Birth of offspring from spermatid or somatic cell by co-injection of PLC ζ -cRNA

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Abstract

Artificial oocyte activation is important for assisted reproductive technologies, such as fertilization with round spermatids (ROSI) or the production of cloned offspring by somatic cell nuclear transfer (SCNT). Recently, phospholipase C ζ (PLC ζ)-cRNA was used to mimic the natural process of fertilization, but this method required the serial injection of PLC ζ -cRNA and was found to cause damage to the manipulated oocytes. Here we tried to generate offspring derived from oocytes that were fertilized using round spermatid or somatic cell nuclear transfer with the co-injection of PLC ζ -cRNA. After co-injecting round spermatids and 20 ng/ μ L of PLC ζ -cRNA into the oocytes, most of them became activated, but the activation process was delayed by more than 1 h. With the co-injection method, the rate of blastocyst formation in ROSI embryos was higher (64%) compared with that of the serial injection method (55%). On another note, when SCNT was performed using the co-injection method, the cloned offspring were obtained with a higher success rate compared with the serial-injection method. However, in either ROSI or SCNT embryos, the birth rate of offspring via the co-injection method was similar to the Sr activation method. The epigenetic status of ROSI and SCNT zygotes that was examined showed no significant difference among all activation methods. The results indicated that although the PLC ζ -cRNA co-injection method did not improve the production rate of offspring, this method simplified oocyte activation with less damage, and with accurate activation time in individual oocytes, it can be useful for the basic study of oocyte activation and development.

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Introduction

For assisted reproductive technology or animal reproductive biotechnology, oocytes are often activated artificially before or after injecting defective spermatozoa, round spermatids or somatic cell nuclei. To date, several oocyte activation methods have been reported. For example, strontium chloride (SrCl₂) is one of the most efficient activation methods reported for mouse oocytes (Kline & Kline 1992, Bos-Mikich *et al.* 1997); electric pulses or calcium (Ca²⁺) ionophores are more efficient for porcine, bovine or human oocytes. However, artificially activated oocytes usually show poor embryonic development and a lower birth rate compared with natural fertilisation with spermatozoa. The reason is unclear, but this may suggest that, compared with spermatozoa, these artificial activation methods are not suitable for ensuing embryo development.

In mammals, a diffusible sperm-specific phospholipase C ζ (PLC ζ) triggers a series of Ca²⁺ oscillations (Swann & Ozil 1994, Saunders *et al.* 2002, Ozil *et al.* 2005, 2006, Matsu-Ura *et al.* 2019) following sperm–egg plasma membrane fusion. Ca²⁺ oscillation resumes the

second meiosis and extrudes a second polar body from the activated oocytes and forms the male and female pronuclei. It is known that this Ca²⁺ oscillation is derived from the inositol 1,4,5-triphosphate (IP3)-signalling pathway in oocytes, which stimulates IP3 receptors of the endoplasmic reticulum as the intracellular storage space for Ca²⁺ (Miyazaki *et al.* 1992, 1993, Jellerette *et al.* 2000, Yoneda *et al.* 2006). Recently, in the mouse, human, monkey and chicken models, PLC ζ was isolated as a sperm-specific PLC (Cox *et al.* 2002, Saunders *et al.* 2002, Coward *et al.* 2005). Furthermore, the microinjection of PLC ζ derived from sperm extracts or from cRNA into the mouse oocytes was able to trigger Ca²⁺ oscillation similar to that observed during fertilisation by spermatozoa (Saunders *et al.* 2002) and was able to initiate embryonic development to term (Cox *et al.* 2002, Coward *et al.* 2005, Miura *et al.* 2018, Yamamoto *et al.* 2020).

If PLC ζ or PLC ζ -cRNA can activate manipulated oocytes more naturally compared with the chemical activation method, it might be possible to increase the chances of success of the birth rate of offspring from

those oocytes. Previously, it was reported that full-term offspring from round spermatid (ROSI)-derived embryos could be obtained by PLC ζ -cRNA injection (Nakanishi *et al.* 2008, Miura *et al.* 2018). However, the birth rate of offspring from the PLC ζ -cRNA-activated oocytes was very low. One possibility is that the low birth rate may be because of technical reasons, where oocytes were injected with round spermatid and PLC ζ -cRNA separately, which may cause additional damage to the oocytes. If we can inject PLC ζ -cRNA and spermatid or somatic cell nuclei at the same time, it will simplify the manipulation and reduce oocyte damage. Moreover, this method allows us to display the accurate activation process of each individual oocyte because those individual oocytes were activated at the time of PLC ζ -cRNA injection. In this study, we examined whether manipulated oocytes could be activated by PLC ζ -cRNA co-injection and offspring could be obtained from oocytes fertilised with ROSI or somatic cell nuclear transfer (SCNT).

Materials and methods

Animals

ICR mice (8–10 weeks of age) were obtained from SLC Inc. (Hamamatsu, Japan). The surrogate pseudopregnant ICR females that were used as recipients of the embryos were mated with vasectomised ICR males, whose sterility was previously demonstrated. On the day of the experiments or after finishing all experiments, the mice were killed by CO₂ inhalation or cervical dislocation and used for further experiments. All animal experiments followed the Guide for the Care and Use of Laboratory Animals and were approved by the Institutional Committee of Laboratory Animal Experimentation of the University of Yamanashi.

Oocyte preparation

Female mice were super-ovulated by administering 5 IU of equine chorionic gonadotropin, followed by 5 IU of human chorionic gonadotropin (hCG) after 48 h. Cumulus–oocyte complexes (COCs) were collected from the oviducts of females 14–16 h later and were transferred to a Falcon dish containing HEPES–CZB medium (Kimura & Yanagimachi 1995a). To disperse the cumulus, the COCs were transferred into a 100 μ L droplet of HEPES–CZB medium containing 0.1% bovine testicular hyaluronidase for 3 min. The cumulus-free oocytes were washed twice and then moved to a 20 μ L droplet of CZB medium (Chatot *et al.* 1989) for culture. The remaining cumulus cells were used as donor cells.

Plasmid construction and *in vitro* transcription for mouse PLC ζ -cRNA synthesis

cDNA sequences encoding PLC ζ were cloned into pCS2 (Ito *et al.* 2008, 2011) and were used as templates for cRNA synthesis. cRNA was synthesised from the linearised template plasmid by *in vitro* transcription using an mMESSAGE MACHINE sp6 kit (Life Technologies) according to the manufacturer's

instructions. The synthesised cRNA was polyadenylated with a poly(A) tailing kit (Life technologies, AM1350). The cRNA with poly(A) tail was precipitated using lithium chloride and dissolved in nuclease-free water. After measuring the concentration using a NanoDrop ND-1000 spectrophotometer (NanoDrop Technologies, Wilmington, DE, USA), aliquots of 500 ng/ μ L were stored at -80°C until further use.

Microinjection of mouse PLC ζ -cRNA

PLC ζ -cRNA was diluted with 12% (w/v) polyvinylpyrrolidone (PVP, Mr 360 kDa; Wako) to 2, 20 and 100 ng/ μ L before use. Microinjection of PLC ζ -cRNA containing PVP into oocytes was performed using 4–5 μ m inner diameter Piezo-driven micropipettes (Prime Tech, Ibaraki, Japan). Briefly, microinjection was performed in HEPES-buffered CZB on an inverted microscope (Olympus) with a micromanipulator (Narishige, Tokyo, Japan) (Ooga & Wakayama 2017, Yamamoto *et al.* 2020). The zona pellucida and cytosolic membrane were penetrated with a piezo drive. Control oocytes were activated using 5 mM SrCl₂ in Ca²⁺-free CZB medium for 30 min before or after spermatid injection, or without male germ cell.

Diploidization of parthenogenetically activated oocyte

The oocytes that were activated for observing parthenogenetic development were diploidised by adding 5 μ g/mL cytochalasin B (CCB; Calbiochem) to the culture medium for 5–6.5 h after PLC ζ -cRNA injection. After washing, they were further cultured in CZB medium for 96 h as described previously. The oocyte activation rate was determined by the pronuclear formation at 6 h after PLC ζ -cRNA injection and then activated oocytes were cultured for 4 days to parthenogenetically examine blastocyst development.

ROSI with PLC ζ -cRNA

To collect spermatids, the seminiferous tubules of the testes were minced as described previously in a previous study (Ogura *et al.* 1994), except that the cells were suspended in HEPES-buffered CZB medium. A 2 μ L aliquot of the spermatogenic cell suspension was mixed with 18 μ L of PLC ζ -cRNA containing PVP in a micro-manipulation chamber. For ROSI, a round spermatid (which was characterised by its small size (approximately 10 μ m) and the presence of a centrally located, distinct nucleolus) was injected into each oocyte with a small amount of PVP solution, as described previously in a previous study (Kimura & Yanagimachi 1995b, Kishigami *et al.* 2006b). As controls, oocytes were activated by strontium solution before injection (pre-activation), after injection (post-activation) and 1 h after injection (post-late-activation) (Fig. 2A). In some cases, PLC ζ -cRNA was injected separately before ROSI injection (Fig. 2B).

SCNT with PLC ζ -cRNA

The SCNT procedure was performed as described earlier (Wakayama *et al.* 1998, Kishigami *et al.* 2006a, Terashita *et al.* 2012), but the process of injecting donor cumulus cell nuclei

was slightly modified. A 2 μ L aliquot of the cumulus cell suspension was mixed with 18 μ L of PLC ζ -cRNA containing 12 % (w/v) PVP in a micro-manipulation chamber. Then, the cumulus cell nuclei were injected into each oocyte with small amount of PVP solution, as described in a previous study (Wakayama *et al.* 1998). As controls, reconstructed oocytes were activated 30 min to 1 h after cumulus cell injection by strontium solution (original method) or separate PLC ζ -cRNA injections (serial-activation) (Fig. 2C).

Measurement of PLC ζ -cRNA injected volume

When PLC ζ -cRNA was injected into the oocytes, it was immediately dispersed inside the cytoplasm, making it difficult to measure the sphere diameter of injected liquid. Therefore, the injected volume was calculated using the distance between the border line of the medium inside the micropipette and the inner diameter, before and after injection (Fig. 1A). The injected volume was obtained by subtracting the remaining conical volume from the initial volume. However, the volume of PLC ζ -cRNA with spermatid or cumulus cell nucleus cannot be calculated due to the complicated manipulation of the injected pipette. When the spermatid or cumulus cell nuclei were injected into the oocytes using a large pipette (4–5 μ m for spermatid, 5–6 μ m for cumulus cell), because the top priority is to make the oocytes survive after drilling a hole using a large pipette, the co-injected PVP with PLC ζ -cRNA was recovered into the pipette and the border line of the medium

was moved to a different position. To measure the volume of the co-injected liquid, fluorescence dye 'phycoerythrin', (Yamagata *et al.* 2012) instead of PLC ζ -cRNA, was used, and we observed the fluorescence image of the oocytes using fluorescence microscopy (IX71; Olympus Corp.) immediately after the phycoerythrin injection (Fig. 1B). Then, the relative brightness levels in the oocytes were measured using Image J software. The injected nuclei were stained with Hoechst 33342 before injection.

Production of fertilized embryos by intracytoplasmic sperm injection

Intracytoplasmic sperm injection (ICSI) was performed as described previously (Kimura & Yanagimachi 1995a, Ito *et al.* 2019). The sperm head derived from an ICR male mouse was separated from the tail by the application of several piezo-pulses, and the head was then injected into the oocyte derived from an ICR female mouse. After 10 min of recovery at room temperature, the oocyte was cultured in CZB medium for preimplantation development.

In vitro culture and embryo transfer

The oocytes that survived ROSI or SCNT were incubated in CZB medium at 37 C in 5% CO₂. Pronucleus formation was checked 6 h after activation/injection. Embryos at the two-cell stage were transferred to a day 0.5 pseudopregnant mouse that

Figure 1 Measurement of injected volume of PLC ζ -cRNA into oocyte. To measure the volume of the injected liquid, phycoerythrin, rather than PLC ζ -cRNA, was injected into the oocyte and observed using fluorescence microscopy. (A) For the serial injection method, the tip of the pipette was less than 1 μ m and the distance between the border line of the medium inside the micropipette before and after injection was used as the height of the pipette cone. The injected volume was calculated by subtracting the remaining conical volume from the initial volume. (B) For the co-injection method, the injected volume was detected using the brightness of phycoerythrin, using Image J software. The white arrow shows the border line of the liquid before and after injection. The white arrowhead shows the brightness of the injected phycoerythrin inside the oocytes. The blue arrowhead shows the cumulus cell nucleus stained by Hoechst.

Figure 2 Schematic diagram of activation procedure in each experiment and morphology of male nucleus in each activation stage. (A, B and C) Schematic diagram of activation procedure. (A) Four different activation methods before (Pre-activation) or after ROSI (Post-activation and Post-late-activation) by Sr treatment or co-injection of PLC ζ -cRNA with round spermatid (Co-injection). Those zygotes were then stained and observed at 30 min, 1 h or 4 h after activation treatment. Arrows indicated start time of activation treatment. (B) Three different activation methods after ROSI by Sr treatment (Post-activation), co-injection of PLC ζ -cRNA with round spermatid (Co-injection) or serial-injection of PLC ζ -cRNA after ROSI (Serial-injection). Those zygotes were cultured for 4 days to examine the rate of blastocyst formation and its quality or they were transferred into the recipient at the two-cell stage for determining whether the full-term development will occur. (C) Three different activation methods after SCNT by Sr treatment (Original) co-injection of PLC ζ -cRNA with somatic cell nucleus (Co-injection) or serial-injection of PLC ζ -cRNA after SCNT (Serial-injection). The SCNT embryos were examined and the epigenetic status was determined at one-cell-stage, or they were transferred into a recipient during the two-cell stage to determine the occurrence of full-term development. (D, E and F) Morphology of male nucleus of ROSI zygote at four different activation methods (see A). Those ROSI zygotes were stained at 30 min (D), 1 h (E) or 4 h (F) after activation. All experiments were repeated at least twice and involved 18–44 zygotes, respectively.

had been mated with a vasectomized male the night before transfer. Approximately 10–15 embryos were transferred into each oviduct. At day 18.5 of gestation, the offspring were delivered by caesarean section and allowed to mature. The remaining unused embryos were cultured up to 4 days to evaluate their potential to develop toward the blastocyst stage.

Observation of nuclear status by Aceto-orcein staining

To observe the nuclear status of the injected round spermatid nucleus after 30 min, 1 h or 4 h, some zygotes were removed from the dish, mounted between a slide and coverslip, fixed with 2.5% (v/v) glutaraldehyde for 5 min, stained with aceto-orcein and examined under a phase-contrast microscope (Yanagida *et al.* 1991) (Fig. 2A).

Blastocyst immunostaining

The immunofluorescence staining of blastocysts was performed as described to evaluate the quality of blastocysts derived from PLCζ-cRNA injected embryos (Wakayama *et al.* 2017, Yamamoto *et al.* 2020). The following primary antibodies were used for immunostaining: anti-CDX2 rabbit MAB (1:500; BioGenex, Fremont, USA) to detect the TE cells and an anti-NANOG mouse polyclonal antibody (1:500; Abcam Inc) to detect the ICM cells. The following secondary antibodies were used for immunostaining: Alexa Fluor 568-labelled goat anti-rabbit IgG (1:500; Thermo Fisher Scientific) and Alexa Fluor 488-labelled goat anti-mouse IgG (1:500; Thermo Fisher Scientific). DNA was stained with DAPI (2 µg/mL; Molecular Probes).

Observation of epigenetic abnormalities in ROSI and SCNT embryos

The oocytes at 10 h after activation were fixed and permeabilized in PBS containing 4% paraformaldehyde and 0.2% (v/v) Triton X-100 (Nacalai Tesque, Kyoto, Japan) for 20 min. The activated zygotes were washed twice in PBS containing 0.25% (w/v) BSA (Nacalai Tesque, Kyoto, Japan) (PBS–BSA) for 15 min each. The embryos were then incubated with the primary antibodies: rabbit polyclonal anti-H3K4 me3 for SCNT embryos (1:1000 dilution; Abcam Inc), rabbit polyclonal anti-H3K9 me3 for ROSI and SCNT embryos (1:1000 dilution; Abcam Inc) or rabbit polyclonal anti-H3K27 me3 for SCNT embryos (1:1000 dilution; Millipore Inc) in PBS–BSA overnight at 4°C. After the embryos were washed twice in PBS–BSA for 15 min, they

were incubated at 25°C for 1 h with dye-conjugated secondary antibodies, namely Alexa Fluor 568-labelled goat anti-rabbit IgG (Molecular Probes Inc). After washing the embryos twice in PBS–BSA, they were mounted on a glass slide in Vectashield anti-bleaching solution (Vector Laboratories, Burlingame, CA, USA) containing 3 µg/mL of DAPI (Molecular Probes Inc). Subsequently, the serial images were obtained using fluorescence confocal microscopy (FV-1000; Olympus Corp.). For the ROSI zygote, because the female pronucleus did not show any difference, only the male pronucleus was observed, and the brightness of its perinucleolar region was judged as 0–3. For the SCNT zygote, the relative H3K4 me3, H3K9 me3 and H3K27 me3 levels were measured using Image J software.

Statistical analysis

The rates of blastocyst formation and offspring development were evaluated using Chi-squared tests, and a *P* value of <0.01 was considered to represent a statistically significant difference.

Results

Appropriate concentration of PLCζ-cRNA for oocytes activation and development of parthenogenetic or cloned embryo

To examine the optimal concentration of PLCζ-cRNA for the parthenogenetic activation of mouse oocyte, 2, 20 and 100 ng/µL of PLCζ-cRNA dissolved in PVP were injected into oocytes using a relatively large injection pipette (4–5 µm of inner diameter), which is usually used for round spermatid injection (Table 1). When 2 ng/µL of PLCζ-cRNA was injected, only 48% of oocytes were activated; however, when 20 or 100 ng/µL of PLCζ-cRNA was injected, 82% or 88% of oocytes were activated with two pronuclei, respectively. The parthenogenetically activated oocytes were then cultured for 4 days to examine the developmental potential *in vitro*. When oocytes were activated by 2 or 100 ng/µL PLCζ-cRNA injection, only 36% or 16% of embryo developed to the blastocyst, respectively. However, when 20 ng/µL of PLCζ-cRNA was injected into mouse oocytes, 63% of the embryos continued and developed into blastocysts.

Table 1 Activation and developmental rate of parthenogenetic or cloned oocytes using different concentration of PLCζ-cRNA.

	Concentration of cRNA (ng/µL)					
	Parthogenesis			Somatic cell nuclear transfer		
	2	20	100	2	20	100
No. of oocytes injected	54	54	55	45	54	46
No. of survived oocytes (%) [*]	46 (85)	50 (93)	51 (93)	33 (67)	42 (75)	25 (56)
No. of PN formed embryos (%) [*]	22 (48)	41 (82)	45 (88)	27 (82)	40 (95)	24 (96)
No. of two-cell embryos at 24 h (%) ^{**}	22 (100)	38 (93)	37 (82)	26 (96)	36 (90)	21 (88)
No. of 4–8 cell embryos at 48 h (%) ^{**}	19 (86)	37 (90)	33 (73)	12 (44)	27 (68)	13 (54)
No. of morula/blastocysts at 72–96 h (%) ^{**}	8 (36)	26 (63)	7 (16)	6 (22) ^b	19 (48) ^a	5 (21) ^b

^{*}Percentage relative to no. of oocytes injected; ^{**}Percentage relative to no. of PN formed embryos. a vs b: *P* < 0.05. PN, pronucleus.

Similar results were obtained when somatic cell nuclei were injected using a relatively large injection pipette (5–6 μm of inner diameter) with 2, 20 or 100 ng/ μL of PLC ζ -cRNA into the enucleated oocytes (Table 1). Although the activation rate was high in all concentrations of PLC ζ -cRNA (82–96%), the rate of blastocyst development was higher when 20 ng/ μL of PLC ζ -cRNA was used (48%) compared with 2 or 100 ng/ μL of PLC ζ -cRNA (21–22%).

In the authors' previous study, although the volume of the injected PLC ζ -cRNA varied between oocytes due to the hand of the experimentalist and the slightly different sizes of pipettes in each experiment, the distance between the border line of the medium inside the micropipette before and after injection was 100 μm to 200 μm (Fig. 1A showed maximum volume of injection). The inner diameter of the pipette was 5–6 μm . From these data, the injected volume of PLC ζ -cRNA was 3–5 pL. On another note, when PLC ζ -cRNA was co-injected with spermatid or cumulus cell nuclei using a large pipette, extra PVP with PLC ζ -cRNA was recovered for the survival of the injected oocytes and only a small amount of PLC ζ -cRNA remained in the oocytes. As shown in Fig. 1A and B, when the injected amount was observed with phycoerythrin, the brightness of the co-injected oocytes was much less than the serial-injected oocytes. When the brightness of the whole oocytes was compared between serial and co-injection method (Fig. 1A and B) by Image J software, the co-injected oocytes exhibited a brightness that was 27 times lower than the serial-injected oocytes. Based on these results, although our previous study showed that 1 ng/ μL of PLC ζ -cRNA was the appropriate concentration for oocyte activation and the same lot of PLC ζ -cRNA was used for both previous and present studies, we decided to use 20 ng/ μL for the following experiments.

Activation and pronuclear formation speed of ROSI zygote by PLC ζ -cRNA co-injection

To compare the rate of activation and pronuclear formation of the co-injection method, ROSI zygotes were activated by Sr activation method before (Pre-activation), immediately after (Post-activation) or 1 h after (Post-late-activation) spermatid injection (Fig. 2A). Then, those activated zygotes were stained at 30 min, 1 h and 4 h after activation.

When the ROSI zygotes were observed 30 min after Sr activation or PLC ζ -cRNA injection, the rate of premature chromosome condensation (PCC) was lower in the oocytes that underwent the co-injection method (64%) than the post- or post-late activation method (71–85%), which suggests that the start of oocyte activation with the PLC ζ -cRNA co-injection was earlier than the other methods and already progressed to the metaphase II stage, which was similar to the pre-activation method

(Fig. 2D). When the ROSI zygotes were observed 1 h after activation, the decondensation rate in co-injected zygotes was lower (17%) than pre- and post-activated zygotes (50–59%), which suggests that the activation process of the oocytes co-injected with PLC ζ -cRNA was delayed and is similar to the post-late activation method (11%) (Fig. 2E). When the pronuclear formation rate was observed at 4 h after activation/injection, the zygotes derived from the co-injection method was lower (6%) than the post-late activated zygotes (41%) (1 h delayed compared with pre-activation method), which suggests that the activation process of the co-injection method was delayed by more than 1 h (Fig. 2F). Note: it is known that the male pronucleus of ROSI zygote by original activation method (pre-activation) were smaller and difficult to identify compared with ICSI zygote (Ogura *et al.* 1994); therefore, the rate of pronuclei in pre-activation method was not high (Fig. 2F).

In vitro and in vivo development of ROSI embryos generated by co-injection of PLC ζ -cRNA

To investigate whether this delay affects the development of embryos, we cultured embryos for 4 days and examined the quality of the zygote and the blastocysts. In this experiment, ROSI zygotes were generated by post-activation using SrCl₂, co-injection of PLC ζ -cRNA with spermatid or PLC ζ -cRNA that was separately injected before spermatid injection (serial-injection) (Fig. 2B).

As shown in Fig. 3A and Table 2, the *in vitro* developmental rate in all stages was always high in the post-activation and co-injection method compared with the serial-injection method. However, when embryos were cultured for 4 days, the rate of blastocyst development in all groups was similar (55–65%) (Fig. 3A and B). When the cell number of blastocysts were examined between post-activation and co-injection method, which is an indicator for detecting the quality of the blastocyst, the cell ratio of ICM and TE of blastocyst were similar irrespective of the activation method used (Fig. 3C and D). Then, the two-cell embryos were transplanted into pseudopregnant females, and the birth rates of offspring were examined. As shown in Fig. 2E, F and Table 3, live offspring were obtained in all groups with similar success rates (26%, 24% and 24% in post-activation, co-injected and serial-injection method, respectively). In addition, we examined whether the activation methods would affect the epigenetic status of the ROSI zygotes. Thus, the levels of H3K9 me3 of ROSI zygotes, which are known to show in ROSI zygotes with specific abnormalities (Kishigami *et al.* 2004), were compared between ICSI, post-activation using Sr Cl₂ and serial- and co-injection methods. As shown in Fig. 3G and H, the level of H3K9 me3 did not differ among all the ROSI zygotes, but largely different compared with the ICSI zygotes.

Figure 3 *In vitro* and *in vivo* development of ROSI embryos among different activation methods. (A and B) *In vitro* development of ROSI embryos. Those embryos were generated by three different activation methods and observed during the two-cell stage (2C: 24 h after activation), 4 or 8-cell stage (4C–8C: 48 h after activation), morula stage (Moru: 72 h after activation) and blastocyst stage (Bla: 96 h after activation). (C and D) The quality of ROSI embryos at the blastocyst stage as shown by immunostaining. These embryos were generated by two different activation methods and cultured for 96 h. (C) Images show a blastocyst stained with DAPI in blue (upper left), inner cell mass (ICM) with anti-Nanog antibody in green (upper right), trophoblast (TE) with anti-Cdx2 antibody in red (lower left) and merged images (lower right). (D) The average number of TE and ICM in blastocysts. (E and F) Full-term development of ROSI zygote. (E) The birth rate was compared between three different activation methods. (F) Live offspring derived from ROSI embryos activated by co-injection. Post-act: oocytes were activated after ROSI; Co-in: co-injection of PLC ζ -cRNA with round spermatid; Serial: serial injection of PLC ζ -cRNA after ROSI. (G and H) The level of H3K9 me3 in one-cell-stage ROSI zygotes was examined by immunofluorescence. (G) The upper left image shows the zygote derived from ICSI, the upper right image shows the zygote derived from post-activation method using strontium, the bottom left image shows the zygote derived from serial activation and the bottom right image shows the co-injection of PLC ζ -cRNA with a round spermatid. (H) The brightness of the perinucleolar region of the male pronucleus. The brightness of this region was judged as 0–3. All experiments were repeated three times and involved 17–25 zygotes, respectively.

Table 2 Preimplantation development of embryos fertilised with a spermatid and activated post-activation using SrCl₂, co-injection or serial injection using PLC ζ -cRNA.

	Treatment		
	Post-activation	Co-injection	Serial- injection
No. of used oocytes	33	60	67
No. survived oocytes after spermatid injection	24 (73)	44 (73)	50 (75)
No. survived oocytes after PLC ζ -cRNA injection	-	-	44 (66)
No. of activated oocyte (%)*	24 (100)	44 (100)	44 (100)
No. of two-PN formed embryos at 6 h (%)*	17 (71)	33 (75)	29 (66)
No. of two-cell embryos at 24 h (%)**	17 (100)	32 (97)	23 (79)
No. of 4–8-cell embryos at 48 h (%)**	16 (94) ^a	27 (82) ^a	17 (59) ^b
No. of morula/blastocysts at 72 h (%)**	14 (82)	25 (76)	16 (55)
No. of expanded blastocysts at 96 h (%)**	11 (65)	21 (64)	16 (55)

*Percentage relative to no. of survived oocytes; **Percentage relative to no. of PN formed embryos. a vs b: $P < 0.05$. PN, pronucleus.

In vitro and in vivo development of SCNT embryos generated by co-injection of PLC ζ -cRNA

Finally, we examined whether PLC ζ -cRNA co-injection method improved the success rate of mouse cloning. As shown in Fig. 2C and Table 4, when PLC ζ -cRNA was co-injected with cumulus cell nuclei, live and healthy offspring were obtained (Fig. 4A and B). The success rate was similar (1.8%) to the original Sr activation method (2.0%), which was similar to a previous report where ICR mouse was used (Tanabe *et al.* 2017). However, when PLC ζ -cRNA was injected separately following SCNT, only one cloned offspring (0.5%) was obtained. The body and placenta weight were not different between all groups.

In addition, we examined whether the activation methods will affect the epigenetic status of SCNT embryos. Thus, the levels of H3K4 me3, H3K9 me3 and H3K27 me3 of SCNT zygotes, which are known to show in cloned embryos with specific abnormalities (Matoba *et al.* 2014, Xie *et al.* 2016, Hormanseder *et al.* 2017), were compared between the co-injection and original methods. As shown in Fig. 4F and G, the level of H3K4 me3, H3K9 me3 and H3K27me3 did not differ between both methods.

Discussion

Recently, many artificial activation methods, including the use of ethanol, electrical stimulation, and calcium ionophore, have been developed (Marcus 1990), but most parthenogenetic agents cause a monotonic increase in Ca²⁺. Moreover, Sr²⁺-containing media induce repetitive intracellular Ca²⁺ oscillations similar to those that occur during normal fertilization (Kline & Kline 1992, Bos-Mikich *et al.* 1995); hence, the artificial methods do not completely mimic the process induced by spermatozoa (Jellertette *et al.* 2000). Therefore, the method of PLC ζ -cRNA injection into oocytes is considered as the best candidate to mimic the fertilization process completely. Previously, we reported that the optimal concentration of mouse PLC ζ -cRNA for mouse oocyte activation was 1 ng/ μ L (Yamamoto *et al.* 2020). In the present study, the optimal concentration of mouse PLC ζ -cRNA for oocyte activation was 20 ng/ μ L, which was 20 times higher than what was previously reported. Moreover, the PLC ζ -cRNA was injected using 4–6 μ m inner diameter (ID) injection pipette, which was larger than the one used in the previous study (less than 1 μ m ID) because round spermatid or somatic cell nuclei were injected at the same time. It is known

Table 3 Full-term development of embryos fertilised with a spermatid and activated post-activation using SrCl₂, co-injection or serial-injection using PLC ζ -cRNA.

	Treatment		
	Post-activation	Co-injection	Serial-injection
No. of oocytes injected	177	200	75
No. of survived oocytes after spermatid injection	128 (72)	155 (78)	55 (73)
No. of survived oocytes after PLC ζ -cRNA injection	-	-	50 (67)
No. of activated oocytes (%)*	128 (100)	155 (100)	50 (100)
No. of two-PN formed embryos (%)*	58 (45)	91 (59)	34 (68)
No. of two-cell embryos at 24 h (%)**	50 (86)	69 (76)	31 (91)
No. of transferred embryos (recipient)	50 (5)	67 (6)	25 (3)
No. of offspring (%)***	13 (26)	16 (24)	6 (24)

*Percentage relative to no. of survived oocytes; **Percentage relative to no. of PN formed embryos; ***Percentage relative to no. of transferred embryos. PN, pronucleus.

Table 4 Full-term development of cloned embryos activated by Original using SrCl₂, co-injection or serial injection using PLC ζ -cRNA.

	Treatment		
	Original	Co-injection	Serial-injection
No. of enucleated oocytes	187	259	505
No. of oocytes surviving after nuclear injection (%)	117 (63)	190 (73)	302(60)
No. of oocytes surviving after PLC ζ -cRNA injection (%)	-	-	253(50)
No. of oocytes with PN formed (%)	115 (98)*	179 (94)*	237 (94)*
No. of embryos developed to two-cell stage (%)**	102 (89)	167 (93)	207 (87)
Recipient	6	7	9
No. of offspring (%)***	2 (2.0)	3 (1.8)	1 (0.5)
Body weight (g)	1.82	2.07	2.07
Placental weight (g)	0.34	0.40	0.31

All two-cell embryos were transferred into recipient females.

*Percentage relative to no. of survived oocytes; **Percentage relative to no. of PN formed embryos; ***Percentage relative to no. of transferred embryos.

PN, pronucleus.

that a high concentration of PLC ζ -cRNA injection can cause embryonic developmental arrest (Cox *et al.* 2002). Thus, it seems that the co-injection method used excessively high concentration and volume of PLC ζ -cRNA for oocyte activation compared with the previous method. When the injected volume was measured, the previous method (Yamamoto *et al.* 2020) and the serial injection method were approximately 3–5 pL to each of the oocytes (Fig. 1A). However, in the ROSI or SCNT experiments, the excess amount of the PVP solution was removed before pulling out the pipette to ensure the survival of the oocytes. Although this skill depended on the experimentalist, it is probable that most of the injected PLC ζ -cRNA was removed with the PVP, as the remaining PVP was approximately 27 times lower than the serial injection method (Fig. 1). In addition, PLC ζ -cRNA was dissolved in a PVP solution rather than in water, which may have affected the function of PLC ζ -cRNA for activating the oocytes. Therefore, the optimal concentration of PLC ζ -cRNA was more than that used in the previous study.

On another note, the PLC ζ -cRNA co-injection method showed that the oocyte activation started normally; however, the process speed was slow and delayed by more than 1 h compared with the 'original' pre-activation method, which used strontium. One possibility is that the translation of the PLC ζ protein from the PLC ζ -cRNA started immediately after co-injection and the oocytes progressed from the metaphase to the metaphase II stage; therefore, the rate of PCC of spermatid nuclei was lower than that in the post-activation method. However, it may take time to translate enough concentrations of PLC ζ protein from the PLC ζ -cRNA. Moreover, Ca²⁺ oscillation regulated the formation of pronuclei (Marangos *et al.* 2003, Kashir *et al.* 2014). The lower concentration of the PLC ζ -cRNA caused lower frequencies of Ca²⁺ oscillation (Saunders *et al.* 2002) and delayed the pronuclear formation (Nakanishi *et al.* 2008), and the formation of pronuclei of PLC ζ -cRNA-co-injected oocytes at an early stage was delayed compared with the original or even the post-late activation method. However, the rate of

pronuclear formation in the co-injection method leveled with the post-activation or serial-activation method when observed at 6 h after injection (Table 2). This may suggest that, even if the pronuclear formation was delayed by the co-injection of PLC ζ -cRNA, the nuclei or chromatin was already loose and easy to decondense because it can avoid the formation of solid PCC at the time of nuclear injection (Fig. 2D). This result suggests that the timing between activation and pronuclear formation is not so important at least within the timing shown in this study, because healthy offspring were obtained by the co-injection method with the same success rate as those of the control and serial-injected group.

It is known that the gap between the activation of oocytes and fertilisation with spermatozoa critically affects the success rate of offspring. When the gap between activation and fertilisation was increased up to 2 h, the birth rate significantly decreased (Kishigami *et al.* 2004). Therefore, to increase the success rate of offspring, it might be better to reduce the gap between activation and spermatid injection. How do we reduce the gap and accelerate the activation process speed using the PLC ζ -cRNA co-injection method? Previously, we tried to activate mouse oocytes by equine PLC ζ -cRNA because it is known that equine PLC ζ -cRNA has the strongest potential for activation in mammalian oocytes. Although the activation potential of equine PLC ζ -cRNA in mouse oocytes were ten times higher than that in the mouse PLC ζ -cRNA, the activated embryos sustained damage due to unknown reasons (Yamamoto *et al.* 2020). On the other hand, when the concentration of mouse PLC ζ -cRNA used was ten times higher, most of the oocytes became activated, but the rate of development into the blastocyst stage was significantly decreased because extra-cRNA in zygote caused damage to the embryo (Cox *et al.* 2002, Miura *et al.* 2018). Thus, it is difficult to accelerate the activation speed by changing the type of PLC ζ -cRNA or the concentration of PLC ζ -cRNA. However, in this study, we succeeded in generating offspring with similar birth rates as compared with the original method, which suggest that the delay of

Figure 4 Full-term development of cloned embryos and the epigenetic status of cloned zygote between different activation methods. (A and B) Cloned offspring derived from co-injection method. (C, D and E) The rate of full-term development of cloned mouse (C), body weight (D) and placenta weight (E) compared between three different activation methods. (F and G) The level of H3K4 me3, H3K9 me3 and H3K27 me3 in one-cell-stage cloned embryos were examined by immunofluorescence. (F) The upper showed the original activation method using strontium, and the bottom showed the co-injection of PLC ζ -cRNA with somatic cell nuclei. (G) The intensities of immunofluorescence relative to those of DAPI. The intensities of cloned embryos generated by co-injection method were compared to the original strontium activation method. All experiments were repeated at least twice and involved 11–28 embryos, respectively.

activation by PLC ζ -cRNA co-injection method (more than 1 h; Tables 3 and 4) was within range and had no effect on producing offspring.

For the *in vitro* development of ROSI embryos, the co-injection method showed higher developmental rate compared with the serial-injection method (Fig. 3A), which suggest that the serial-injection method not only increases the need for manipulation of oocytes, but also causes damage to the embryos. However, when embryos were transferred to recipient females, the birth rate of embryos between the co-injection method and the serial-injection method were not different, which may

suggest that the damage caused by the serial-injection did not affect fetal development. On the other hand, the offspring rate of ROSI embryos by the PLC ζ -cRNA co-injection method was similar to the original method, and oocytes were activated more naturally without sustaining damage (Table 3). Thus, the lower birth rate of ROSI embryos was not because of the method of oocyte activation used.

In the SCNT experiments, the co-injection method showed higher birth rate compared with the serial-injection method, but it was slightly lower than the original method (Table 4). Previously, we tried to

understand the importance of oocyte activation method for mice cloning (Kishikawa *et al.* 1999). In that study, reconstructed oocytes were activated by spermatozoa and live cloned fetuses were obtained, but there was a decrease in the success rate compared with the original method. However, oocytes were manipulated four times in that study, which included enucleation, somatic cell nuclear injection, fertilisation with spermatozoa and removal of male pronucleus after full activation. It is likely that those four instances of serial manipulation caused a lot of damages to the oocytes. Moreover, it was difficult to distinguish between male pronuclei and cloned pseudo-pronuclei, and in some cases, it is possible that the cloned pseudo-pronuclei were removed instead of the male pronuclei. Therefore, that study failed to demonstrate whether the method used for oocyte activation is important for embryonic development or not. In this study, reconstructed oocytes were activated by PLC ζ -cRNA co-injection, similar to sperm injection but without causing damage to the oocytes. However, the success rate of cloned mice did not increase compared with the original Sr activation method.

In addition, we examined whether the difference among the oocyte activation methods will alter the epigenetic status of the ROSI and SCNT zygotes, because epigenetic abnormalities observed in the ROSI and SCNT zygotes are considered as the main reason for low birth rate (Kishigami *et al.* 2006b, Matoba *et al.* 2014, Xie *et al.* 2016, Hormanseder *et al.* 2017). However, unfortunately, the epigenetic abnormalities of the ROSI and SCNT zygotes did not correct when it was activated by the PLC ζ -cRNA co-injection method. This suggests that the activation process is not related to the abnormality of the ROSI and SCNT zygotes.

Thus, in this study, we failed to increase the success rate of ROSI or SCNT offspring; however, the co-injection of PLC ζ -cRNA has several merits compared with the original method. For example, in the original ROSI protocol, 5–10 oocytes were usually moved to the activation medium and kept for 30 min and moved to a culture medium for 15 min as rest time. Then, round spermatid were injected into those activated oocytes within 15 min, and this process was repeated until all the oocytes have been used (Kimura & Yanagimachi 1995b, Kishigami *et al.* 2006b). Moreover, the frequent treatment of the activation medium carries the risk of activating the remaining oocytes before use. However, as shown here, the PLC ζ -cRNA co-injection method simplified the ROSI techniques considerably. It does not require any additional treatment for oocyte activation, which can be achieved with only one injection. For SCNT, reconstructed oocytes were usually pooled until the nuclear transfer of all oocytes are finished and then activated by Sr medium at the same time, irrespective of the period of reprogramming in oocytes cytoplasm. On the other hand, the co-injection method activates each reconstructed oocyte one by one. Although there is about a 1 h lag between co-injection and oocyte activation,

this relatively accurate activation time of each oocyte might contribute to the understanding of the mechanisms associated with nuclear reprogramming and embryo development. Thus, the co-injection method can be useful for assisted reproductive technology and for the basic study of oocyte activation and embryonic development.

Declaration of interest

The authors declare that there is no conflict of interest that could be perceived as prejudicing the impartiality of the research reported.

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Author contribution statement

J I, M O and T W conceived and designed the study. N H, S W, R I, M O and T W performed experiments, analysed the data and interpreted the results. S W, J I, M O and T W obtained funding. T W wrote the manuscript. All authors read and edited the manuscript.

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