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—Original Article—

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 Ca^{2+} 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

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The fertilization of oocytes by spermatozoa is the earliest event of the life cycle. Following fertilization, the oocyte initiates repetitive rises of intracellular Ca^{2+} concentration (Ca^{2+} oscillations) and resumes its meiotic cell cycle to exit from metaphase II (MII). This dynamic event, which is called oocyte activation, is known to be crucial for subsequent proper embryonic development [1–3]. Because spermatogenic cells acquire their oocyte-activating capacity as they develop in the testes, earlier spermatogenic cells, such as round spermatids, have little or no such capacity, depending on the species. Therefore, when immature spermatogenic cells are used for fertilizing oocytes by microinjection, oocyte activation by artificial stimuli is necessary in many cases. In mice, for example, several methods have been used to activate oocytes, such as electric pulses or strontium chloride treatment [4–6].

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In 2002, Saunders *et al*. [7] identified the novel sperm-specific phospholipase C zeta (PLCζ) as a candidate for a sperm-borne oocyte-activating factor that is capable of inducing Ca^{2+} oscillations [8]. Subsequent studies revealed that PLCζ is conserved across mammalian species and mutations in the gene encoding PLCζ are linked to human infertility [9–12]. Indeed, sperm extracts depleted of PLCζ or sperm derived from *Plcz1*$^{-/-}$ male mice failed to trigger Ca^{2+} oscillations [7, 13, 14]. Importantly, the major structural basis for the function of PLCζ seems to be common across mammalian species because PLCζ mRNA from one species can induce the activation of oocytes of different species [10, 15]. However, it is interesting to note that the potential of inducing Ca^{2+} oscillations and nuclear-cytoplasmic localization patterns of this factor appear to be species-dependent [15, 16]. The absolute amount of PLCζ protein has been suggested to be critical for the proper oocyte activation and embryonic development. A low amount of PLCζ was unable to induce oocyte activation, whereas a high concentration of PLCζ mRNA injection caused developmental arrest of embryos [17].

Auxin-inducible degron (AID) technology is an auxin-inducible protein degradation system that enables temporal control of protein stability [18]. In plants, auxin binds to the transport inhibitor response 1 (TIR1) protein and promotes binding of a functional Skp1-Cullin-

F-box (SCF) ubiquitin ligase, which forms a complex with TIR, to target proteins harboring a small tag, called the AID tag [19, 20]. The targeted proteins are polyubiquitinated by SCF-TIR1 complexes and are quickly degraded by proteasomes. Nishimura *et al*. [18] converted the plant-specific AID system to a technology enabling auxin-dependent degradation of target proteins tagged with AID even in non-plant cells, including mammalian cells, contributing to the analysis of protein function [21].

Here, we applied AID technology to the degradation of the PLCζ protein translated from mRNA injected into mouse oocytes and examined the effects of its degradation on embryonic development. We found that the AID technology could induce degradation of the targeted PLCζ protein and that embryonic demise because of a high concentration of PLCζ protein could be rescued by auxin-dependent PLCζ degradation.

Materials and Methods

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.

Media

Potassium-modified simplex optimized medium (KSOM) [22] was used for embryo culture in a humidified atmosphere of 5% CO_2 in air at 37°C. HEPES-buffered KSOM was used for gamete handling and round spermatid injection (ROSI) at room temperature. Ca^{2+}-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 Ca^{2+} 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% CO_2 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 10^0 ng/µl hPLCζ-AID-EGFP and 1000 ng/µl 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/µl) 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/µl) 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/µl 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/µl hPLCζ mRNA, consistent with a previous report [17]. Among them, those injected with moderate concentrations of mRNA (10^{-1} and 10^0 ng/µl) 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 (10^1 and 10^2 ng/µl) 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/µl or higher, an excessive amount of the hPLCζ protein compromises subsequent embryonic development.

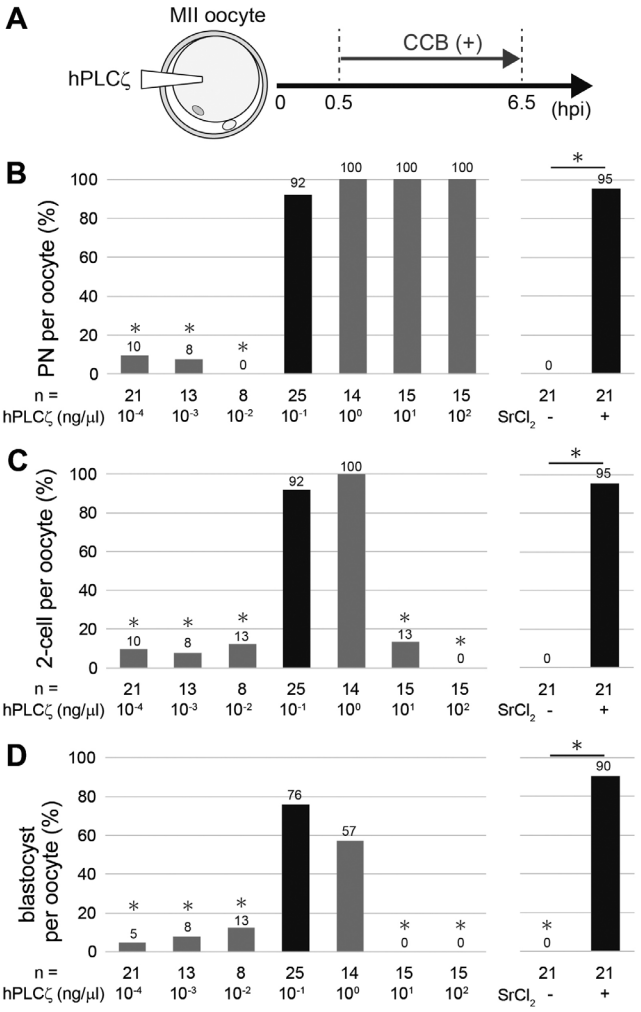

Fig. 1. Activation and development rates of mouse oocytes injected with hPLCζ mRNA. A) Schematic representation of intact hPLCζ mRNA injection into metaphase II (MII) stage mouse oocytes and their culture with CCB from 0.5–6.5 h post mRNA injection (hpi) to induce parthenogenesis. B–D) Rates of 2-pronuclei (PN; 6.5 hpi), 2-cell (24 hpi), and blastocyst (96 hpi) formation per oocytes that survived at 6.5 hpi. The oocytes were injected with 10^{-4}–10^2 ng/µl hPLCζ mRNA. * $P < 0.05$, *vs*. 10^{-1} ng/µl group. Key: $SrCl_2$ (–), oocytes without any activation treatment; $SrCl_2$ (+), oocytes activated with strontium chloride. * $P < 0.05$.

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

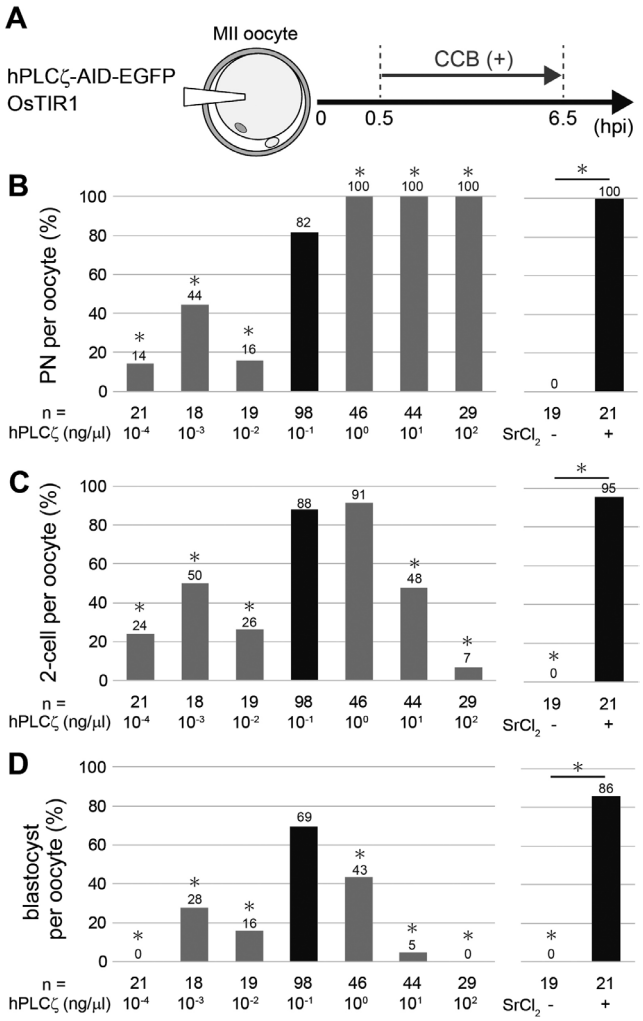

Fig. 2. Activation and development rates of mouse oocytes injected with fused hPLCζ-AID-EGFP mRNA. A) Schematic representation of hPLCζ-AID-EGFP and OsTIR1 mRNA injections into MII oocytes and their culture with CCB from 0.5–6.5 hpi for inducing parthenogenesis. B–D) Rates of 2-PN formation (6.5 hpi), 2-cell (24 hpi), and blastocyst (96 hpi) development per oocytes that survived at 6.5 hpi. Oocytes were injected with 10^{-4}–10^{2} ng/μl hPLCζ-AID-EGFP and 1000 ng/μl OsTIR1 mRNAs. * $P < 0.05$, *vs*. 10^{-1} ng/μl group. Key: $SrCl_2$ (–), oocytes without any activation treatment; $SrCl_2$ (+), oocytes activated with strontium chloride. * $P < 0.05$.

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 (10^{1} and 10^{2} 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 10^{0} 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 10^{1} 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 (10^{0} 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

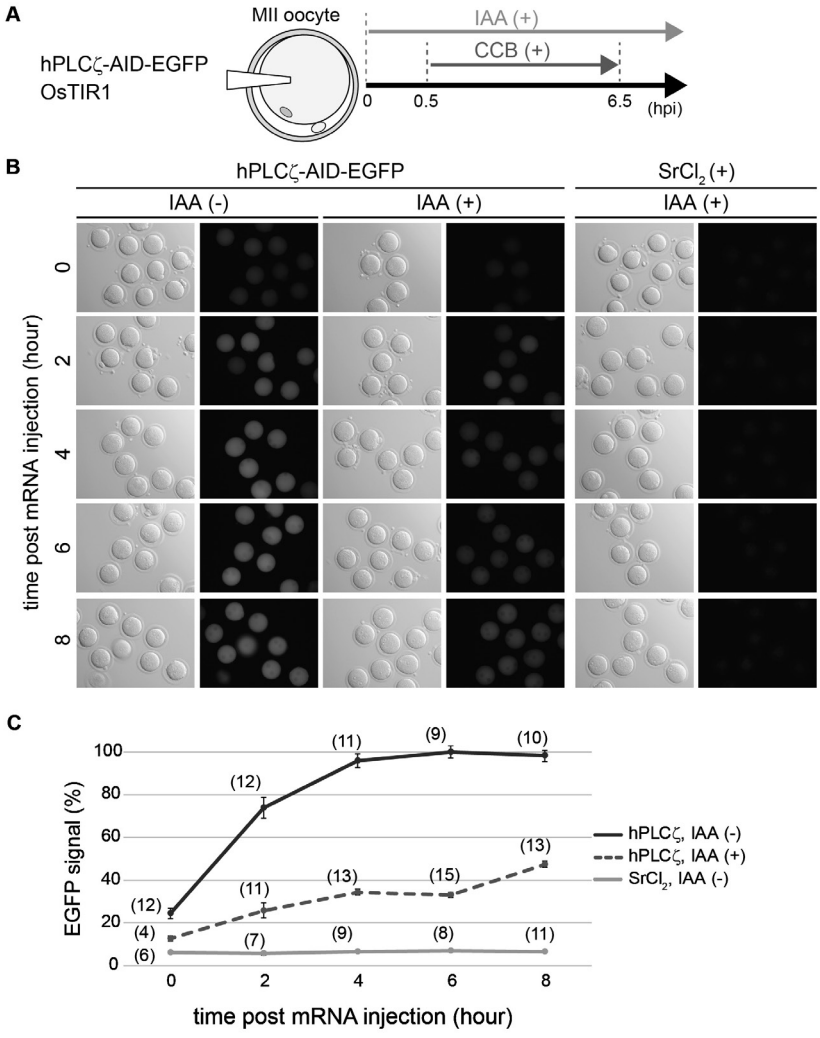

Fig. 3. Auxin-induced degradation of hPLCζ-AID-EGFP protein in mouse oocytes *in vitro*. A) Schematic representation of hPLCζ-AID-EGFP and OsTIR1 mRNA injections into MII oocytes and their culture with CCB from 0.5–6.5 hpi and with IAA from 0–8 hpi. B) Microscopic observations over time of EGFP fluorescence in oocytes injected with 10^2 ng/µl hPLCζ-AID-EGFP and 1000 ng/µl OsTIR1 mRNAs and cultured with or without IAA [IAA (+) or IAA (–)]. The concentration of hPLCζ-AID-EGFP mRNA was higher than the optimal concentration for oocyte activation to detect the changes of the EGFP signal intensity. (C) EGFP signal intensities in oocytes injected with hPLCζ-AID-EGFP and OsTIR1 mRNAs and cultured with or without IAA. $SrCl_2$ (+), oocytes activated with strontium chloride. The numbers in parentheses indicate the number of oocytes examined at each stage. The intensity level of the hPLCζ, IAA (–) group at 6 hpi was set as 100% on the y-axis because it showed the highest level of EGFP intensity among the experimental groups.

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

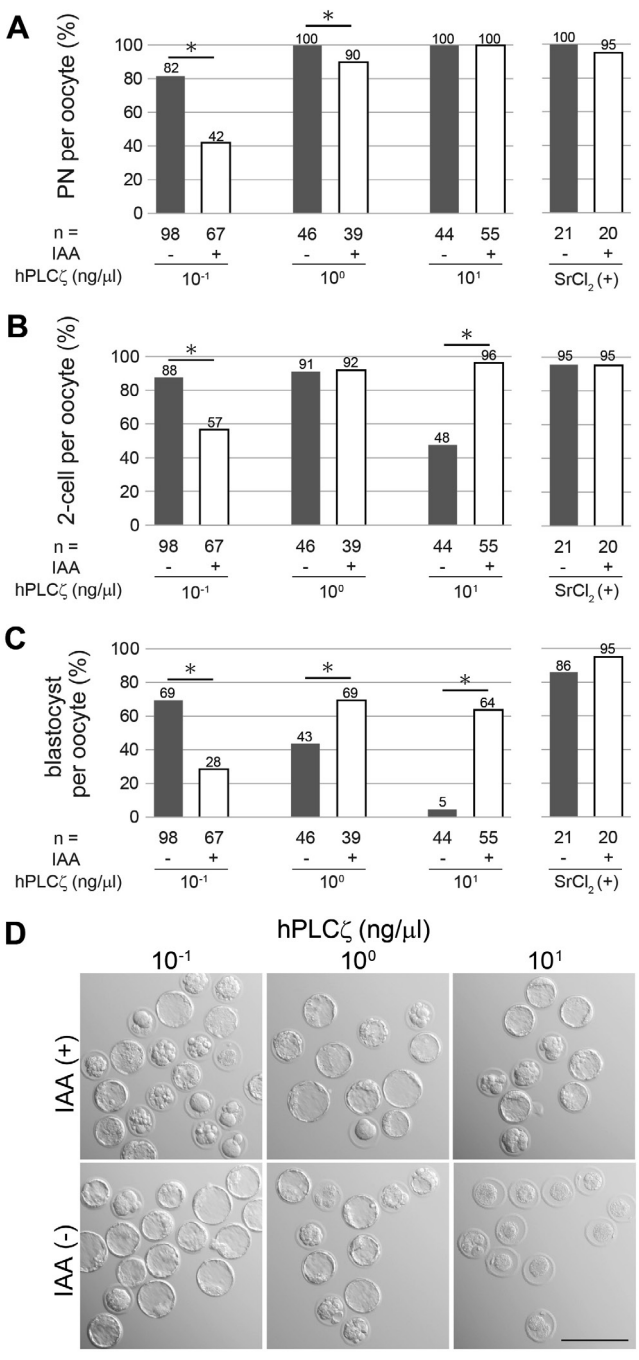

Fig. 4. Activation and development rates of mouse oocytes injected with hPLCζ-AID-EGFP and cultured with IAA. A–C) Rates of 2-PN (6.5 hpi), 2-cell (24 hpi), and blastocyst (96 hpi) formation per oocytes that survived at 6.5 hpi. Oocytes were injected with 10^{-1}–10^{1} ng/μl hPLCζ-AID-EGFP and 1000 ng/μl OsTIR1 mRNAs and cultured with CCB (from 0.5–6.5 hpi) and IAA (from 0–96 hpi). $SrCl_2$ (+), 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.

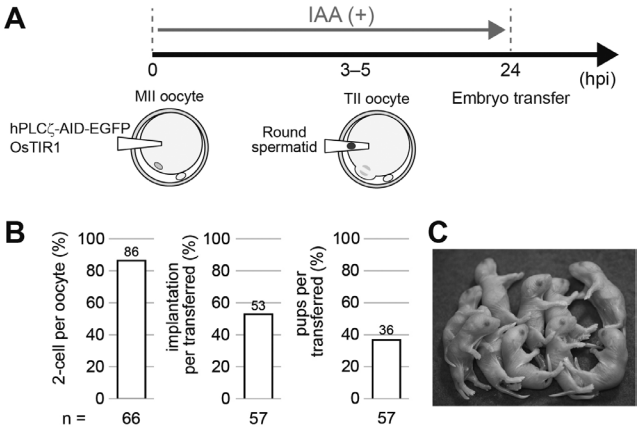

Fig. 5. Round spermatid injection (ROSI) into mouse oocytes injected with hPLCζ-AID-EGFP mRNA and cultured with IAA. A) Schematic representation of round spermatid injection into mouse telophase II (TII) oocytes injected with 10^{0} ng/μl hPLCζ-AID-EGFP and 1000 ng/μl OsTIR1 mRNAs and cultured with IAA. 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.

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.

Importantly, we succeeded in obtaining live pups by ROSI coupled with PLCζ-AID-mediated oocyte activation at relatively high efficiency (36%; B6D2F1 × C57BL/6J; Fig. 5). This pup form rate is similar to or even better than those in previous studies obtained via ROSI using electric pulses (28%; B6D2F1 × B6D2F1) [4] or strontium chloride (29%; B6D2F1 × B6D2F1) [31] for activation of the B6D2F1 oocytes as in the present study. Also, this pup rate was higher than that of ROSI with the intact hPLCζ mRNA (2×10^{0} ng/μl; 16% per transfer; B6D2F1 × C57BL/6J or ICR, data not shown).

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 Ca^{2+} 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.

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