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RESEARCH ARTICLE

TECHNIQUES AND RESOURCES

The birth of quail chicks after intracytoplasmic sperm injection

Shusei Mizushima[1], Gen Hiyama[1], Kogiku Shiba[2], Kazuo Inaba[2], Hideo Dohra[3], Tamao Ono[4], Kiyoshi Shimada[5] and Tomohiro Sasanami[1,*]

ABSTRACT

Intracytoplasmic sperm injection (ICSI) has been successfully used to produce offspring in several mammalian species including humans. However, ICSI has not been successful in birds because of the size of the egg and difficulty in mimicking the physiological polyspermy that takes place during normal fertilization. Microsurgical injection of 20 or more spermatozoa into an egg is detrimental to its survival. Here, we report that injection of a single spermatozoon with a small volume of sperm extract (SE) or its components led to the development and birth of healthy quail chicks. SE contains three factors – phospholipase Cζ (PLCZ), aconitate hydratase (AH) and citrate synthase (CS) – all of which are essential for full egg activation and subsequent embryonic development. PLCZ induces an immediate, transient Ca^{2+} rise required for the resumption of meiosis. AH and CS are required for long-lasting, spiral-like Ca^{2+} oscillations within the activated egg, which are essential for cell cycle progression in early embryos. We also found that co-injection of cRNAs encoding PLCZ, AH and CS support the full development of ICSI-generated zygotes without the use of SE. These findings will aid our understanding of the mechanism of avian fertilization and embryo development, as well as assisting in the manipulation of the avian genome and the production of transgenic and cloned birds.

KEY WORDS: Intracytoplasmic sperm injection, Physiological polyspermy, Quail, Phospholipase Cζ, Aconitate hydratase, Citrate synthase

INTRODUCTION

Fertilization is crucial for zygote formation in sexual reproduction. In most animals, a single fertilizing spermatozoon evokes a temporal rise in intracellular Ca^{2+} ($[Ca^{2+}]i$) in an egg upon gamete fusion, and this $[Ca^{2+}]i$ plays essential roles in egg activation (Stricker, 1999; Runft et al., 2002). In birds, many (20-60) spermatozoa enter each egg before activating it (Fofanova, 1965; Nakanishi et al., 1990; Wishart, 1997). This polyspermic fertilization is a key characteristic of some oviparous animals, such as birds and reptiles, with large eggs. In mammals, phospholipase Cζ (PLCZ) has been identified as the sperm-borne egg-activating factor, as it induces a series of $[Ca^{2+}]i$ oscillations in the egg (Saunders et al., 2002). Interestingly, microinjection of PLCZ collected from chicken (Coward et al., 2005) or medaka (Coward et al., 2011) spermatozoa can also induce inositol trisphosphate (IP_3)-dependent Ca^{2+} oscillations in the mouse egg. In the newt, citrate synthase (CS) has been identified as another sperm-borne egg-activating factor (Harada et al., 2007).

Intracytoplasmic sperm injection (ICSI) has been very useful for studying the mechanisms of egg activation in mammals and urodeles (Yanagimachi, 2005; Morozumi et al., 2006; Iwao, 2012). Furthermore, this technology has contributed to the production of live offspring, and has also been applied clinically to humans. By contrast, ICSI has not yet been successful in producing offspring in birds because the natural polyspermic fertilization is difficult to mimic (Hrabia et al., 2003; Mizushima et al., 2009; Mizushima et al., 2010). All quail embryos produced by the injection of a single spermatozoon die before embryonic stage 6 (Mizushima et al., 2008; nomenclature as used in Hamburger and Hamilton, 1951). As mentioned above, during normal fertilization in birds, multiple spermatozoa enter one egg before activation is complete (Fofanova, 1965; Nakanishi et al., 1990; Wishart, 1997). Therefore, we reasoned that a single spermatozoon does not contain sufficient egg-activating material to induce full activation of an egg. To test this hypothesis, we studied whether avian sperm extract (SE) was able to enhance the development of ICSI-generated quail zygotes. We also tried to identify the chemical nature of SE components that would activate the quail egg and support subsequent embryonic development of the zygotes.

RESULTS

We first observed spatiotemporal changes in the $[Ca^{2+}]i$ rise in Fluo-8H AM-loaded quail eggs. Microinjection of 50 fmol IP_3 into an unfertilized egg evoked an immediate increase in $[Ca^{2+}]i$; the Ca^{2+} signal propagated over the germinal disk and then peaked ∼5 min after the injection (Fig. 1A,B; supplementary material Movie 1). Thereafter, $[Ca^{2+}]i$ decreased gradually and returned to the basal level within 30 min after injection. Microinjection of 2 ng SE per egg, which is equivalent to 200 spermatozoa per egg, evoked multiple, long-lasting spiral-like Ca^{2+} waves that followed an initial transient Ca^{2+} rise resembling the $[Ca^{2+}]i$ increase in IP_3-injected eggs (Fig. 1C,D; supplementary material Movie 2). These repetitive spiral-like Ca^{2+} waves each originated from the injection site and continued for at least 1 h. The Ca^{2+} waves did not have a simple propagation pattern, but had irregular and complicated waveforms (supplementary material Movie 2). The mean fluorescence intensity of the entire germinal disk area oscillated with a mean interspike interval of ∼1 min (Fig. 1D). Although the injection of 50 fmol IP_3 into the egg caused very small oscillations within ∼20 min of injection, these oscillations differed from the spiral-like Ca^{2+} oscillations in that there were significant differences in the mean amplitude of these oscillations (supplementary material Fig. S1). When fluorescence intensities were captured from different areas within the germinal disk, reciprocal repeating oscillations were observed (inset in Fig. 1D). These results indicated that SE contains a novel egg-activating factor that induces the spiral-like Ca^{2+}

[1]Department of Applied Biological Chemistry, Faculty of Agriculture, Shizuoka University, 836 Ohya, Shizuoka 422-8529, Japan. [2]Shimoda Marine Research Center, University of Tsukuba, 5-10-1 Shimoda, Shizuoka 415-0025, Japan. [3]Research Institute of Green Science and Technology, Shizuoka University, 836 Ohya, Shizuoka 422-8529, Japan. [4]Department of Food Production Science, Faculty of Agriculture, Shinshu University, 8304 Minamiminowa, Kamiina, Nagano 399-4598, Japan. [5]WCU Major in Biomodulation, Department of Agricultural Biotechnology, College of Agriculture and Life Sciences, Seoul National University, Seoul, Republic of Korea.

*Author for correspondence (atsasan@ipc.shizuoka.ac.jp)

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DEVELOPMENT

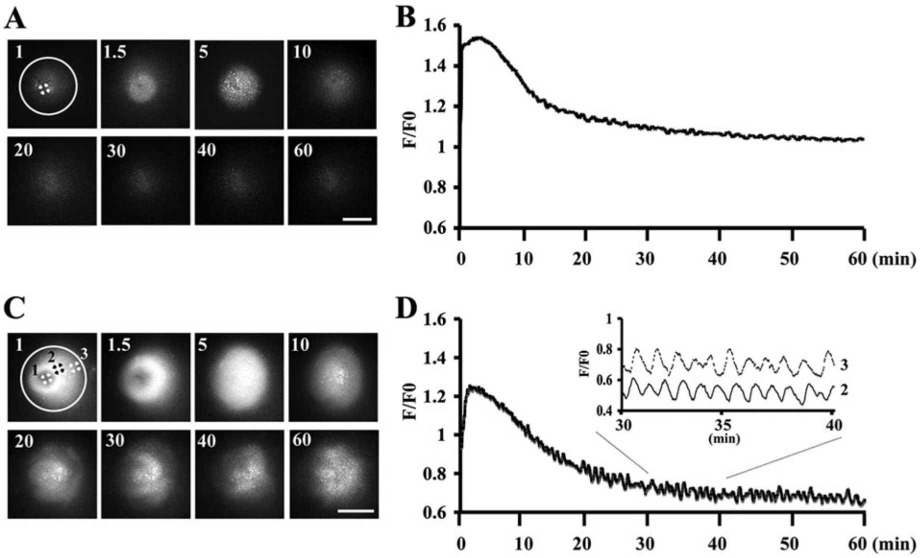

Fig. 1. Changes in [Ca^{2+}]*i* in quail eggs injected with IP_3 or SE. (A,C) Fluorescent images of eggs injected with 50 fmol IP_3 (A) or 2 ng SE (C). Time (min) after injection is indicated top left. The solid circle outlines the germinal disk. Area 1, the microinjection site; 2 and 3, the areas used for the [Ca^{2+}]*i* timecourse measurements in D. Scale bars: 1 mm. (B,D) Timecourse measurements of the average Ca^{2+} levels in the germinal disk (solid circles) in A and C are shown. The inset in D shows the timecourse measurements of Ca^{2+} levels in areas 2 and 3 in C. A and B are representative of results obtained from eight experimental repeats and C and D from six experimental repeats. See also supplementary material Movies 1 and 2.

oscillations, which differ from those seen with PLCZ/IP_3-induced [Ca^{2+}]*i* increases. We repeated the SE injection experiment six times (*n*=6) and found that the fundamental patterns in each egg were similar to each other.

To identify the active components that evoked the spiral-like Ca^{2+} oscillations, gel filtration chromatography was used to fractionate SE (Fig. 2A). Only materials in fraction 3 could induce the spiral-like Ca^{2+} oscillations (Fig. 2B,C). The spiral-like Ca^{2+} oscillation-inducing activity in fraction 3 was adsorbed onto *Lens culinaris* agglutinin (LCA)-coated agarose beads (Fig. 2D). SDS-PAGE was then used to compare the components before and after LCA absorption (Fig. 2E). We found that three bands (70, 45 and 30 kDa) bound to the LCA-agarose beads. Liquid chromatography tandem mass spectrometry (LC-MS/MS), *de novo* protein sequencing, and protein identification software (PEAKS) (Ma et al., 2003) were used to identify the proteins. The 70 and 45 kDa proteins were identified as aconitate hydratase (AH) and CS, respectively (supplementary material Table S1). The 30 kDa protein was found to be a mixture of superoxide dismutase (SD), malate dehydrogenase (MD) and AH (supplementary material Table S1).

Neither porcine AH nor porcine CS induced any significant Ca^{2+} release when microinjected individually into quail eggs (data not shown). However, simultaneous injection of these factors induced long-lasting repetitive Ca^{2+} waves similar to those induced by injection of SE (Fig. 2F). Nevertheless, dual microinjections of AH and CS did not generate an immediate (i.e. within 5 min) elevation

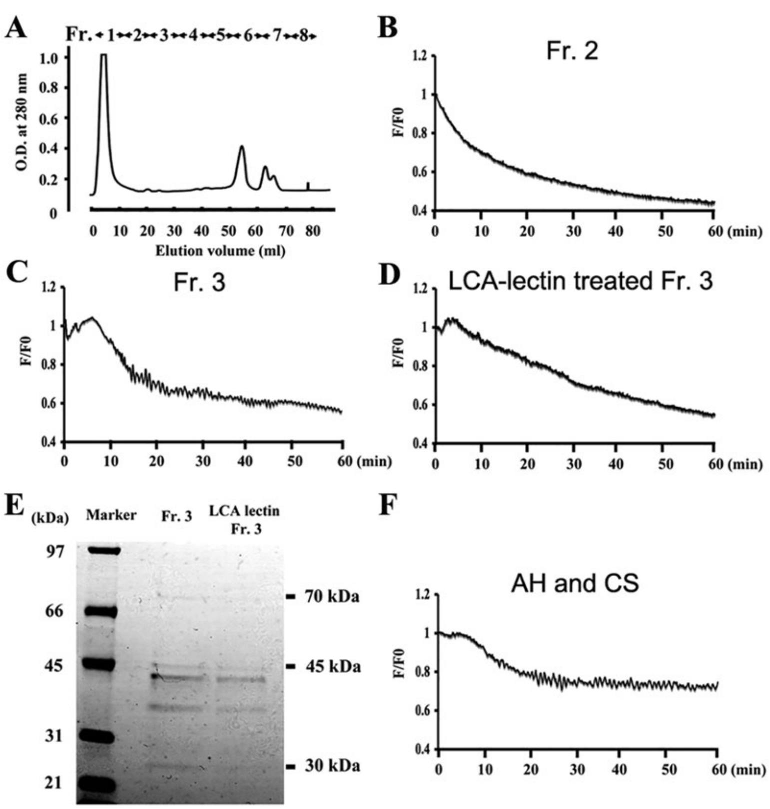

Fig. 2. Identification of avian-specific egg-activating factors responsible for spiral-like Ca^{2+} oscillations. (A) SE was subjected to separation on a Superdex 200 pg column and eight 10 ml fractions were collected. (B-D) Changes in [Ca^{2+}]*i* in quail eggs injected with fraction 2 (B), fraction 3 (C) or LCA-agarose-treated fraction 3 (D). (E) SDS-PAGE analysis of fraction 3, or fraction 3 treated with LCA-agarose. Proteins are stained with Coomassie Brilliant Blue. (F) Changes of [Ca^{2+}]*i* in quail egg injected with a mixture of porcine AH (100 pg) and porcine CS (100 pg). A and E are the results from a single experiment; B-D are typical examples of measurements of [Ca^{2+}]*i* from two experimental repeats; F shows representative results from eight experimental repeats.

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of Ca^{2+} (Fig. 2F). Immunodepletion of SE with anti-AH or anti-CS antibodies, but not with normal rabbit IgG, abolished the spiral-like oscillations (Fig. 3A-C), suggesting that both AH and CS were necessary for this phenomenon. To confirm that AH and CS could together induce spiral-like Ca^{2+} oscillations in quail eggs, we synthesized cRNAs encoding quail AH and CS and microinjected these into unfertilized quail eggs. Co-injection of quail AH and CS cRNAs, as with the co-injection of porcine AH and CS, induced spiral-like Ca^{2+} oscillations in unfertilized quail eggs (Fig. 3D). Notably, onset of the cRNA-induced spiral-like Ca^{2+} oscillations was delayed by ∼15 min relative to the onset of the protein-induced oscillations (i.e. SE or porcine AH plus CS microinjection). This delay probably reflected the time required for cRNA translation. By contrast, injection of PLCZ cRNA induced an immediate $[Ca^{2+}]i$ rise (Fig. 3E). Furthermore, double microinjections of PLCZ and CS or PLCZ and AH cRNAs induced a transient rise in $[Ca^{2+}]i$ without inducing spiral-like Ca^{2+} oscillations (Fig. 3F,G). Importantly, when PLCZ cRNA was injected with the AH and CS cRNAs, both the transient Ca^{2+} rise and the subsequent spiral-like Ca^{2+} oscillations occurred (Fig. 3H).

We employed heparin (Yue et al., 1995) and 2-aminoethoxydiphenyl borate (2-APB), a selective inhibitor of IP_3 receptor (IP_3-R) (Martin-Romero et al., 2008), to investigate the molecular events leading to the induction of spiral-like Ca^{2+} oscillations. Injection of 1 ng heparin with a mixture of PLCZ, AH and CS cRNAs diminished the transient rise in $[Ca^{2+}]i$ without disturbing the spiral-like Ca^{2+} oscillations (supplementary material Fig. S2B). The pre-incubation of eggs with 100 μM 2-APB before the injection had no effect on the induction of spiral-like Ca^{2+} oscillations (supplementary material Fig. S2C). These results indicated that IP_3-R does not participate in signal transduction for the induction of spiral-like Ca^{2+} oscillations in quail eggs.

When a microinjection of 25 fmol cyclic ADP ribose (cADPR), which has been shown to activate ryanodine receptors in sea urchins (Whitaker and Swann, 1993) and bovine eggs (Yue et al., 1995), was performed, irregular patterns of Ca^{2+} waves were observed that were distinctly different from the IP_3- or PLCZ-generated transient rise in Ca^{2+} (supplementary material Fig. S2D). Although the mean amplitude of these Ca^{2+} waves did not differ from those of the CS plus AH-induced Ca^{2+} oscillations, the mean interval of the oscillations in cADPR-injected eggs was significantly longer (supplementary material Fig. S3). Furthermore, the removal of extracellular Ca^{2+} by adding 20 μM BAPTA to the culture medium did not affect the amplitude or duration of spiral-like Ca^{2+} oscillations, indicating that extracellular Ca^{2+} is not required for this event (supplementary material Fig. S2H).

When quail SE proteins on western blots were probed with anti-AH antibody, a 70 kDa protein was evident; this AH in SE was ∼10 kDa smaller than the AH in unfertilized egg, liver or kidney extracts (supplementary material Fig. S4A). Several immunoreactive bands of ∼45 kDa were also detected; however, the nature of these bands remains unknown (supplementary material Fig. S4A). These 45 kDa proteins were not involved in the process of egg activation because they were not detected in fraction 3 obtained by gel filtration

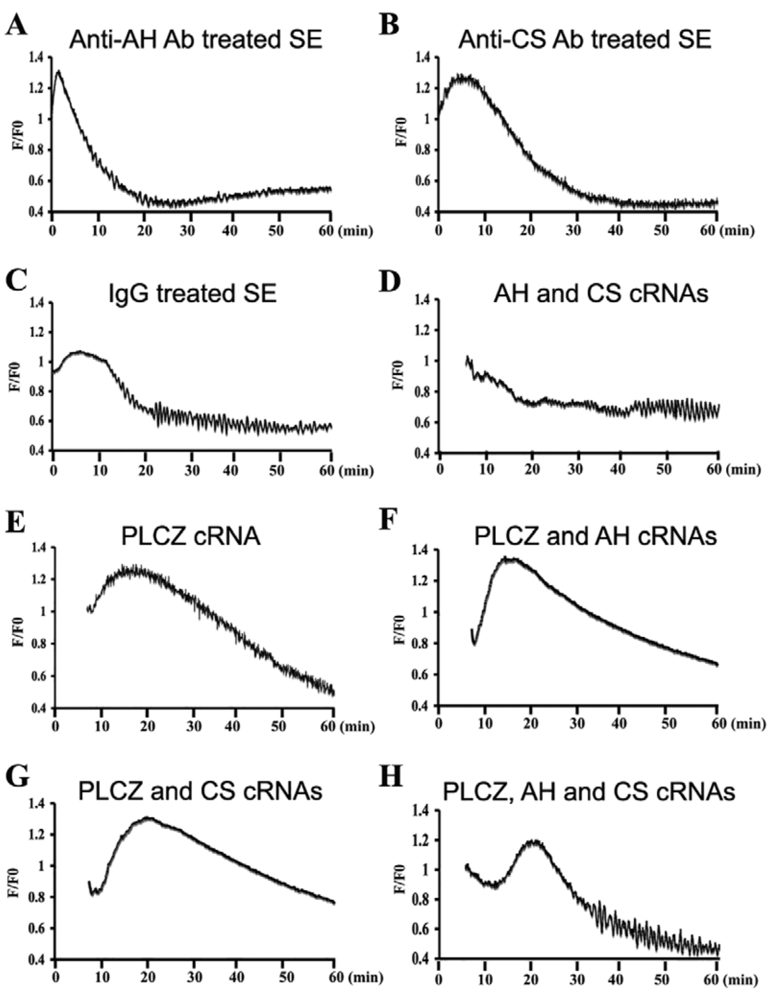

Fig. 3. Changes in $[Ca^{2+}]i$ in quail egg after microinjection of egg-activating factors. Eggs were microinjected with anti-AH antibody-treated SE (A), anti-CS antibody-treated SE (B), normal rabbit IgG-treated SE (C), a mixture of AH and CS cRNAs (D), PLCZ cRNA alone (E), a mixture of PLCZ and AH cRNAs (F), a mixture of PLCZ and CS cRNAs (G) or a mixture of PLCZ and cRNAs for AH and CS (H). Note that UV irradiation was not performed during the first 5 min after injection of cRNA aliquots to avoid decomposition of the injected cRNA. A and B are representative of results from two, C from three, D from four, E from six, F from four, G from four and H from 13 experimental repeats.

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Table 1. Blastoderm development produced by ICSI at 24 h of culture

Injected sample	No. of eggs		No. of embryos						
			Developed to stage*						
	Injected	Developed (%)	IV	V	VI	VII	VIII	IX	X
In vivo fertilized egg	6	6 (100)							6
Sperm alone	26	5 (19)	2		2	1			
Sperm+60 pg PLCZ cRNA	13	6 (46)	2	1	1	2			
Sperm+50 fmol IP3	29	25 (86)		3	6	9	7		
Sperm+2 ng SE	19	15 (79)	1	2			3	4	5
Sperm+100 pg AH cRNA+100 pg CS cRNA	3	0 (0)							
Sperm+60 pg PLCZ cRNA+100 pg AH cRNA	9	5 (56)		2	1	2			
Sperm+60 pg PLCZ cRNA+100 pg CS cRNA	8	5 (63)	1	1		3			
Sperm+60 pg PLCZ cRNA+100 pg AH cRNA+100 pg CS cRNA	24	17 (71)	3	3	1		2	1	7
Sperm+25 fmol cADP ribose	5	1 (20)	1						
Sperm+60 pg PLCZ cRNA+25 fmol cADP ribose	11	5 (46)			2	1	1	1	

*Developmental stages were determined according to Eyal-Giladi and Kochav (1976).

chromatography (data not shown). Anti-CS antibody detected a 45 kDa band in quail SE on western blots, but a slightly smaller molecule (44 kDa) was detected in the egg, liver and kidney extracts (supplementary material Fig. S4B). Ejaculated sperm were used to clone cDNAs encoding quail AH or CS; notably, a sperm-specific AH cDNA lacked 105 bp that encoded 35 amino acids at the N-terminus, and a sperm-specific CS cDNA contained a 3 bp insert encoding an arginine at position 314 (supplementary material Fig. S5). Although such structures are not predicted to be required for egg activation because AH and CS derived from porcine heart induce spiral-like Ca^{2+} oscillation, these results suggest the existence of a specific form of AH and CS in quail sperm.

To investigate the relationship between quail egg activation and subsequent embryonic development, we examined the effects of three factors (PLCZ, AH and CS) on the development of ICSI-generated zygotes. ICSI-treated eggs co-injected with 60 pg PLCZ cRNA or 50 fmol IP_3 initiated the first cleavage at ∼4.5 h (data not shown). Notably, this first cleavage was delayed by 1.5 h relative to the developmental timecourse that follows *in vivo* fertilization (data not shown). Moreover, the development of these ICSI-derived zygotes was further delayed after 24 h in culture (Table 1; nomenclature as used in Eyal-Giladi and Kochav, 1976). By contrast, when ICSI-treated eggs were co-injected with 2 ng SE, 9 of 15 embryos (60%) underwent the first cleavage normally (data not shown) and developed to the IX and X stages after 24 h in culture (Table 1). Furthermore, when ICSI-treated eggs were co-injected with a mixture containing all three factors (cRNAs encoding PLCZ, AH or CS), 8 of 17 embryos (47%) underwent the first cleavage normally (data not shown) and developed to stages equivalent to those of eggs fertilized *in vivo* (Table 1). By contrast, no embryo developed normally if ICSI was performed without any of these factors (Table 1). These results indicated that all three factors are essential for the normal development of ICSI-derived embryos. The co-injection of PLCZ cRNA together with cADPR also improved embryonic development (stage IX) after 24 h in culture to a greater extent than treatment with either PLCZ cRNA or cADPR alone (Table 1).

Microinjection of 50 fmol IP_3 into ICSI-treated quail eggs improved the rate of blastoderm development (to 86%, Table 1), but the development of each embryo was arrested at H&H stage 6 (Table 2, Fig. 4A). Likewise, the development of embryos generated by dual injection of PLCZ and AH cRNAs, PLCZ and CS cRNAs, or PLCZ cRNA and 25 fmol cADPR into ICSI eggs died at H&H stages 5, 6 or 8, respectively (Table 2). Ultimately, we produced a live chick by co-injecting 2 ng SE and a single spermatozoon into an unfertilized quail egg (Table 2, Fig. 4B). The final hatchability of

Table 2. Viability and hatchability of quail embryos produced by ICSI

Injected sample	No. of embryos transferred to surrogate shell culture (%)	No. of embryos developed to stage*												
		VII	X	XIII	3	4	5	6	8	16	25	30	43	Hatch (%)
Sperm alone	1 (20)	1												
Sperm+60 pg PLCZ cRNA	2 (33)			1				1						
Sperm+50 fmol IP3	16 (64)		2	3	2	1	3	5						
Sperm+2 ng SE	12 (80)		3		2	2				1	1	1	1	1 (8)
Sperm+100 pg AH cRNA +100 pg CS cRNA	0 (0)													
Sperm+60 pg PLCZ cRNA +100 pg AH cRNA	2 (40)				1		1							
Sperm+60 pg PLCZ cRNA +100 pg CS cRNA	3 (60)		1		1			1						
Sperm+60 pg PLCZ cRNA +100 pg AH cRNA+100 pg CS cRNA	10 (59)		3	1		1		1			2		1	1 (10)
Sperm+25 fmol cADP ribose	0 (0)													
Sperm+60 pg PLCZ cRNA +25 fmol cADP ribose	3 (60)				1		1		1					

*Developmental stages were determined according to Eyal-Giladi and Kochav (1976) (Roman numerals) or Hamburger and Hamilton (1951) (Arabic numerals).

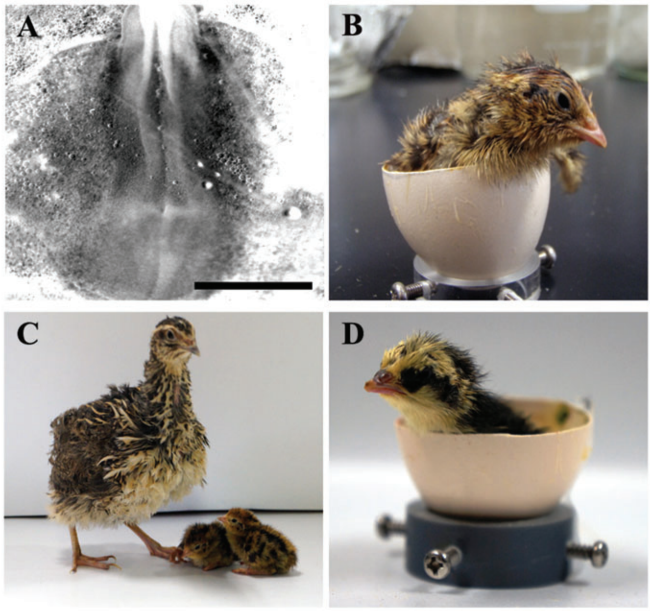

Fig. 4. Development of a quail embryo derived by ICSI. (A) Quail embryo at H&H stage 6 resulting from ICSI and co-injection of 50 fmol IP_3. Scale bar: 1 mm. (B) Hatchling quail (named Megumi) resulting from ICSI and co-injection of 2 ng SE. Note that Megumi hatched from a surrogate chicken eggshell. (C) Megumi and her offspring produced by natural mating. (D) Another female quail was produced by ICSI with co-injection of a mixture containing PLCZ and cRNAs for CS and AH.

the embryos transferred to the surrogate shell culture was 8%. A total of 5/12 (42%) embryos developed past H&H stage 6, and one of these developed to just before hatching (H&H stage 43). The resulting chick was female; after sexual maturation, she produced healthy offspring by natural mating (Fig. 4C). It should be noted that the onset of her sexual maturation (the onset of egg laying) occurred at 7 weeks and 3 days of age, which was within the normal range for female Japanese quail (6-8 weeks of age; Stein and Bacon, 1976). Another healthy female offspring also hatched following co-injection of a single spermatozoon and a mixture of the three PLCZ, AH and CS cRNAs (Table 2; Fig. 4D). The final hatchability of the embryos in this treatment group was 10%, with 4/10 (40%) embryos developing past H&H stage 6, and one developing to just before hatching (H&H stage 43). Unfortunately, the hatching quail chick died before sexual maturation due to an unknown reason. Because PLCZ, AH and CS were found to be solely essential for egg activation and the full-term development of ICSI-derived zygotes, we concluded that SD and MD, as identified by LC-MS/MS analysis, were not required for these events and did not analyze them further.

DISCUSSION

Role of Ca^{2+} waves in quail development

To our knowledge, this is the first description of the pattern of Ca^{2+} oscillation during *in vitro* fertilization in birds. In mammals, including mice (Saunders et al., 2002), humans (Cox et al., 2002), pigs (Yoneda et al., 2006) and cattle (Yoon and Fissore, 2007), PLCZ has been identified as a sperm-borne egg-activating factor. Thus, microinjection of PLCZ cRNA (Saunders et al., 2002) or recombinant protein (Kouchi et al., 2004) elicited long-lasting Ca^{2+} oscillations, similar to those observed in mouse eggs fertilized *in vitro*. In addition, the PLCZ-generated Ca^{2+} oscillations are sufficient to trigger the resumption of meiosis, pronucleus formation, and subsequent normal blastocyst development (Saunders et al., 2002; Cox et al., 2002; Yoneda et al., 2006). Here, we showed that quail eggs require two different kinds of Ca^{2+} waves to enable full-term development following ICSI: (1) PLCZ induced a transient Ca^{2+} rise and (2) AH and CS together induced long-lasting, spiral-like Ca^{2+} oscillations.

The difference between mammals and quail does not seem to arise because of any weakness in the egg activation activity of avian PLCZ, as the Ca^{2+} oscillation-inducing activity of chicken PLCZ for mouse eggs is equivalent to that of its mammalian counterpart (Coward et al., 2005). We suggest that the egg activation mechanisms in quail are different from those in mammals, as supported by the following findings: (1) neither PLCZ nor IP_3 had the ability to induce spiral-like Ca^{2+} oscillations; (2) spiral-like Ca^{2+} oscillations occurred irrespective of the presence or absence of a PLCZ-induced transient rise in Ca^{2+}; and (3) heparin or 2-APB, an antagonist for IP_3-R, did not interfere with the spiral-like Ca^{2+} oscillations. Although the underlying mechanisms have not yet been elucidated in detail, the induction of spiral-like Ca^{2+} oscillations might be mediated, at least in part, via ryanodine receptors rather than via IP_3-R because cADPR, an activator for ryanodine receptors, potently induced irregular patterns of Ca^{2+} waves in eggs (supplementary material Fig. S2D). Other unidentified receptors responsible for the signal transduction that induces the spiral Ca^{2+} oscillations might exist given that the pattern of Ca^{2+} waves (i.e. the mean interval; supplementary material Fig. S3B) as well as embryonic development (Tables 1 and 2) induced by PLCZ and cADPR differed from those induced by PLCZ, AH and CS.

In mice, a single transient rise in $[Ca^{2+}]i$ caused by artificial stimuli, such as electrical pulses or exposure to ethanol, could induce partial egg activation (Tatone et al., 1999; Ducibella et al., 2002; Jones, 2005). The eggs underwent second polar body extrusion, but the chromatin rearrested on a monopolar third spindle. However, repeated stimulations were able to lead the eggs to full activation (Ducibella et al., 2002; Jones, 2005). This was because the inactivation of cytostatic factor, which is the cytosolic protein responsible for meiotic arrest at metaphase II, is achieved by repetitive Ca^{2+} spikes and not by a single transient Ca^{2+} rise (Ducibella et al., 2002; Jones, 2005). In addition, 24 electrical pulses induced an increase in cortical granule exocytosis (Ducibella et al., 2002), which triggers the zona reaction preventing polyspermy (Jones, 2007). Although the exact mechanism for the induction of Ca^{2+} oscillations remains to be uncovered, the theory that egg-derived PLCβ or PLCγ might enhance IP_3 generation via a positive feedback of transient Ca^{2+} rise after the introduction of PLCZ has been proposed for mammalian somatic cells and ascidian eggs (Dupont and Dumollard, 2004; Igarashi et al., 2007; Swann and Yu, 2008). Thus, the Ca^{2+} oscillation in mammalian eggs is thought to be required for the completion of meiosis as well as for blocking polyspermic fertilization.

What is the role of the AH- and CS-derived spiral-like Ca^{2+} oscillations in quail eggs? Unlike mammals, treatment of the ICSI-activated quail egg by introducing PLCZ cRNA or IP_3 did not lead to full-term development, whereas eggs that were microinjected with a mixture of PLCZ, AH and CS cRNAs reached the hatching stage. In addition, double injections of CS and AH, but not a single injection of PLCZ, induced spiral-like Ca^{2+} oscillations (Fig. 3D,E), whereas these oscillations alone did not stimulate the first cleavage of the eggs (Table 1). These results indicate that spiral-like Ca^{2+} oscillations and PLCZ/IP_3-generated Ca^{2+} signaling might contribute independently to different cellular events during fertilization. Thus, the PLCZ-induced transient Ca^{2+} rise is

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indispensable for the resumption of meiosis, and the AH- and CS-induced long-lasting, spiral-like Ca^{2+} oscillations work as the major driving force for cell cycle progression in early embryos. Because there is no block to polyspermy before membrane fusion in birds (Wong and Wessel, 2006), the role of Ca^{2+} oscillations in polyspermy blockage has been lost in quail eggs. How the spiral-like Ca^{2+} oscillations are induced by CS and AH and how they enhance development of the early embryo must be answered by future studies. As described above, ryanodine receptors may be responsible for the induction of spiral-like Ca^{2+} oscillations; however, the signal derived from ryanodine receptors alone appears to be insufficient to support full-term development of the bird *in vitro*.

Comparison of ICSI and normal polyspermic fertilization

Here, SE containing 2 ng of proteins – equivalent to ∼200 spermatozoa – was found to be required for the full-term development of ICSI-generated quail zygotes. Previous reports demonstrated that chicken SE equivalent to a single or half of a spermatozoon induced pronucleus formation in mouse eggs (Dong et al., 2000), whereas a single quail or chicken spermatozoon could not activate the quail egg (Takagi et al., 2007; Mizushima, 2012). These results indicated that many spermatozoa are necessary to provide sufficient amounts of PLCZ, AH and CS proteins to ensure successful egg activation in the quail. Unlike mammals, polyspermy is normal in fertilized avian eggs. Here, many (20-60) spermatozoa penetrate the perivitelline membrane, which is homologous to the mammalian zona pellucida, and enter the egg germinal disk (Fofanova, 1965; Nakanishi et al., 1990; Wishart, 1997). Wishart and Staines (1999) demonstrated that fewer than 20 sperm-generated holes in the perivitelline membrane over the germinal disk were associated with reduced fertility in both chickens and turkeys. These reports indicate that at least 20 spermatozoa seem to be necessary to activate the avian egg.

Why a much larger amount of SE factors is required for full-term development following ICSI might be accounted for by the difference between *in vitro* insemination and our ICSI system. In the polyspermic newt egg, studies of *in vitro* insemination showed that a few spermatozoa enter successively at different points, and small wave-like increases in $[Ca^{2+}]i$ occur sequentially at each sperm entry site (Harada et al., 2011; Iwao, 2012). The Ca^{2+} wave induced by one spermatozoon propagated over only one-eighth to a quarter of the egg surface, which suggests that many spermatozoa must enter to induce a Ca^{2+} increase throughout the entire egg (Harada et al., 2011). Complete activation of newt eggs by a single microinjection of newt SE required a protein content equivalent to 330 spermatozoa (Harada et al., 2011). These results are consistent with our observations (Fig. 1C,D; Table 1). Although we did not assess the yield of PLCZ, AH and CS proteins in the present study, the successive entry of multiple spermatozoa into the avian germinal disk seems to be essential for full egg activation in birds.

In our current ICSI system, the hatchability was low (8-10%). Some of the ICSI-assisted embryos might have been rescued by improving the surrogate shell culture system (system III in the present study), such as optimizing the oxygen supply. In fact, the previous studies (Ono et al., 1994, 1996) demonstrated that the hatchability of intact *in vivo* fertilized eggs obtained from the anterior part of the magnum was 19-25% after the surrogate shell culture was performed and ∼30-50% of embryos died within 2 days of surrogate shell culture. This result implies that one of the reasons for the low hatchability in our current ICSI system is a defect in the surrogate shell system. However, we anticipate that this lower rate might also be explained by an inability of our current ICSI system to reproduce polyspermic fertilization.

Further studies are needed to explore the mechanism of avian polyspermic fertilization. Unfortunately, no *in vitro* insemination systems are currently available because avian eggs are too large to handle in culture systems.

Conclusions

The ICSI technique is well developed in mammals and has been successfully used to produce healthy offspring in humans, mice, hamsters, rats, rabbits, cattle, sheep, horses, cats, pigs and monkeys (Yanagimachi, 2005), but no chicks have been generated so far. To our knowledge, this is the first demonstration of full-term (zygote-to-adult) development of a bird following ICSI. Importantly, the resulting two offspring were female and they were not the result of parthenogenesis because the ZW sex-determining system in birds does not allow for parthenogenetic production of female chicks (Harada and Buss, 1981). The successful production of healthy chicks after ICSI has enormous implications for industrial, agricultural and conservation applications, including avian transgenesis, cloning technology and in protecting endangered bird species. Furthermore, the discovery that sperm-borne AH and CS function as egg-activating factors responsible for embryonic development and the unique pattern of Ca^{2+} oscillations during egg activation in birds provides new insights into the molecular mechanisms of egg activation in vertebrates. Our results will also advance our understanding of the detailed molecular mechanisms that underlie polyspermic fertilization in birds.

MATERIALS AND METHODS

Animals

Male and female Japanese quail, *Coturnix japonica*, of 8-20 weeks of age (Motoki Corporation) were maintained individually under a photoperiod of 14 hours light:10 hours dark (lights on at 05:00) with *ad libitum* access to water and a commercial diet (Motoki Corporation). In domestic birds, including quail, ovulation occurs ∼30 min after egg laying, with fertilization taking place within 15 min of ovulation (Woodard and Mather, 1964). In order to anticipate the time of fertilization *in vivo*, the egg laying times of individual birds were recorded every day. All experimental procedures for the care and use of animals were approved by the Animal Care and Use Committee of Shizuoka University, Japan (approval number 24-12).

ICSI and *ex vivo* culture

Ejaculated semen was collected from individual birds immediately before copulation (Kuroki and Mori, 1997). To prepare SE, spermatozoa were washed repeatedly in phosphate-buffered saline (PBS) and collected by centrifugation at 800 ***g*** for 3 min; fully washed spermatozoa were suspended in PBS. Spermatozoa were disrupted by homogenization and sonication; clarified supernatant was collected by centrifugation at 20,400 ***g*** for 10 min and then stored as SE. Bicinchoninic acid (BCA) protein assay kits (Pierce) were used to measure protein concentrations in the SE.

Unfertilized eggs were recovered from the anterior magnum within 1 h after oviposition (Mizushima et al., 2008). Each egg was microinjected with a single ejaculated spermatozoon together with either 50 fmol IP_3 or 2 ng SE. The total injected volume was ∼1 nl. All procedures used for ICSI were performed as described by Hrabia et al. (2003) and Mizushima et al. (2008). Briefly, under a Hoffman modulation contrast microscope (IX70, Olympus), IP_3 or SE solution was first drawn into an injection micropipette, followed by a single ejaculated spermatozoon in the same micropipette. The ovum was placed into Dulbecco's modified Eagle's medium (DMEM) in a plastic dish (35×18 mm; six-well multidish, Nunclon) and both were then injected into the central area of the germinal disk of the egg (∼30-50 µm in depth) using a micromanipulator connected to the injector (IM-9B, Narishige) with silicon tubing filled with silicon oil under a stereomicroscope (SZ11, Olympus). A rough estimate of the injection speed is ~6 nl/min. Because the germinal disk of quail eggs is opaque, the completion of the injection was confirmed visually by observing a swelling of the injection site under a stereomicroscope. This manipulation was performed with the aid of an image-processor system (Image Σ-III, Nippon Avionics). To produce the pipettes for ICSI, borosilicate glass capillary tubing (1 mm outer diameter, 0.75 mm inner diameter; Sutter) was drawn with a pipette puller (P-97/IVF, Sutter), and the tip of the pipette was cut with a microforge (MF-900, Narishige) such that the inner diameter at the tip was ~5-7 µm.

Each egg was cultured in DMEM in a plastic cup at 41.5°C in an atmosphere containing 5% CO_2 (Ono et al., 1994). Individual embryos were then transferred to a large surrogate Japanese quail eggshell. The shells were filled with thin chicken egg albumen and tightly sealed with cling film. The shell was secured by a pair of plastic rings and elastic bands. Embryos were then cultured for 63 h at 37.5°C and 70% relative humidity, with rocking at a 90° angle every 30 min. Finally, individual embryos were transferred to a small surrogate chicken eggshell [a generous gift from the Avian Bioscience Research Center (ABRC) of Nagoya University, Japan]. These were sealed with cling film using thin chicken egg albumen as a glue, and cultured at 37°C with rocking at a 30° angle until hatching (Ono et al., 1994). For *in vivo* fertilized eggs, a zygote obtained from the anterior magnum ~1 h after the expected time of fertilization was cultured using the same procedure as used for ICSI-derived zygotes.

Measurement of $[Ca^{2+}]i$ in quail egg

The Ca^{2+}-sensitive indicator dye Fluo-8H AM (AAT Bioquest) was used to measure all changes in $[Ca^{2+}]i$. Dye-loaded unfertilized eggs were injected with 50 fmol IP_3 (Sigma-Aldrich), 2 ng SE, 100 pg porcine AH (Wako Pure Chemical Industries), 100 pg porcine CS (Sigma-Aldrich), 25 fmol cADPR (Sigma-Aldrich), 60 pg quail PLCZ cRNA, 100 pg quail AH cRNA, 100 pg quail CS cRNA, or a defined combination thereof using a micromanipulator connected to the injector as described above. In cases of co-injection, the final concentration of each component of a mixture was equivalent to the concentration of that component in the respective single-injection experiments.

Rabbit anti-chicken AH polyclonal antibody (20 µg/ml; GeneTex, GTX114233), rabbit anti-chicken CS polyclonal antibody (20 µg/ml; GeneTex, GTX110624) or normal rabbit IgG (20 µg/ml; Sigma-Aldrich, I5006) was mixed with 2 mg/ml SE; each mixture was incubated overnight to neutralize the respective antigen in the SE. To examine the effects of heparin on $[Ca^{2+}]i$, eggs were pre-injected with 1 ng heparin before the microinjection of each test substance. To evaluate the effects of 2-APB on $[Ca^{2+}]i$, the microinjection and subsequent culture were performed in medium supplemented with 100 µM 2-APB. To remove extracellular Ca^{2+} from the medium, 20 µM BAPTA was included in Ca^{2+}-deficient DMEM (Gibco) and the microinjection and subsequent culture were performed in this medium.

Fluorescent images of each injected egg were taken with a digital CCD camera (ImagEM, C9100-13, Hamamatsu Photonics) connected to a fluorescence stereomicroscope (M165 FC, Leica). AQUACOSMOS (Hamamatsu Photonics) imaging software was used to measure background fluorescence from outside of the germinal disk and to then calculate the average fluorescence intensity of the germinal disk region (~7 mm^2). The F0 value was set as fluorescence intensity at the time of injection and timecourse measurements in the same area were continued for at least 60 min (F value). F/F0 values were plotted as $[Ca^{2+}]i$ in the eggs. When the F/F0 value was more than 0.05 at 20 min after the injection, we interpreted this to indicate that spiral Ca^{2+} oscillations had been induced. To analyze the spiral-like Ca^{2+} waves, the fluorescence intensities at two regions (~150 μm^2) within the germinal disk were quantitated as described above.

Cloning of AH and CS cDNAs

SE was subjected to separation on a Superdex 200 pg column (GE Healthcare); in total, eight 10 ml fractions were collected. Fraction 3 was treated with LCA-agarose beads overnight at 4°C and the supernatant was collected by centrifugation at 20,400 ***g*** for 10 min. Fraction 3, or fraction 3 treated with LCA-agarose beads, was resolved by SDS-PAGE (Laemmli, 1970) and subjected to Coomassie Brilliant Blue staining. For *de novo* protein sequencing analysis, sequencing-grade trypsin was used as suggested by the manufacturer (Promega) to prepare and digest the proteins within the gel. The peptides recovered from the gel were analyzed by LC-MS/MS (NanoFrontier eLD, Hitachi High-Technologies) according to the manufacturer's instructions. A *de novo* sequencing software package, PEAKS, was used to identify proteins from the MS/MS data (Ma et al., 2003).

We used primers designed from *de novo* sequence analysis and cDNA templates prepared from ejaculated quail spermatozoa and quail liver to amplify *AH* and *CS* sequences. The full-length sequences encoding quail AH and CS were obtained from ejaculated spermatozoa and liver using 5′ and 3′ RACE kits (Invitrogen) according to the manufacturer's instructions. The PCR products of quail *AH* or *CS* were cloned into the pGEM-T Easy vector (Promega) and digested with *Spe*I to linearize each recombinant plasmid. The mMESSAGE mMACHINE kit (Ambion) was used according to the manufacturer's instructions to synthesize each cRNA. Microinjection of cRNA was performed as described above.

Immunoblotting

An ejaculated sperm and an unfertilized egg were collected as described above. Ejaculated sperm, germinal disk of unfertilized egg, liver and kidney were homogenized, sonicated and the supernatant was collected by centrifugation at 20,400 ***g*** for 10 min. The protein concentration was measured by BCA protein assay kit (Pierce). Each extract (10 µg protein per lane) was resolved by SDS-PAGE (Laemmli, 1970) on a 12% polyacrylamide gel and then transferred onto PVDF membrane (Millipore). Following transfer and blocking for 30 min with 5% skimmed milk, the membrane was incubated for 1 h with rabbit anti-chicken AH polyclonal antibody or rabbit anti-chicken CS polyclonal antibody (both GeneTex, see above) and was subsequently incubated for 30 min with goat anti-rabbit secondary antibodies conjugated with horseradish peroxidase (Millipore, 12-348).

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Competing interests

The authors declare no competing financial interests.

Author contributions

S.M. and T.S. conceived and designed the study and wrote the manuscript. H.D. performed the LC-MS/MS and analyzed all LC-MS/MS data. Ko.S. and K.I. assisted with the Ca^{2+} imaging. S.M., G.H. and T.S. performed other experiments and analyzed the data. Ki.S. performed the pilot study on ICSI with a Hoffman modulation contrast microscope. T.O. performed the pilot study on image enhancement of ova and *ex vivo* embryo culture. All authors approved the final manuscript.

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Supplementary material

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