
Embryo development

## Introduction

At fertilization, mammalian oocytes show repetitive transient increase in intracellular calcium ion concentration ($[Ca^{2+}]_i$), known as $Ca^{2+}$ oscillations. Each of which is due to $Ca^{2+}$ release from the endoplasmic reticulum (ER) mainly through type 1 inositol 1,4,5-trisphosphate receptor (InsP3R) [1-4]. $[Ca^{2+}]_i$ each rise lasts about 1 min and the $Ca^{2+}$ transients occur at intervals of 5–30 min [1,5]. The $Ca^{2+}$ oscillations are a pivotal signal for egg activation and embryo development [6-7]. They cause resumption of the second meiosis and subsequent formation of male and female pronuclei (PN).

Repetitive $Ca^{2+}$ release is induced by a cytosolic sperm factor driven into the ooplasm upon sperm-egg fusion [8]. Several lines of evidence indicate that a sperm-specific isozyme "zeta" of InsP3-producing enzyme phospholipase C (PLCZ1) is a strong candidate to be the sperm factor [1,9-12]. Depleting PLCZ1 from sperm extract by anti-PLCZ1 antibody abolished the $Ca^{2+}$ oscillation-inducing activity [9]. Expressing PLCZ1 in the oocyte by RNA injection induced $Ca^{2+}$ oscillations and the egg activation [9,12]. Recombinant PLCZ1 protein injected in the oocyte could elicit $Ca^{2+}$ oscillations [13,14]. Knocking down PLCZ1 in transgenic mice resulted in the deficiency of $Ca^{2+}$ oscillation inducing activity of the sperm and no offspring [15].

At present, one of the most powerful therapeutic procedures for male factor infertility is to inject a single sperm directly into the egg, known as ICSI.

$Ca^{2+}$ oscillations have been observed in human oocytes after ICSI [16]. However, 1–5% of all ICSI treatments resulted in failure, and the main cause for this was shown to be deficiencies in the egg activation process [17-19]. Several cases of male factor infertility are probably results of dysfunctional isoforms or reduced expression levels of PLCZ1 [20-22]. Egg activation failure can be treated by methods to elevate $[Ca^{2+}]_i$, such as applying $Ca^{2+}$ ionophore, as currently used in most clinics, but such chemicals cannot mimic the pattern of $[Ca^{2+}]_i$ rises at normal fertilization and can be potentially cytotoxic or mutagenic for eggs and embryos [23]. On the other hand, PLCZ1 is a native physiological egg activating factor. When expressed by the injection of *in vitro*

transcribed RNA, PLCZ1 can induce fertilization-like $Ca^{2+}$ oscillations, resulting in parthenogenetic development up to blastocysts in mice, cows, pigs, monkeys and humans [9,24-26]. In addition to ICSI, round spermatid injection (ROSI) is developing as an alternative treatment for patients who have defects in spermatogenesis. Recently, it has been shown that ROSI is applicable to men with azoospermia, who possess only round spermatids; this application resulted in the birth of healthy babies [27]. The expression of egg-activating sperm factor could be detected at primary spermatocyte and round spermatid in monkeys and humans, respectively [28-30]. On the contrary, it had also been shown that round spermatids injected into mouse eggs could not induce egg activation [31]. In human, the precise stage of spermatogenesis from which PLCZ1 starts to express remains unknown. Moreover, the expression level of PLCZ1 or $Ca^{2+}$ oscillation-inducing activates is considered to be different among the men with azoospermia. From these reason, stimulation for egg activation should be conducted to resume the cell cycle progression after ROSI. Thus, the effective egg activation method is also useful for ROSI as well as ICSI failure.

In this study, to establish the best egg activation method and improve development rates after ICSI or ROSI, we compared the pattern of $[Ca^{2+}]_i$ elevation after stimulation by cytosolic aspiration, electrical stimulation, ionomycin treatment and PLCZ1 RNA injection. We found that the pattern of $Ca^{2+}$ oscillations after PLCZ1 RNA injection exhibited similar characteristics to that after ICSI treatment. And, in terms of the developmental rates, we determined the optimal concentration of PLCZ1 RNA to activate the human oocytes. Our data provide the basis for future studies to apply PLCZ1 RNA injection to clinical use to rescue human oocytes from failed activation.

## Materials and Methods

### Ethical aspects

This study was conducted with the informed consent of all participating patients. The

Institutional Review Boards of the Saint Mother Obstetrics and Gynecology Clinic approved this study on January 17, 2016. This study was registered and adhered to International Committee of Medical Journal Editors criteria. The University Hospital Medical Information Network Clinical Trials Registry is UMIN000020860.

**Preparation of PLCZ1 RNA**

cDNA encoding human PLCZ1 (GenBank accession number NM_033123) was prepared using PCR techniques from human testis cDNA library (PCR Ready First Strand cDNA; C1234260; BioChain Institute, Hayward, CA), and cloned into pTNT vector (Promega, Madison, WI). The 30 nucleotides of poly (A) region of pTNT vector was substituted with 168 nucleotides of poly (A) tail. The constructed plasmids were purified with NucleoBond Xtra Midi Plus EF kit (Takara, Shiga, Japan) and digested with *Bam*HI, and resulting fragment was used as templates for *in vitro* transcription ([32] for details). Briefly, RNA was synthesized by T7 polymerase using mMessage mMachine Kit (Thermo Fisher Scientific, Waltham, MA) and purified by RNeasy Mini Kit (Qiagen, Venlo, Netherlands). Dried RNA was resolved in 150mM KCl solution (final concentration, ~1.5 μg/μl) and checked the quality by electrophoresis. RNA was diluted to the range between 0.01 and 1000 ng/μl and injected into oocytes.

**Procedure for egg activation**

Human M-II oocytes were obtained from IVF patients who had consented to participate in this study. The number of provided oocytes was limited to two at most. Oocytes were activated by one of the following four procedures.

1. PLCZ1 RNA injection

Various concentrations of PLCZ1 RNA were injected into oocyte by using a Piezo manipulator in the fluorescence microscope. In order to minimize damage to the egg, RNA was injected very mildly. Piezo settings were intensity 2 and speed 2. In the dish, the pipette was washed in 12% PVP drop, PLCZ1 RNA was aspirated, and approx. 4 pl of PLCZ1 were injected into the oocyte in the HTF medium (10% SPS contained) covered with mineral oil. The injected volume of PLCZ1 is the same to that of ROSI.

2. Cytosolic aspiration and injection

2-3 times of cytosolic aspiration as the same way of ICSI was performed following Tesarik's method [33]. Then oocytes were cultured in the HTF medium with 10% SPS.

3. Electrical stimulation

Oocytes were placed in 295-mM mannitol solution with 0.1 mM $CaCl_2$ and 0.05 mM $MgCl_2$, and stimulated with an alternating current of 5 V/cm at 1 MHz for 10 s followed by a single 1.2-kV/cm pulse of direct current for 99 ms using an electro-cell fusion generator (LF201; Nepagne). After electrical stimulation, oocytes were returned to the HTF medium with 10% SPS.

4. Ionomycin treatment

Oocytes were culture in the microdrop of HTF with 10% SPS containing 10 μM of Ionomycin, for 5 min.

**$[Ca^{2+}]_i$ measurement**

Oocytes were placed in SPS medium and loaded with $Ca2^+$-sensitive fluorescent dye Fluo8H (Molecular Probes, Inc., Eugene, OR) for 30 min at room temperature in the dark box. After loading, oocytes were activated by one of the above mentioned four procedures, and subjected to $[Ca^{2+}]_i$ measurement with a 20 X objective lens for 2 h at 37°C, with 5% $CO_2$ gas in the chamber(Olympus IX-71, Yokogawa CSU-X1 and CSU-Frontier ). $Ca^{2+}$ images were acquired at intervals of 1 sec by using an image processor (8 bit; DP73, Olympus, Tokyo) and analyzed by cellSens dimension (Olympus).

**Evaluation of egg activation**

Appearance of polar body and formation of pronuclei were confirmed 12-14 hours after egg activation with an inverted microscope (Olympus IX-70). For comparative studies, morphological changes, pattern of $Ca^{2+}$ oscillations and cleavage rates were analyzed.

**Statistical Analysis**

Data were analyzed using the Microsoft Excel Add-in software (MacToukei-kaiseki v2.0) and statistical significances were evaluated by Student's T test.

## Results

To compare the pattern of $Ca^{2+}$ oscillations induced by PLCZ1 RNA with that by other egg activation methods, $[Ca^{2+}]_i$ was monitored by Fluo8H, fluorescent $Ca^{2+}$ indicator, after the treatment by various egg activation methods. In the cytosolic aspiration and injection method, $Ca^{2+}$ oscillations were not observed (Fig.1A). In the ionomycin activation method, fluorescent intensity gradually decreased from 5 min after treatment. Broad $[Ca^{2+}]_i$ increase was observed from 40 to 60 min after treatment, but $Ca^{2+}$ oscillations did not occur (Fig.1B). Figure 1C showed that a series of $Ca^{2+}$ oscillations after ICSI. In this egg, 1st $Ca^{2+}$ spike occurred at 130 min after ICSI treatment. Average interspike interval was 9.09 ± 3.20 min. In the electrical stimulation, increase of $[Ca^{2+}]_i$ was observed immediately after treatment. After that, low amplitude of $Ca^{2+}$ spikes were induced at random intervals. It was different from the pattern of $Ca^{2+}$ oscillations induced by ICSI (Fig.1C and Fig.1D). In the PLCZ1 RNA activation method, PLCZ1 RNA (100 ng/μl) was injected into oocytes and fluorescent intensity measured by conventional fluorescence microscopy (Fig.1E) and confocal microscopy (Fig.1F). 1st $Ca^{2+}$ spike occurred at 17 min and 28 min respectively. In comparison with conventional fluorescence microscopy, higher amplitude and longer lasting of $Ca^{2+}$ oscillations in low background level could be recorded by confocal microscopy. These results showed that the pattern of $Ca^{2+}$ oscillations by PLCZ1 RNA injection was quite similar to that of $Ca^{2+}$ oscillations seen in ICSI eggs

For adaptation of egg activation by PLCZ1 RNA injection to clinical use, optimal concentrations of PLCZ1 RNA for preimplantation development were examined. Various concentrations of PLCZ1 RNA (0.01-1000 ng/μl) were injected into eggs by ICSI needle and the developmental rate evaluated (Table 1). The result showed that eggs injected with 100 ng/μl PLCZ1 RNA exhibited the highest rate in extrusion of 2nd polar body (66.7%, n=18), formation of female pronucleus (66.7%, n=18) and developmental capability.

Frequency of $Ca^2$ spikes and accumulated time of elevated $[Ca^{2+}]_i$ are important factors for egg activation. The patterns of $Ca^{2+}$ oscillations induced by 100 ng/μl PLCZ1 RNA (Table 2) and ICSI (Table 3) were analyzed. The latency time of $Ca^{2+}$ oscillations in PLCZ1 (18.88 ± 4.26 min) was different from that of ICSI (87.8 ± 35.54 min). Number of spikes per 2 h and interspike interval were not significantly different. Furthermore, duration of 1st $Ca^{2+}$ spike was 2.69 ± 0.68 min and 2.83 ± 0.97 min, and mean duration of $Ca^{2+}$ spikes (2nd to 6th) was 1.16 ± 0.06 min and 1.68 ± 0.53 min in PLCZ1 RNA injected oocytes and ICSI oocyte, respectively (not shown data). These results suggested that the pattern of $Ca^{2+}$ oscillations induced by PLCZ1 RNA (100 ng/μl) were comparable with that induced by ICSI.

To reveal the usefulness of PLCZ1 RNA injection method for the egg-activation after ROSI, the effect of various activation methods on development of embryos was examined. After treatment of ionomycin, PLCZ1 RNA and electrical stimulation, the timing of PN formation and developmental rate were evaluated (Table 4). The result indicated that embryo development induced by PLCZ1 RNA was better than the other activation methods. At day 3, approx. 50% embryos in which PN were observed grew to over 7 cells after PLCZ1 RNA injection.

## Discussion

### $Ca^{2+}$ oscillations pattern in human oocyte induced by PLCZ1 RNA injection.

$Ca^{2+}$ mobilization is essential for egg-activation and prerequisite for normal embryo development [7,34]. In this research, to find the most effective egg activation method for preimplantation development, we examined the $Ca^{2+}$ oscillations pattern induced by cytosolic aspiration and injection, ionomycin treatment, electric stimulation and PLCZ1 RNA injection, compared with that induced by ICSI. In the cytosolic aspiration and injection method, $Ca^{2+}$ oscillations did not occur, but a sustained low-amplitude $[Ca^{2+}]_i$ response was observed 20 min after stimulation. This may be caused by $Ca^{2+}$ influx from

222 culture medium or $Ca^{2+}$ leak form intracellular $Ca^{2+}$ stores, such as endoplasmic reticulum
223 (ER) or mitochondria [35]. In the ionomycin treatment, long lasting $[Ca^{2+}]_i$ increase was
224 observed 20 min after stimulation. Ionomycin is a calcium ionophore with reported faster
225 effects than A23187 [36]. It has been shown that human oocytes activated with ionomycin
226 exhibited one or two large $[Ca^{2+}]_i$ increase followed by prompt return to baseline levels
227 [37-39]. In our study, ionomycin treatment lead to development to over 7 cells at Day3.,
228 but it was less effective than either electrical stimulation or PLCZ1 RNA injection (Table
229 4). Adequate duration of a sustained $[Ca^{2+}]_i$ elevation is necessary for accomplishing the
230 successful egg-activation [40]. Thus, this may be the reason for the lowest developmental
231 rate in ionomycin treatment among activation methods tested in this experiment. $Ca^{2+}$
232 oscillations could be observed in ICSI eggs as previously reported [16]. Latency time is
233 around 88 min after ICSI treatment (Table 3). In mature spermatozoa, PLCZ1 can be
234 found localized to the acrosomal, equatorial and/or post-acrosomal regions [41-43]. The
235 latency from the sperm-egg fusion to the beginning of $[Ca^{2+}]_i$ rise takes several minutes
236 in mouse eggs [44,45]. Assuming that PLCZ1 proteins present in the equatorial region
237 are mostly soluble, they can readily diffuse out of the head upon the fusion with the egg,
238 whereas PLCZ1 may diffuse gradually from injected sperm to egg cytoplasm. In the
239 electrical stimulated eggs, rapid increase of $[Ca^{2+}]_i$ and subsequent low amplitude of
240 random $Ca^{2+}$ spikes were observed (Fig. 1D). This is the first report for $[Ca^{2+}]_i$
241 measurement after electrical stimulation, not during electrical stimulation. To reveal its
242 underlying mechanism, further experiments may be required. When 100ng/μl of PLCZ1
243 RNA was injected into oocytes, amplitude, duration and time integral of $Ca^{2+}$ oscillations
244 were similar to that in the ICSI oocytes (Table 2 and 3). This $Ca^{2+}$ oscillatory behavior
245 was probably due to the positive feedback of $Ca^{2+}$ to PLCZ1 activity. Dissociation
246 constant for $Ca^{2+}$ activation of PLCZ1 is very low at around the resting $[Ca^{2+}]_i$. This idea
247 of $Ca_{2+}$-on-PLCZ1 mechanism is supported by some experimental results [46]. Our
248 comparative analysis among egg-activating methods indicated that PLCZ1 RNA was
249 expected to be a better therapeutic agent as an artificial but physiological activator,

leading to normal and efficient preimplantation development.

**Number of $Ca^{2+}$ spikes and preimplantation development**

The number of $[Ca^{2+}]_i$ rise has pivotal roles on the embryo development [3]. When the embryos experienced either too few or too many $Ca^{2+}$ oscillations, the growth rates to blastocyst stage were not significantly changed *in vitro* fertilized mouse eggs, but ~ 20% of the transcripts were mis-regulated and fewer offspring were born following embryo transfer [47], indicating that the pattern of $Ca^{2+}$ oscillations is significant for normal preimplantation development. Moreover, for full activation of $Ca^{2+}$-bound calmodulin (CaM) activates $Ca^{2+}$/CaM-dependent protein kinase II (CaMKII) and mitogen activated protein kinase (MAPK), total time of $[Ca^{2+}]_i$ rises is quite important [48,49]. CaMKII inactivates Emi2, one of the CSF constituents, resulting in release of APC/C and degradation of cyclin B1 to resume the second meiotic division [52,53]. The number of $Ca^{2+}$ oscillations is involved in a decrease in MAPK activity and PN formation [6]. In this research, we showed that PLCZ1 RNA injection could mimic the number and total time of $Ca^{2+}$ spikes induced by sperm

**Safety of PLCZ1 RNA injection for clinical use**

One of the problems for RNA injection methods is that the protein is continuously expressed by existence of the injected RNA. Therefore, it becomes difficult to control the expression level of PLCZ1 to elicit appropriate frequency of $Ca^{2+}$ oscillations. In mouse eggs, with high concentration of PLCZ1 RNA, a burst of $Ca^{2+}$ spikes occurred 120–180 min after RNA injection, and prevented the 1st mitotic division [32]. In the present experiments, we determined the optimal concentration of injected RNA for normal embryo development (Table 4). Short type variant of PLCZ1 (s-PLCζ) has been reported to be expressed in the mouse testis. $Ca^{2+}$ oscillation-inducing activity of s-PLCζ was estimated to be roughly two orders of magnitude lower than that of PLCZ1 [32]. For removal of the cytotoxicity and easy handling, s-PLCζ RNA was injected into mouse oocytes following ROSI. As a result, healthy offspring were born and all grew to be normal adults and reproduced healthy second-generation mice [52]. However, injection

278 with high concentration of human PLCZ1 RNA did not induce a burst of $Ca^{2+}$ spikes in
279 human oocytes (Table 1). Previous reports showed that the frequency of human sperm-
280 and PLCZ1 RNA-induced $Ca^{2+}$ oscillations was higher than observed at fertilization in
281 mouse oocytes [53-55]. This is probably because the down-regulation or sensitivity to
282 $Ca^{2+}$ of InsP3 receptor is different between human and mouse oocytes [56,57]. Generally,
283 the stability of mRNA depends on the length of poly (A) tail at the 3' untranslated region.
284 [58], but average of RNAs half-lives is about 9 hours [59]. Then, it is not likely that
285 injected PLCZ1 RNA could exist in the cells for a long time. Introduction of mRNA into
286 cells has been also applied for generating iPS cells, because mRNA has no ability to
287 integrate into the host genome [60-62]. Thus, our findings provide the basis for clinical
288 use of PLCZ1 RNA as an egg-activating agent and will be beneficial in further
289 experiments studying the mechanisms of human egg activation.
290

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**Contributions**

T.Y., M.I., I.T., K.K., S.T., and A.T. designed the research; T.Y., M.I., Y.T., and A.T. performed the experiments; T.Y., M.I., Y.T., and A.T. analyzed data; T.Y., and M.I prepared the figures; and T.Y., M.I., A.T. wrote the manuscript; and all authors commented on the manuscript, and approved the final version.

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**Competing interests**

The authors declare that they have no conflicts of interest.

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**Figure legends**

Figure 1.

$Ca^{2+}$ oscillations in human oocyte induced by cytosolic aspiration (A), Ionomycin treatment (B), ICSI (C) and electrical stimulation (D) were observed by conventional fluorescence microscopy. PLCZ1 RNA (100 ng/μl) induced $Ca^{2+}$ oscillations were observed by conventional fluorescence microscopy (E) and confocal laser scanning microscopy (F). The timing of stimulation was set as the zero time. At least 3 sets of experiments were performed. Representative results are shown.

Table 1. Optimal concentration of PLCZ1 RNA for preimplantation development

Table 2. Profile of $Ca^{2+}$ oscillations induced by PLCZ1 RNA

Table 3. Profile of $Ca^{2+}$+ oscillations induced by ICSI

Table 4. Effect of activation method on development of ROSI embryos

# Figure 1

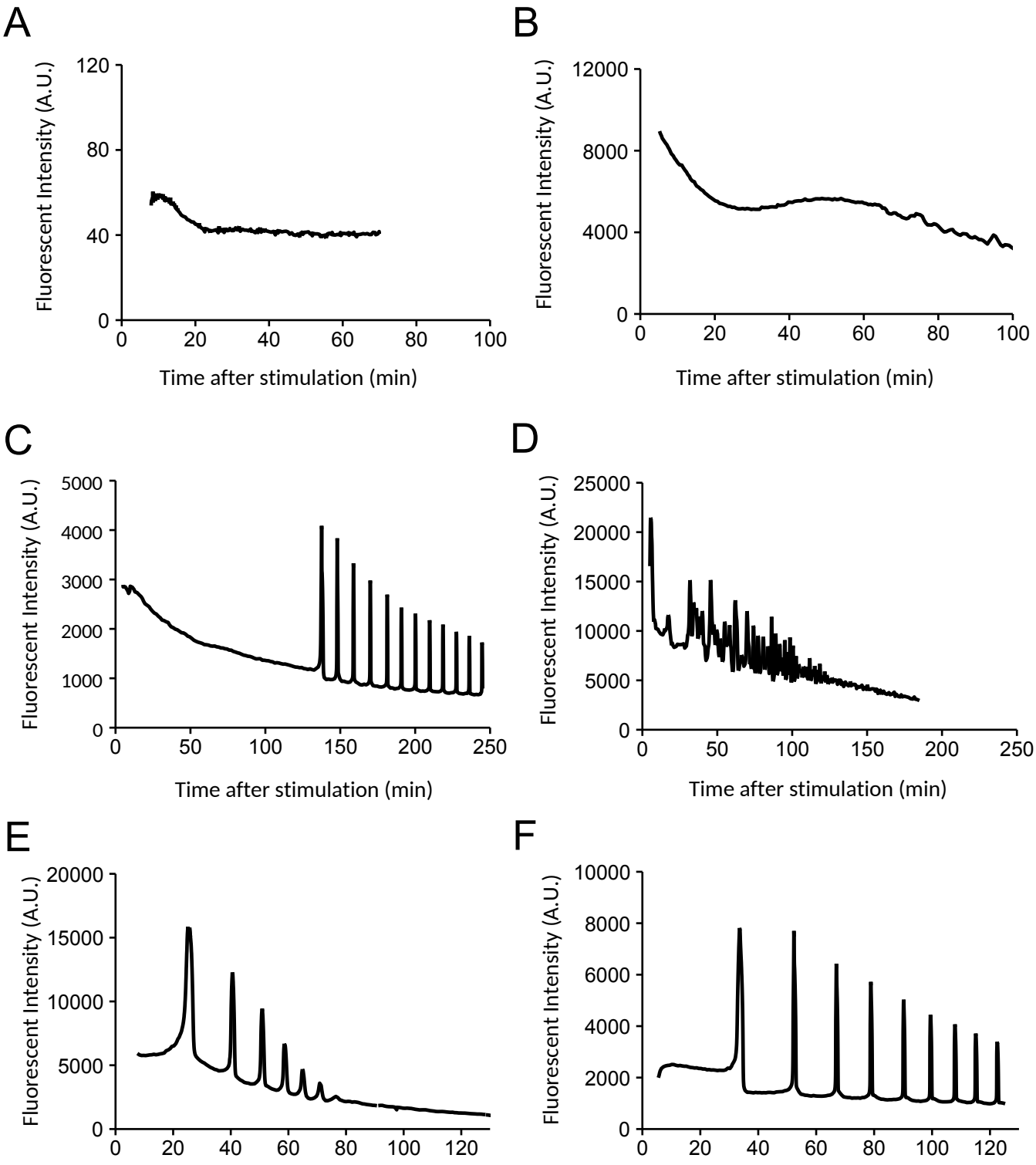
A
Fluorescent Intensity (A.U.)
120
80
40
0
0
20
40
60
80
100
Time after stimulation (min)
B
Fluorescent Intensity (A.U.)
12000
8000
4000
0
0
20
40
60
80
100
Time after stimulation (min)
C
Fluorescent Intensity (A.U.)
5000
4000
3000
2000
1000
0
0
50
100
150
200
250
Time after stimulation (min)
D
Fluorescent Intensity (A.U.)
25000
20000
15000
10000
5000
0
0
50
100
150
200
250
Time after stimulation (min)
E
Fluorescent Intensity (A.U.)
20000
15000
10000
5000
0
0
20
40
60
80
100
120
F
Fluorescent Intensity (A.U.)
10000
8000
6000
4000
2000
0
0
20
40
60
80
100
120

Table 1. Optimal concentration of PLCZ1 RNA for preimplantation development

Table 1

| Conc.of PLCZ1 RNA(ng/μl) | Day1 | | Day2[1] | Day3[1] |
|---|---|---|---|---|
| | 2nd PB(%) | 1PN(%) | | |
| **0.01 (n=13)** | 23.1 | 23.1 | mono cell(1), 2 cell(1), 3 cell(1) | mono cell(1), 2 cell(1) |
| **0.05 (n=14)** | 14.3 | 14.3 | 2 cell(1), 5 cell(1) | - |
| **0.1 (n=4)** | 0 | 0 | - | - |
| **1 (n=6)** | 0 | 0 | - | - |
| **5 (n=25)** | 16.0 | 8.0 | 2 cell (2) | 2 cell (2) |
| **10 (n=11)** | 9.1 | 9.1 | 2 cell (1) | 3 cell (1) |
| **100 (n=18)** | 66.7 | 66.7 | mono cell(1), 2 cell(6), 3 cell(2), 4cell(3) | 3 cell(4), 4 cell(3), 5 cell(2), 6cell(1), 8cell(1), 10 cell(1) |
| **1000 (n=3)** | 66.7 | 66.7 | mono cell (2) | mono cell (2) |

[1]No. of Eggs in parenthesis.

Table 2. Profile of $Ca^{2+}$ oscillations induced by PLCZ1 RNA

Table 2

| Egg No. [1] | 1 | 2 | 3 | 4 | 5 | 6 | 7 | 8 | Average[2] |
|---|---|---|---|---|---|---|---|---|---|
| **Latency (min)** | 17 | 14 | 16 | 21 | 16 | 22 | 17 | 28 | 18.88±4.26 |
| **No. of Spikes/2hr** | 7 | 6 | 29 | 27 | 10 | 6 | 11 | 15 | 13.88±8.64 |
| **Interspike interval (min)** | 8.57 | 6.17 | 1.76 | 2.78 | 3.60 | 10.00 | 4.00 | 3.80 | 5.09±2.71 |

[1]Eggs were injected with 100 ng/μl PLCZ1 RNA. [2]means±SD

Table 3. Profile of $Ca^{2+}$ oscillations induced by ICSI

Table 3

| Egg No. | 1 | 2 | 3 | 4 | 5 | Average[1] |
|---|---|---|---|---|---|---|
| **Latancy (min)** | 88 | 130 | 37 | 67 | 120 | 87.8±35.54 |
| **No. of Spikes/2hr** | 42 | 12 | 8 | 15 | 7 | 16.8±12.92 |
| **Interspike interval (min)** | 3.69 | 9.42 | 26.70 | 11.87 | 16.71 | 13.7±7.74 |

[1]means±SD

Table 4. Effect of activation method on development of ROSI embryos

Table 4

| Egg activation method | No. of eggs | Day1 | | Day3(□7cell) | |
|---|---|---|---|---|---|
| | | 2PN(%) | 1PN(%) | 2PN(%) | 1PN(%) |
| **Ionomycin** | 65 | 33.8 (22/65) | 26.2 (17/65) | 50.0 (11/22) | 17.6 (3/17) |
| **PLCZ1 RNA** | 35 | 31.4 (11/35) | 34.3 (12/35) | 54.5 (6/11) | 50.0 (6/12) |
| **Electrical stimulation** | 535 | 26.2 (140/535) | 28.0 (150/535) | 36.4 (51/140) | 38.0 (57/150) |

No. of Eggs in parenthesis.

Highlights

►Pattern of $Ca^{2+}$ oscillations induced by several egg-activating methods is examined in human oocytes.

►Optimal concentration of PLCZ1 RNA for embryo development is determined.

►The characteristics of $Ca^{2+}$ oscillations after PLCZ1 RNA injection are similar to that after ICSI treatment.

►Developmental rate of embryo activated by PLCZ1 RNA is the highest, compared with ionomycin and electrical stimulation.