Application of auxin-inducible degron technology to mouse oocyte activation with PLCζ

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Abstract. In mammals, spermatozoa activate oocytes by triggering a series of intracellular Ca²⁺ oscillations with phospholipase C zeta (PLCζ), a sperm-borne oocyte-activating factor. Because the introduction of PLCζ alone can induce oocyte activation, it might be a promising reagent for assisted reproductive technologies. To test this possibility, we injected human PLCζ (hPLCζ) mRNA into mouse oocytes at different concentrations. We observed the oocyte activation and subsequent embryonic development. Efficient oocyte activation and embryonic development to the blastocyst stage was achieved only with a limited range of mRNA concentrations (0.1 ng/μl). Higher concentrations of mRNA caused developmental arrest of most embryos, suggesting that excessive PLCζ protein might be harmful at this stage. In a second series of experiments, we aimed to regulate the PLCζ protein concentration in oocytes by applying auxin-inducible degron (AID) technology that allows rapid degradation of the target protein tagged with AID induced by auxin. Injection of the hPLCζ protein tagged with AID and enhanced green fluorescent protein (hPLCζ-AID-EGFP) demonstrated that high EGFP expression levels at the late 1-cell stage were efficiently reduced by auxin treatment, suggesting efficient hPLCζ degradation by this system. Furthermore, the defective development observed with higher concentrations of hPLCζ-AID-EGFP mRNA was rescued following auxin treatment. Full-term offspring were obtained by round spermatid injection with optimized hPLCζ-AID activation. Our results indicate that this AID technology can be applied to regulate the protein levels in mouse oocytes and that our optimized PLCζ system could be used for assisted fertilization in mammals.

Key words: Auxin-inducible degron technology, Mouse, Oocyte activation, Round spermatid injection, Sperm-specific phospholipase C zeta
Animals
B6D2F1 (C57BL/6N × DBA/2) strain mice were obtained from Japan SLC (Hamamatsu, Japan) and C57BL/6J and ICR strain mice were obtained from CLEA Japan (Tokyo, Japan) at 8–10 weeks of age. They were housed with food and water ad libitum under controlled temperature (24 ± 1°C), humidity (55 ± 2%), and lighting conditions (daily light period, 0700 to 2100 h). The animal experiments described here were approved by the Animal Experimentation Committee at the RIKEN Tsukuba Institute, and were performed in accordance with the committee’s guiding principles.

Materials and Methods

Animals
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Media
Potassium-modified simplex optimized medium (KSOM) [22] was used for embryo culture in a humidified atmosphere of 5% CO₂ in air at 37°C. HEPES-buffered KSOM was used for gamete handling and round spermatid injection (ROSI) at room temperature. Ca²⁺-free KSOM containing 3 mM strontium chloride was used for activating oocytes without using human PLCζ (hPLCζ) injection as a positive control.

Oocyte collection
Female B6D2F1 mice were induced to superovulate by an injection of 7.5 IU equine chorionic gonadotropin (Nippon Zenyaku Kogyo, Fukushima, Japan), followed 48 h later by 7.5 IU of human chorionic gonadotropin (hCG) (ASKA Pharmaceutical, Tokyo, Japan). At 16 h after injecting hCG, cumulus-oocyte complexes were collected from the oviducts and placed in a KSOM droplet containing 0.1% bovine testicular hyaluronidase (#385931; Calbiochem, La Jolla, CA, USA) for 3 min. The cumulus-free oocytes were washed five times and cultured in fresh KSOM until use.

Plasmid construction and in vitro transcription for human PLCζ mRNA synthesis
We amplified a cDNA sequence encoding hPLCζ [10, 15] and subcloned it into pcDNA3.1-p(A)83 vectors [23]. According to previous reports, hPLCζ had a stronger Ca²⁺ oscillation-inducing potential than mouse PLCζ [10, 15, 17]. In the present study, therefore, we used hPLCζ mRNA, expecting that the smaller amount of the PLCζ protein required for oocyte activation would cause rapid elimination of PLCζ from auxin-treated oocytes. OsTIR1 (TIR1 derived from Oryza sativa) and an AID fragment [1–132 amino acids (aa) of the 1–229 aa full-length AID) [24, 25] from OsTIR1-9Myc::IRES::aid-CENPH (pMK106; RDB08469; RIKEN BioResource Center, Tsukuba, Japan) [18] were also ligated to pcDNA3.1-p(A)83 vectors. These pcDNA3.1-p(A)83 vectors and pcDNA3.1-EGFP-p(A)83 [23] were used as template plasmids for in vitro transcription. To generate the template plasmid of a fused hPLCζ-AID–EGFP mRNA, DNA fragments of hPLCζ, AID, and EGFP were amplified by polymerase chain reaction (PCR) from the linearized template plasmids and cloned into a pcDNA3.1-poly(A)83 plasmid by using a Gibson Assembly Cloning Kit (# E5510; New England BioLabs, Beverly, MA, USA). RNA was synthesized from the linearized template plasmids by in vitro transcription using a mMESSAGE mMACHINE T7 Ultra Kit (Life Technologies # AM1345; Thermo Fisher Scientific, Waltham, MA, USA) following the manufacturer’s instructions. The synthesized mRNAs were precipitated by lithium chloride and dissolved in nuclease-free water. After measuring the concentration using a NanoDrop ND-1000 spectrophotometer (NanoDrop Technologies, Wilmington, DE, USA), aliquots were stored at −80°C until use.

mRNA injection
Microinjection of mRNA was carried out using a Piezo-driven micropipette (Prime Tech, Ibaraki, Japan). Several droplets (~4 µl) of HEPES-buffered KSOM with or without 10% polyvinylpyrrolidone (PVP) were placed on the bottom of the microinjection chamber and covered with mineral oil. The PVP-containing droplets were used for washing the tip of the micropipette for mRNA injection. We applied Piezo pulses to break the zona pellucida and oolemma [4]. Intact hPLCζ or fused hPLCζ-AID-EGFP mRNA (10⁻⁴–10⁻² ng/µl) together with OsTIR1 mRNA (1000 ng/µl) were injected into oocytes in HEPES-buffered KSOM. Each oocyte received ~10 pl mRNA. After mRNA injection, the oocytes were kept in HEPES-buffered KSOM at room temperature (24°C) for 10 min. Degradation of the hPLCζ-AID-EGFP protein was induced by 500 μM indole-3-acetic acid (IAA, I5148; Sigma-Aldrich, St Louis, MO, USA), a natural auxin. The mRNA-injected oocytes were placed in fresh KSOM with or without IAA and cultured for 96 h at 37°C under 5% CO₂ in air. The oocytes activated for observation of parthenogenetic development were diploidized by adding 5 μg/ml cytochalasin B (CCB; #250223; Calbiochem) to the culture medium for 0.5–6.5 h after mRNA injection. Control parthenogenetically activated embryos were generated by culturing oocytes in activation medium containing 5 μg/ml CCB for 1 h followed by KSOM containing 5 μg/ml CCB for 5 h. After washing, they were further cultured in KSOM for 90 h as described above.

Fluorescence microscopy
To examine the levels of EGFP expression, fluorescent images were acquired using an inverted fluorescence microscope (IX70; Olympus, Tokyo, Japan). Oocytes or embryos were transferred to HEPES-buffered KSOM with or without IAA in a glass-bottomed dish on a thermo plate (Tokai Hit, Shizuoka, Japan) on the microscope stage. Fluorescence intensity was measured using ImageJ software (https://imagej.nih.gov/ij/download.html).
**Round spermatid injection**

Spermatogenic cells were isolated mechanically from the seminiferous tubules of C57BL/6J males as previously described [26]. Cell suspensions were placed in a PVP droplet prepared on the manipulation chamber as described above. B6D2F1 oocytes were injected with \(10^9\) ng/\(\mu l\) hPLCζ-AID-EGFP and 1000 ng/\(\mu l\) OsTIR1 mRNAs and cultured in KSOM containing IAA. At 3–5 h later, oocytes proceeded to telophase II with protrusion of the second polar body. They were then injected with round spermatids in HEPES-buffered KSOM containing IAA. The injected oocytes were kept in HEPES-buffered KSOM with IAA at room temperature (24°C) for 10 min before reincubation in KSOM with IAA at 37°C under 5% CO\(_2\) in humidified air.

**Embryo transfer**

Two-cell embryos produced by ROSI were transferred into the oviducts of day 1 pseudopregnant females of ICR strain, which had been mated with a vasectomized male the night before transfer. For anesthesia, 2.5% tribromoethanol (0.014 ml/g of body weight) was administered by intraperitoneal injection. In the evening on days 18 and 19, each female mouse was injected subcutaneously with 2 mg of progesterone to avert spontaneous delivery. On the morning of day 20, the recipient mice were examined for the numbers of implantation sites, and live offspring were retrieved by Caesarean section.

**Statistical analysis**

Fisher’s exact test, followed by Benjamini-Hochberg procedure [27] for multiple comparison tests, was performed for statistical analysis in Figs 1 and 2. Statistical significance was assessed at \(P < 0.05\). Quantitative data in Fig. 3C are shown as the mean ± standard error of the mean.

**Results**

**Injection of hPLCζ mRNA into mouse oocytes induces activation and embryonic development in a dose-dependent manner**

First, to examine the dose-dependent effect of hPLCζ mRNA on oocyte activation and embryonic development, we microinjected different concentrations of hPLCζ mRNA (\(10^{-4}\)–\(10^2\) ng/\(\mu l\)) into mouse MII oocytes (Fig. 1A). Oocyte activation was determined by pronuclear (PN) formation at 6.5 h after mRNA injection. Injection of low concentrations of hPLCζ mRNA (\(10^{-4}\)–\(10^{-2}\) ng/\(\mu l\)) induced activation in only 0–10% of the oocytes injected (Fig. 1B). Consequently, these oocytes showed very low 2-cell (8–13%) and blastocyst (5–13%) formation rates per injected oocytes (Fig. 1C, D). In the \(10^{-2}\) ng/\(\mu l\) group, the 2-cell rate was higher than the activation rate, probably reflecting delayed activation of oocytes by a low concentration of PLCζ mRNA, as previously reported [15]. In contrast, 92–100% of oocytes were activated after the injection of \(10^{-1}\)–\(10^2\) ng/\(\mu l\) hPLCζ mRNA, consistent with a previous report [17]. Among them, those injected with moderate concentrations of mRNA (\(10^{-1}\) and \(10^0\) ng/\(\mu l\)) showed the highest 2-cell and blastocyst formation rates in all the experimental groups. However, most (87–100%) of the oocytes injected with higher concentrations of mRNA (\(10^0\) and \(10^2\) ng/\(\mu l\)) showed arrested development at the 1-cell stage even though they underwent 100% activation. These results indicated that, although injection of hPLCζ mRNA can efficiently induce activation of mouse oocytes at concentrations of \(10^{-1}\) ng/\(\mu l\) or higher, an excessive amount of the hPLCζ protein compromises subsequent embryonic development.

**Effect of fused hPLCζ–AID–EGFP mRNA injection without auxin**

To evaluate whether the fusion of AID and EGFP tags to the hPLCζ protein or injection of an additional factor, OsTIR1, would affect the activation property of hPLCζ, we performed a series of
injection experiments without auxin treatment. Injections of different concentrations of hPLCζ-AID-EGFP (10⁻⁴–10⁻² ng/μl) together with OsTIR1 (1000 ng/μl) mRNAs into mouse MII oocytes resulted in activation and embryonic development in the patterns nearly identical to those with intact hPLCζ alone (Fig. 2). Specifically, the activation efficiency and subsequent developmental rate remained low by injection of 10⁻⁴–10⁻² ng/μl hPLCζ-AID-EGFP mRNA (Fig. 2B–D). In these groups, the 2-cell rates were higher than the activation rates, as observed in the group of 10⁻² ng/μl hPLCζ mRNA injection above (Fig. 1B, C). However, 82–100% of oocytes were activated upon injection of ≥ 10⁻¹ ng/μl hPLCζ-AID-EGFP mRNA and those injected with 10⁻¹ ng/μl showed a significantly higher blastocyst formation rate (69%) than that of other groups. Oocytes injected with higher concentrations of mRNA (10⁰ and 10² ng/μl) arrested before the blastocyst stage. These results indicate that neither fusion of hPLCζ with AID and EGFP, nor coinjection with OsTIR1 mRNA affected the activity of the hPLCζ protein.

**EGFP monitoring for auxin-induced degradation of hPLCζ-AID-EGFP in mouse oocytes**

Next, we tested whether the degradation of hPLCζ-AID-EGFP in the mRNA-injected oocytes could be induced by auxin using EGFP fluorescence as an indicator, according a method previously reported (Fig. 3A) [18, 25]. We used purified IAA to induce AID degradation [18, 25]. In the absence of IAA, the EGFP fluorescence level in the injected oocytes increased with time and plateaued at around 4 h after mRNA injection (Fig. 3B, C). The EGFP signal was detected in the cytoplasm (Fig. 3B), consistent with a previous report [15]. In contrast, treatment of the mRNA-injected oocytes with IAA greatly reduced the EGFP signals, although slight EGFP signals continued to be detectable until at least 8 h after injection. Noninjected control oocytes activated with strontium chloride did not show any EGFP fluorescence. These data suggest that the hPLCζ-AID-EGFP protein can be degraded efficiently by auxin treatment in mouse oocytes.

**Auxin treatment improved the development rate of oocytes injected with high concentrations of hPLCζ-AID-EGFP mRNA**

To examine the biological consequences of auxin-induced degradation of hPLCζ-AID-EGFP protein, we cultured the mRNA-injected oocytes with or without IAA and observed their subsequent embryonic development until the blastocyst stage. We first confirmed that IAA treatment does not affect the development of oocytes activated by strontium chloride (Fig. 4A–C). When oocytes were activated with 10⁻¹ ng/μl hPLCζ-AID-EGFP mRNA, IAA treatment significantly reduced the rates of oocyte activation and development into 2-cells and blastocysts (Fig. 4). The oocytes injected with 10⁰ ng/μl fused mRNA and cultured with IAA also showed a significantly reduced activation rate from 100% to 90% (Fig. 4A). However, after in vitro culture, they developed into blastocysts at a rate of 69%, which was significantly higher than that of the non-IAA controls (43%; Fig. 4C and D). This was also the case with oocytes injected with 10² ng/μl fused mRNA; the 2-cell and blastocyst rates were significantly improved by IAA treatment (96% and 64%, respectively; Fig. 4C and D).

**Full-term development of embryos generated by ROSI using oocytes activated with hPLCζ-AID-EGFP and cultured with IAA**

Finally, to examine the feasibility of using this hPLCζ-AID system for assisted fertilization, we performed ROSI coupled with hPLCζ-AID-mediated oocyte activation and observed postimplantation development following embryo transfer. We microinjected mRNAs for hPLCζ-AID-EGFP (10⁰ ng/μl) and OsTIR1 to oocytes and treated them with IAA for 3–5 h. We performed ROSI using the oocytes that had proceeded to telophase II with extrusion of the second...
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polar body (Fig. 5A). Most (86%) of the oocytes surviving ROSI developed to the 2-cell stage on the following day (Fig. 5B). After transfer of these 2-cell embryos into pseudopregnant female mice, 53% (30/57) were implanted and 36% (24/57; 11 males and 13 females) developed into normal offspring at day 19.5 (Fig. 5B, C). These data suggest that this hPLCζ–AID system could be applied practically for assisted fertilization in mammals.

Discussion

Here, we showed that although hPLCζ mRNA injection can efficiently induce activation in mouse oocytes, subsequent embryonic development is impaired by excessive accumulation of the hPLCζ protein in the oocytes, for unknown reasons (Figs. 1, 2). We employed the AID technology to facilitate degradation of the excess hPLCζ protein and confirmed its efficacy by the significant improvement in development of embryos injected with high concentrations of hPLCζ mRNA (Figs. 3, 4). We also demonstrated that this hPLCζ–AID strategy could be applied to ROSI to produce pups (Fig. 5).

The AID technology, harnessing an auxin-dependent protein degradation system originally found in plants, has been applied to a wide variety of cell types from many different species, including mammals [18, 25]. AID-mediated degradation requires the SCF E3 ubiquitin ligase, which functions together with OsTIR. We show...
here that this AID-mediated degradation works in mouse oocytes as well, suggesting the presence of SCF E3 ligase in mammalian oocytes. However, our EGFP fluorescence monitoring revealed that AID failed to completely abolish the hPLCζ proteins upon auxin treatment (Fig. 3B, C). This is in clear contrast with previous reports showing almost complete depletion of AID-tagged protein by auxin treatment in NIH3T3 and embryonic stem cells [18, 25]. One potential explanation for this difference is that we introduced PLCζ as a form of mRNA, whereas other studies used plasmids or DNA transfection and established stable lines [18, 25, 28–30]. In contrast to DNA/plasmid transfection approaches, where mRNA is transcribed continuously from the inserted DNA fragment by endogenous transcriptional machinery, our mRNA injection method endowed massive copies of mRNA at a single time point so the amount of proteins translated from injected hPLCζ mRNA might have overcome the degradation ability of the AID system for complete depletion. Alternatively, the SCF E3 ligase in mouse oocytes might be less active than in other cell types. Further optimization of this approach (e.g., injection of AID-tagged protein, coinjection with SCF E3 ligase, and change of the timing of IAA treatment) might allow complete degradation of AID-tagged proteins in mouse oocytes and more efficient embryonic development.

Fig. 4. Activation and development rates of mouse oocytes injected with hPLCζ-AID-EGFP and cultured with IAA. A–C) Rates of 2-PN (6.5 hpi), 2-cell (24 hpi), and blastocyst (96 hpi) formation per oocytes that survived at 6.5 hpi. Oocytes were injected with 10–1–101 ng/μl hPLCζ-AID-EGFP and 1000 ng/μl OsTIR1 mRNAs and cultured with CCB (from 0.5–6.5 hpi) and IAA (from 0–96 hpi). SrCl2 (+), oocytes activated with strontium chloride. The data for oocytes cultured without IAA are the same as those in Fig. 2B–D. * P < 0.05. D) Representative images of blastocysts (96 hpi) derived from oocytes injected with hPLCζ-AID-EGFP and OsTIR1 mRNAs and cultured with or without IAA. Scale bars, 200 μm.

Fig. 5. Round spermatid injection (ROSI) into mouse oocytes injected with hPLCζ-AID-EGFP mRNA and cultured with IAA. A) Schematic representation of round spermatid injection into mouse telophase II (TII) oocytes injected with 106 ng/μl hPLCζ-AID-EGFP and 1000 ng/μl OsTIR1 mRNAs and cultured with IAA. B) Two-cell rate (24 hpi) per oocytes that survived just after ROSI, and the implantation and birth rates per 2-cell embryos transferred at 24 hpi. C) A litter of newborn offspring produced by ROSI from oocytes activated with hPLCζ-AID-EGFP.
Although Nakanishi et al. [32] succeeded in obtaining mouse pups using oocytes activated with a short form of mouse PLCζ mRNA, the efficiency was remarkably low (7% per transfer; B6D2F1 or ICR × B6D2F1). These facts suggest that, compared with these previous systems, our PLCζ-AID system has a similar or even better potential for activating mouse oocytes without compromising subsequent developmental capacity.

It is known that the efficiencies of oocyte activation methods differ between species. For example, strontium chloride is one of the most efficient activation methods for mouse oocytes, but electric pulses or calcium ionophores are more often used for porcine and bovine oocytes [33, 34]. Moreover, in some mammals, including the common marmoset, only little information about oocyte activation methods has been reported [35, 36]. In contrast, because the Ca2+ oscillation-inducing activity of PLCζ is conserved among many vertebrates [10, 15], PLCζ injection could be widely used for oocyte activation. Therefore, our novel AID method could contribute to promoting the efficiency of the PLCζ activation method, advancing the development of mammalian assisted reproductive technology in general.

In summary, we have demonstrated that the AID technology could induce the protein degradation in mammalian oocyte by injecting AID-tagged hPLCζ and OsTIR mRNAs. We also showed that the hPLCζ protein translated from high concentrations of injected mRNA inhibited embryonic development, but this could be rescued by degrading the hPLCζ protein using our AID technology. With this technology, the embryo development and pup formation rates of hPLCζ-injected oocytes could be improved in many species of animals.

Acknowledgments

We thank Dr Azusa Inoue for technical advice about the AID technology. We also thank Toshiko Tomishima for help with maintaining the mouse strains. This research was supported by KAKENHI grants 17H07367 (KM), 15H04854 (JI), and 25112009 (AO), and Brain/MINDS from the Japan Agency for Medical Research and development (AO).

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