Zygote **16** (August), pp. 211–222. © 2008 Cambridge University Press
doi:10.1017/S0967199408004620 First Published Online 26 June 2008 Printed in the United Kingdom

Effect of volume of oocyte cytoplasm on embryo development after parthenogenetic activation, intracytoplasmic sperm injection, or somatic cell nuclear transfer

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Date submitted: 04.06.07. Date accepted: 03.08.07

Summary

Animal cloning methods are now well described and are becoming routine. Yet, the frequency at which live cloned offspring are produced remains below 5%, irrespective of the nuclear donor species or cell type. One possible explanation is that the reprogramming factor(s) of each oocyte is insufficient or not properly adapted for the receipt of a somatic cell nucleus, because it is naturally prepared only for the receipt of a gamete. Here, we have increased the oocyte volume by oocyte fusion and examined its subsequent development. We constructed oocytes with volumes two to nine times greater than the normal volume by the electrofusion or mechanical fusion of intact and enucleated oocytes. We examined their *in vitro* and *in vivo* developmental potential after parthenogenetic activation, intracytoplasmic sperm injection (ICSI) and somatic cell nuclear transfer (SCNT). When the fused oocytes were activated parthenogenetically, most developed to morulae or blastocysts, regardless of their original size. Diploid fused oocytes were fertilized by ICSI and developed normally and after embryo transfer, we obtained 12 (4–15%) healthy and fertile offspring. However, enucleated fused oocytes could not support the development of mice cloned by SCNT. These results suggest that double fused oocytes have normal potential for development after fertilization, but oocytes with extra cytoplasm do not have enhanced reprogramming potential.

Keywords: Giant oocyte, Fertilization, ICSI, Nuclear transfer, Parthenogenesis

Introduction

Nuclear transfer (NT) experiments using adult mammalian somatic cells began 10 years ago (Wilmut *et al.*, 1997). However, the biology underlying this process remains unclear in many respects and cloning by somatic cell nuclear transfer (SCNT) still has a very low success rate. We have developed a microinjection-based nuclear transfer method to generate cloned mice from adult or foetal somatic cells (Wakayama *et al.*, 1998; Wakayama & Yanagimachi, 1999, 2001b), somatic stem cells (Mizutani *et al.*, 2006), or embryonic stem (ES) cells (Wakayama *et al.*, 2001). Recently, we improved the success rate of cloning in mice up to six-fold using dimethyl sulphoxide (DMSO) (Wakayama & Yanagimachi, 2001a), trichostatin A (Kishigami *et al.*, 2006a, 2007) or serial nuclear transfer (Wakayama *et al.*,

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2005). However, the overall efficiency of mouse cloning is less than 6% and the offspring that survive to term have displayed many abnormalities (Wakayama, 2007), which are probably caused by incomplete genomic reprogramming after nuclear transfer into the oocyte cytoplasm. The effect of the donor cell type on the cloning success rate has been reported previously and all studies used hybrid mouse oocytes because they have shown better results in *in vitro* culture. However, the quality, volume and reprogramming potential of the oocyte cytoplasm have not yet been analysed.

In general, the cytoplasm of a single oocyte has surplus potential for fertilizability, including its capacity to support sperm decondensation and male pronucleus formation. For example, a normal oocyte can accept and decondense up to four spermatozoa (Clarke & Masui, 1987), whereas a quarter-size oocyte can accept only one spermatozoon (Tarkowski & Balakier, 1980; Wakayama & Yanagimachi, 1998). Although quarter-size oocytes fail to develop after fertilization, half-size oocytes can develop to full term without a reduction in the birth rate (Wakayama & Yanagimachi, 1998). However, it is not yet clear whether the cytoplasm of an oocyte is sufficient to reprogramme one somatic cell nucleus in SCNT experiments, because in nature, the reprogramming factor(s) of the oocyte cytoplasm is prepared for the receipt of a gamete nucleus, not a somatic cell nucleus. Unmatched reprogramming factor(s) may reprogramme the somatic cell nucleus inefficiently, or simply because of their inadequate reprogramming potential, the somatic cell nucleus may require more reprogramming factors to erase somatic memory and to achieve totipotency. In the latter case, cloning success rates might be improved by the addition of supplementary oocyte cytoplasm before the SCNT procedure.

Occasionally, female mammals ovulate large diploid 'giant oocytes'. The frequency varies from 0.1 to 0.5% in many mammalian species, including humans (Austin & Braden, 1954; Austin, 1960; Funaki & Mikamo, 1980; Balakier *et al.*, 2002). To overcome this low spontaneous ovulation rate, giant oocytes have been constructed artificially. However, there is a consensus that naturally derived mature giant oocytes have abnormal diploid chromosome content and contain either one mixed or two metaphase plates, instead of the normal haploid chromosomes of the mature oocyte. Therefore, to distinguish them, we here refer to them as 'artificial giant (AG) oocytes'. To the best of our knowledge, the production of AG oocytes and embryos was first reported by Soupart (1980), using Sendai virus fusion. However, this approach was replaced with less toxic and more reproducible treatments using polyethylene glycol (PEG) (Eglitis, 1980; Gulyas *et al.*, 1984) and more recently, by electrofusion (Gordo *et al.*, 2002). Several reports of the use of these techniques have been published. Tesarik *et al.* (2000) used mature mouse oocytes fused with germinal vesicle-stage oocytes and activated oocytes, zygotes, or aged oocytes to determine the effects of different stages of the cell cycle on development. Others have applied these techniques to study the mechanisms by which oocytes resume meiosis and regulate their surface membrane functions (Fulka *et al.*, 1989, 1993, 1997); to determine the distribution of mitochondria (Fulka, 2004); to evaluate the nature of the block to polyspermy at the oolemma (Krukowska *et al.*, 1998); and to investigate cytoplasmic deficiencies in aged oocytes (Gordo *et al.*, 2002). *In vitro* developmental potential has also been examined using the fusion of two mature intact oocytes. When those diploid AG oocytes are activated parthenogenetically, they usually extrude two second polar bodies and form two female pronuclei (Gulyas *et al.*, 1984; Vassetzky & Sekirina, 1985; Karnikova *et al.*, 2000), as do naturally ovulated and fertilized giant oocytes (Rosenbusch and Schneider, 1998; Karnikova *et al.*, 2000; Balakier *et al.*, 2002). Such activated AG oocytes can develop to blastocysts *in vitro*, but their potential to develop to full term has never been demonstrated, because parthenogenetic embryos usually die (Gulyas *et al.*, 1984; McGrath & Solter, 1984; Surani *et al.*, 1984).

In this study, we first defined the optimal methods of constructing AG oocytes and examined the *in vitro* developmental potential of these oocytes using parthenogenetic activation. Secondly, we fused intact oocytes with enucleated oocytes and examined their *in vivo* developmental capacity after intracytoplasmic sperm injection (ICSI) with isolated sperm heads. Finally, we examined the nuclear reprogramming potential of the enucleated AG oocytes after SCNT.

Materials and methods

Animals

Oocytes, spermatozoa and cumulus cells were harvested from B6D2F1 (C57BL/6 × DBA/2) strain mice. ICR strain pseudopregnant females mated with vasectomized males of the same strain were used as surrogate mothers for the transferred embryos.

All animals were maintained in accordance with the guidelines of the Laboratory Animal Service at RIKEN Center for Developmental Biology. The protocols for animal handling and treatment were reviewed and approved by the Animal Care and Use Committee at the same institution.

Oocyte collection

Female mice were induced to superovulate with consecutive injections of equine chorionic gonadotropin

https://doi.org/10.1017/S0967199408004620 Published online by Cambridge University Press

(eCG; 5 IU) and human CG (hCG; 5 IU) 48 h apart. Fourteen hours after the human CG injection, cumulus–oocyte complexes (COC) were collected from the oviducts. The oocytes were freed from the cumulus cells by the addition of 0.1% bovine testicular hyaluronidase (ICN Biochemicals) to the COC-containing medium. After the cumulus cells had been dispersed from the oocytes, the oocytes were rinsed twice with CZB medium (Chatot *et al.*, 1990) and stored under 5% CO_2 at 37.5 °C for 30 min until use.

Enucleation

For ICSI and NT experiments, the second meiotic metaphase (MII) spindle of the oocytes had to be removed before the AG oocytes were created. A group of oocytes was transferred to a droplet (about 10 µl) of HEPES–CZB medium containing 5 µg/ml cytochalasin B under mineral oil on a micromanipulation microscope stage at 25 °C. An oocyte was selected and immobilized at the tip of a holding pipette by gentle suction. The zona pellucida of the oocyte was then penetrated by a piezo-actuated (Prime Tech) enucleation pipette (Wakayama *et al.*, 1998). The MII chromosome–spindle complex, distinguished as a translucent region in the ooplasm, was drawn into the pipette with minimal accompanying ooplasm. As the pipette tip was withdrawn through the zona pellucida entry site, its contents were pinched off, leaving behind an enucleated oocyte (ooplast). One batch of 20 oocytes typically took 6 min to enucleate. Immediately following enucleation, the ooplasts were placed under oil in droplets of cytochalasin-B-free CZB medium under 5% CO_2 at 37.5 °C for 1–3 h until use. The detailed protocols have been reported elsewhere.

Removal of the zona pellucida

Intact or enucleated oocytes were treated with acid Tyrode's solution (pH 2.5) for 30 s to dissolve the zona pellucida. Zona-free oocytes were washed with HEPES–CZB medium at least twice and then cultured in CZB medium, as above, for at least 30 min before use.

Oocyte fusion

In this study, we used two fusion methods, mechanical and electrofusion. For mechanical fusion (Fig. 1*a*), zona-free oocytes were transferred to HEPES–CZB medium in the micromanipulation chamber. One oocyte was held by the holding pipette and another was placed next to it. Both oocytes had to be exactly in line between the holding and fusing pipettes. The

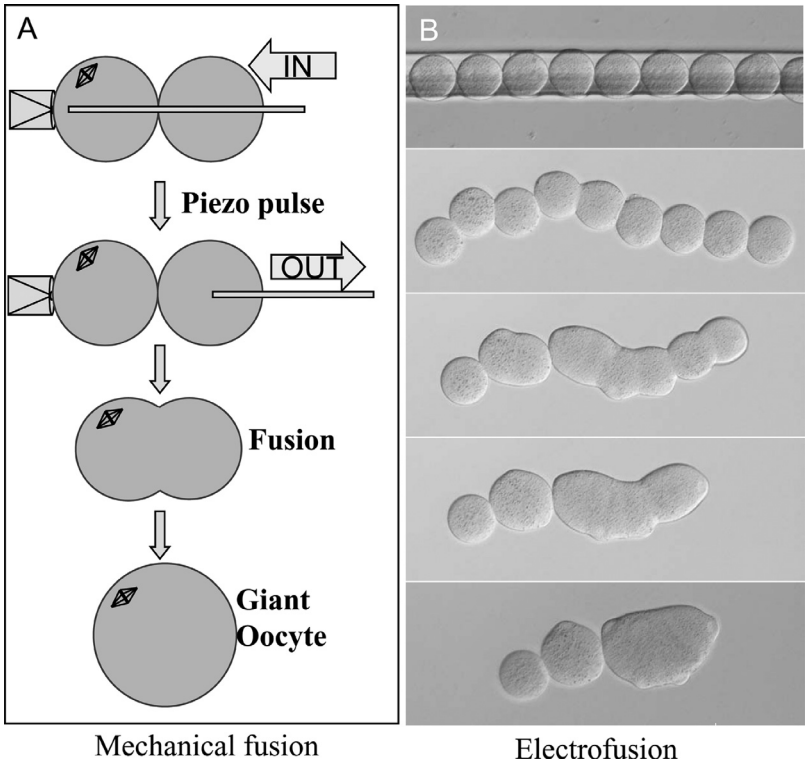

Figure 1 Methods of mechanical and electrofusion. (*a*). Mechanical fusion. (*b*) Electrofusion. Nine oocytes were clustered using phytohaemagglutinin and then given a fusion pulse. About 30 min later, one group of six and one group of two oocytes had fused, but one oocyte remained apart.

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fusing pipette (ID: 3 μm) was then inserted into the cytoplasm of the second oocyte, almost to the membrane of the first oocyte, as shown in Fig. 1*a*. Finally, both oocyte cell membranes were perforated using a piezo-pulse and the fusing pipette was pulled out gently. The paired oocytes were kept on the micromanipulation stage for about 20 min until fusion began and were then transferred and stored in CZB medium for more than 1 h, as above, to recover from fusion damage.

For electrofusion (Fig. 1*b*), pairs of zona-free oocytes were placed together in HEPES–CZB medium containing 0.05 mg/ml phytohaemagglutinin (PHA) for 1 min. They were then moved to new HEPES–CZB medium and pushed together using a micropipette. Once the oocytes had become firmly attached, they were placed into Ca^{2+}-free fusion medium (0.3 M mannitol, 0.1 mM $MgSO_4$ and 0.1% polyvinyl pyrrolidone [PVP]) until they sank to the bottom of the droplet. Within a few minutes, the paired oocytes were picked up and transferred to a fusion chamber with electrodes 0.5 mm apart, covered with fusion medium. A current was then applied (20 V alternate current [AC] at 1 MHz) for 1–2 s to line up the paired oocytes. Immediately thereafter, 70 V, 100 V, or 150 V direct current (DC) pulses were applied for 20 μs to cause fusion. The oocyte pairs were kept in the chamber for 5 min, then were carefully removed, transferred to CZB medium and incubated for at least 1 h to recover from fusion damage. In some experiments, ten oocytes or more were lined up for electrofusion in an attempt to generate even larger AG oocytes, to evaluate the appropriate volume of cytoplasm for the cloning procedure.

After fusion, we examined whether the oocytes were spontaneously activated or not.

Parthenogenetic activation of AG oocytes

In these experiments, AG oocytes were generated from nonenucleated oocytes (Fig. 2*a*). The fused oocytes were transferred to activation medium (10 mM $SrCl_2$ in Ca^{2+}-free CZB medium; (Bos-Mikich *et al.*, 1997). Six hours after activation, those oocytes had extruded their second polar bodies and contained female pronuclei. For the control experiments, single oocytes were activated with cytochalasin B, which prevents the extrusion of the second polar body, resulting in diploid embryos (Bos-Mikich *et al.*, 1997). The parthenogenetically activated zygotes were washed in CZB medium at least twice and cultured in CZB medium, as above, for 96 h to examine the *in vitro* developmental potential of the embryos.

Fertilization of AG oocytes by ICSI

In this experiment, AG oocytes were generated by the fusion of pairs of nonenucleated and enucleated oocytes (Fig. 2*b*). Thus, the AG oocytes possessed only one MII spindle. For sperm preparation, the cauda epididymis was removed from a mature male mouse (B6D2F1, 12–16 weeks old). Blood and adipose tissue were removed from the surface and the caudal (enlarged) part was excised with a pair of fine scissors. This tissue was compressed to release a dense mass of spermatozoa from which drops (about 2 μl) were taken. These spermatozoa were placed in a dish containing 0.4 ml of TYH (Toyoda *et al.*, 1971) and cultured in a CO_2 incubator at 37 °C for 20–30 min. The sperm concentration was approximately 3–5 × 10^6/ml at this stage.

ICSI was carried out using the technique described by Kimura & Yanagimachi (Kimura & Yanagimachi, 1995) with modifications. A droplet of sperm suspension (2–3 μl) was mixed thoroughly with 20 μl of HEPES–CZB medium containing 12% (w/v) PVP (Mr 360 kDa) in the micromanipulation chamber. The sperm head was separated from the midpiece and tail by the application of one or more piezo pulses and injected into an AG oocyte (Fig. 2*b*). Injected oocytes were transferred into CZB medium under mineral oil and incubated as described above. In the control experiments, ICSI was performed on single intact or zona-free oocytes and the zygotes were cultured in the same way.

Nuclear transfer into AG oocytes

In these experiments, AG oocytes were generated by the fusion of two enucleated oocytes, so there were no MII spindles (Fig. 2*c*). Nuclear transfer to cumulus and ES cells (line GRP14, which was established in our laboratory in the BD129F1 genetic background) was performed as described previously (Wakayama *et al.*, 1998, 2005; Kishigami *et al.*, 2006b). Briefly, cumulus cells were collected at the same time as the oocytes were collected. After treatment with hyaluronidase, 2 μl aliquots of the condensed cumulus cell suspension were transferred to PVP medium (as with ICSI, described above). The ES cells were thawed two days before each experiment and collected by trypsinization. To remove the trypsin, the cells were washed at least twice in phosphate-buffered saline (PBS) and 2 μl aliquots of the condensed cell suspension were transferred to PVP medium.

For nuclear injections, AG oocytes were transferred to a micromanipulation chamber. The donor cell was then drawn in and out of the injection pipette until the cell membrane was broken. In some cases, a few piezo pulses were applied to break the plasma membrane. After each cell nucleus had been drawn deep into the pipette, another was drawn into the same pipette. Within a few minutes, several nuclei were lined up within a single pipette and were injected one by

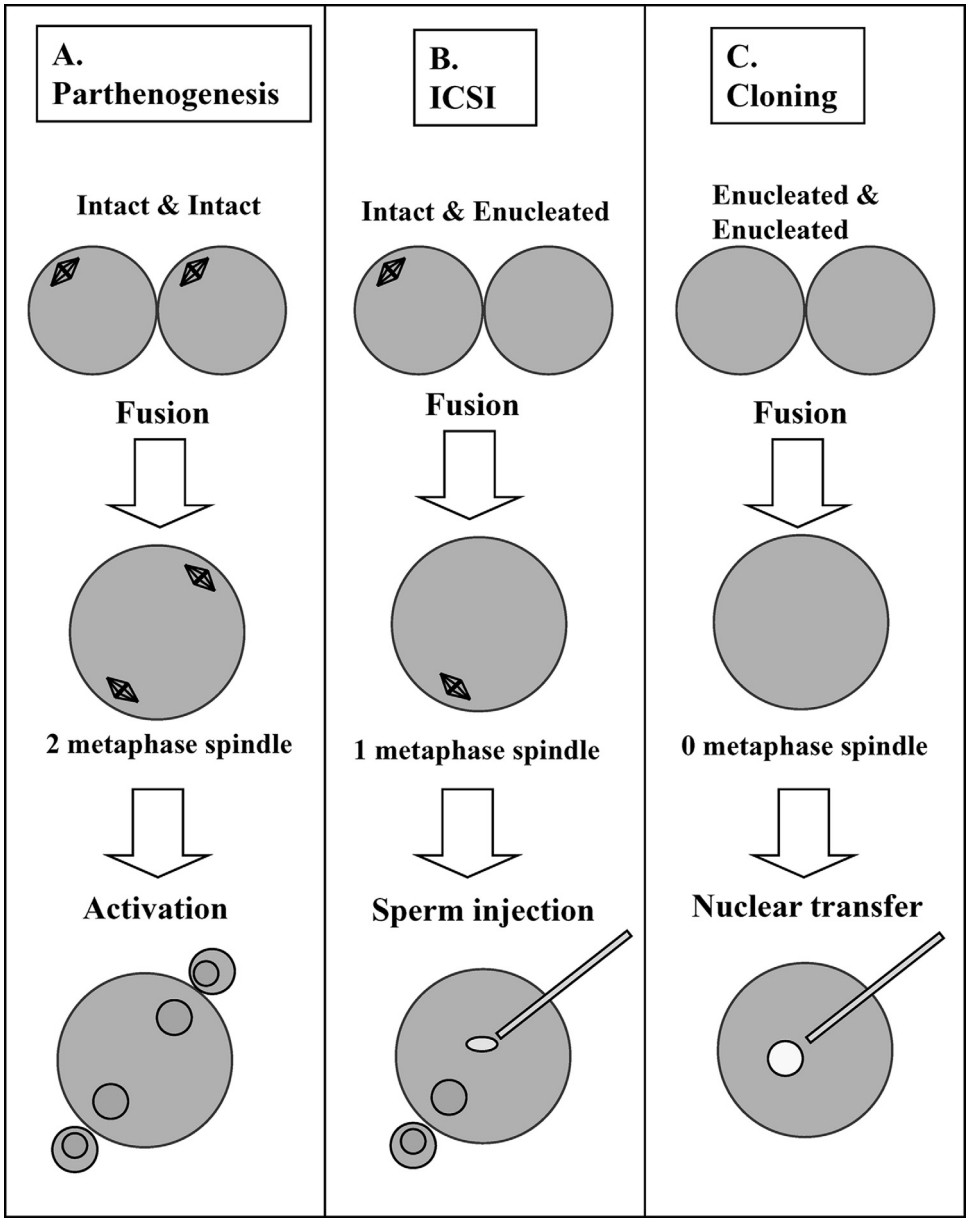

Figure 2 Experimental design. (*a*) Two intact oocytes were fused and activated parthenogenetically to examine their developmental potential *in vitro.* (*b*) Intact and enucleated oocytes were fused and then sperm heads were injected to examine the potential of the fertilized embryos to develop to term *in vivo.* (*c*) Two enucleated oocytes were fused and then somatic or ES cell nuclei were injected to examine the reprogramming potential of the AG oocyte cytoplasm.

one into separate AG oocytes at room temperature (Kishigami *et al.*, 2006b).

After NT, the reconstructed oocytes were activated with 10 mM $SrCl_2$ in Ca^{2+}-free CZB medium in the presence of 5 μg/ml cytochalasin B and 1% DMSO and cultured for four days in KSOM medium (Specialty Media).

In vitro and in vivo development

The oocytes were examined using an inverted microscope between 5 and 7 h after sperm injection or artificial activation. When the AG oocytes contained pronuclei or pseudopronuclei, they were considered to be activated. In the ICSI experiments, only those oocytes with two distinct pronuclei and a single second polar body were considered normally fertilized. These oocytes were cultured for up to 72 h to evaluate embryo development. Because they lacked the zona pellucida, these embryos often aggregated. To prevent this, each embryo was placed individually into a small hole made by pressing a fine needle onto the plastic dish under the medium.

When the embryos reached the morula–blastocyst stage, they were transferred to the uteri of

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pseudopregnant foster mothers (ICR strain, albino), which had been mated with vasectomized ICR males three days previously. All recipient females were euthanized at 19.5 days post coitus (dpc) and their uteri were examined for the presence of fetuses and implantation sites. Live fetuses were raised by lactating foster mothers (ICR strain).

Observation of oocyte spindle morphology by confocal laser microscopy

AG oocytes and control oocytes were fixed for 30 min in PBS–polyvinyl alcohol (0.1 mg/ml PVA) containing 3.5% (w/v) paraformaldehyde and 0.2% (v/v) Triton X-100. The fixed oocytes were washed twice in PBS–PVA and stored overnight at 4 °C in PBS supplemented with 3% (w/v) bovine serum albumin (PBS–BSA; Sigma–Aldrich) and 1% (v/v) Triton X-100 (Nacalai Tesque, Inc.).

To stain their α-tubulin, the oocytes were incubated with anti-α-tubulin antibody conjugated with fluorescein isothiocyanate (FITC; 1:100; Sigma–Aldrich) for 60 min at room temperature. After the oocytes had been washed three times in PBS–BSA for 10 min each, their DNA was stained with 400 μg/ml propidium iodide (Sigma–Aldrich). When they had been washed thoroughly, the oocytes were mounted on slides using Vectashield Mounting Medium (Vector Laboratories Inc.) and observed under a Bio-Rad Radiance 2100 confocal scanning laser microscope (Bio-Rad Laboratories).

Production of chimeric mice

Control embryos with increased size rather than volume were produced by the aggregation of two 8-cell embryos, as described previously (Nagy *et al.*, 2003). Briefly, BDF1 females were mated with BDF1 males and 8-cell embryos were collected at 3 dpc. The embryos were aggregated in a small hole in a culture dish. The next day, the aggregated blastocysts were transferred into 2.5 dpc pseudopregnant ICR females. At 19.5 dpc, the pregnant females were euthanized by cervical dislocation and the chimeric offspring were collected by Caesarean section.

Statistical analysis

Outcomes were evaluated using chi-squared tests and $p < 0.01$ was regarded as statistically significant.

Results

Fusion conditions

It has been reported that mouse AG oocytes produced using PEG fusion cannot be activated consistently. However, in mouse and human oocytes, the stimulation caused by pipette penetration does not, by itself, activate the oocytes (Tesarik & Testart, 1994; Kimura & Yanagimachi, 1995; Ogura *et al.*, 2001). To avoid any undesired oocyte activation caused by the fusion protocol, we developed a new oocyte fusion method by modifying the method of Tesarik *et al.* (2000), in which paired zona-free oocytes were fused using a relatively large pipette. In this experiment, we did not use phytohaemagglutinin P because all the oocytes treated with this agent died just after fusion (data not shown). When two intact zona-free oocytes were arranged in a straight line and fused with a single piezo pulse, 53% of the pairs fused successfully and none of them was activated spontaneously (Table 1). When the AG oocytes were observed by confocal microscopy after immunostaining for α-tubulin, no abnormalities were apparent compared with the control single oocytes (Fig. 3*f*).

This mechanical technique requires specialized skill and it is difficult to construct many AG oocytes, so we developed an electrofusion protocol. In preliminary experiments, we found that 2 MHz and 2 s AC pulse was sufficient for the line up of oocyte and caused minimal damage if the oocytes were pretreated with PHA. We then compared the effects of DC voltages ranging from 70 V to 150 V. As shown in table 1, maximal fusion rates (78.8%) were obtained when a 100 V electrical pulse was applied. We used that setting for all subsequent experiments.

Table 1 Fusion rate of paired mouse oocytes by electro- or mechanical method.

Fusion method	PHA treatment	Volts of DC	No. of used (paired) oocyte	No. of died or non fused oocyte	No. of fused oocyte (% from paired oocyte)	No. of activated oocytes
Mechanical fusion	No	–	166 (83)	78 (47)	44 (53)	0
Electrofusion	Yes	70	130 (65)	98 (75)	16 (25)	0
	Yes	100	66 (33)	14 (21)	26 (79)	0
	Yes	150	60 (30)	46 (77)	7 (23)	0

DC period was fixed at 20 μs.

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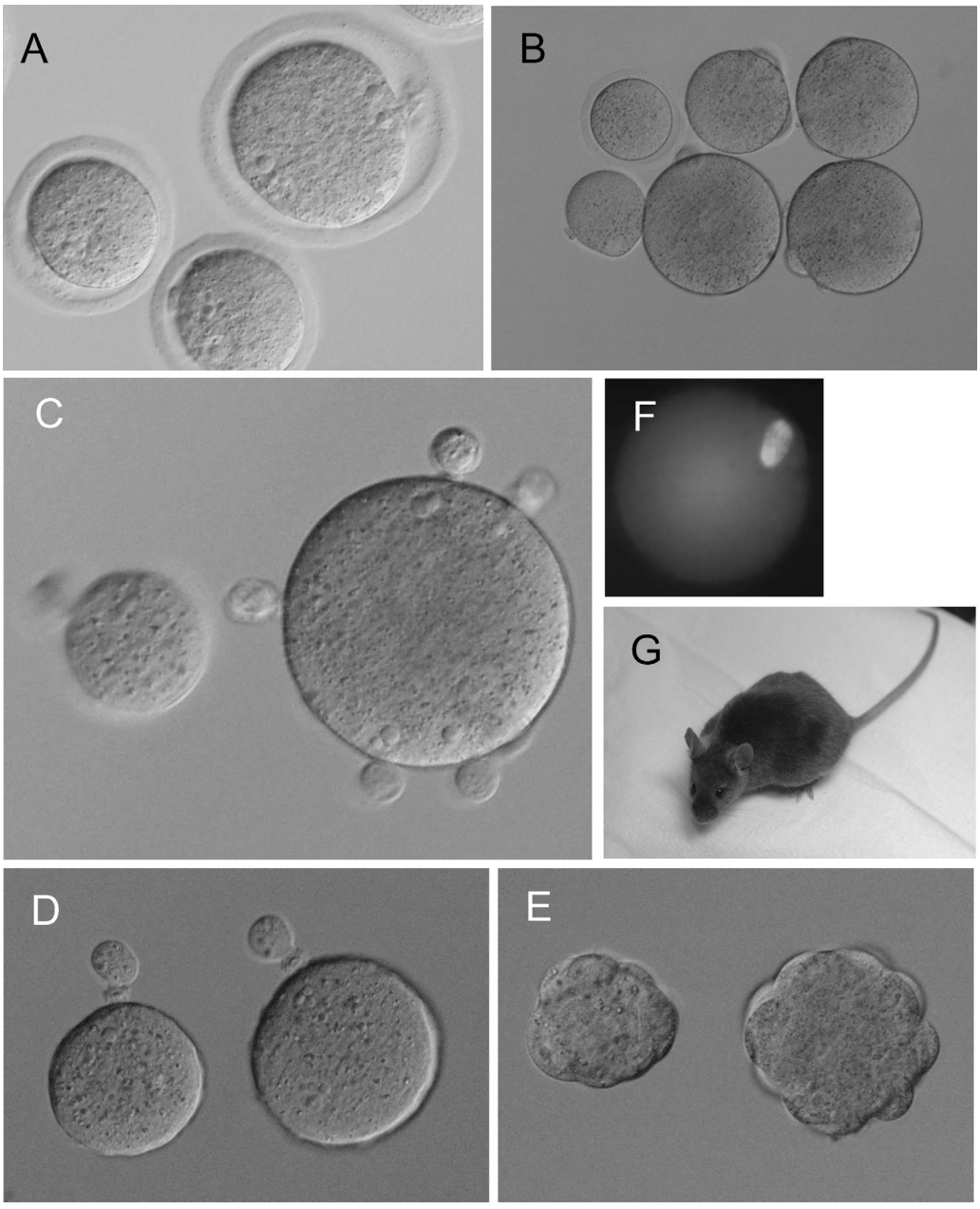

Figure 3 AG oocytes, giant embryos and full-term development. (*a*) Naturally ovulated oocyte, 6 h after strontium activation with cytochalasin B. (*b*) Control intact oocyte and fused oocytes that were two to five times the normal size. (*c*) Activated oocytes extruded the expected number of second polar bodies, equivalent to the number of fused oocytes. (*d*, *e*) Sperm heads were microinjected into single and double-sized AG oocytes. These extruded single second polar bodies (*d*) and had developed to the morula stage (*e*) at 72 h. (*f*) The spindle apparatus of a double-sized oocyte, observed with confocal microscopy. Colours: α-tubulin, green; DNA, red. (*g*) Offspring (named Giko) derived from a mechanically fused AG oocyte microinjected with a sperm head.

Activation and *in vitro* development

Next, we examined the *in vitro* developmental potential of AG oocytes after parthenogenetic activation. In this experiment, we made AG oocytes that were two to nine times larger than normal oocytes by electrofusion and double-sized AG oocytes by mechanical fusion of nonenucleated oocytes. It was rare to get three or more oocytes to fuse electrically at the same time. Usually, only a few oocytes within any line fused. For example, when 10 oocytes were aligned together and an electrical pulse was applied, some oocytes died or did not fuse and only a few remaining oocytes fused with each other (Figs. 1*b*, 3*b*). The largest fusion product we

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Table 2 Preimplantation development rates of mouse AG oocytes followed by parthenogenetic activation.

Method of fusion	Size of AG oocyte (times)	No. fused oocyte	No. activated oocytes (%)	No. (%) of embryos developing to each stage at 96 h after activation			
				1-cell	2-cell	8-cell	Mol/Bla
Control	1	35	35 (100)	0	1	3	31 (89)
Electrofusion	2	72	71 (99)	0	2	14	55 (77)
	3	20	20 (100)	0	0	1	19 (95)
	4	16	16 (100)	0	0	2	14 (88)
	5	9	9 (100)	0	0	1	8 (89)
	6	1	1 (100)	0	0	0	1 (100)
	7	2	2 (100)	0	0	0	2 (100)
	9	2	2 (100)	0	0	1	1 (50)
Mechanical fusion	2	11	9 (90.9)	0	0	0	9 (100)

Table 3 Full term development of AG oocyte after ICSI and embryo transfer.

Size of oocyte (fusion method)	Type of fertilization	Condition of zona pellucida	Used oocytes (*n*)	Fused/ aggregated oocytes (*n*)	Oocyte survived after ICSI (*n*)	Morula/ blastocyst (*n*)	Embryo transfer (*n*)	Offspring (*n*)
Double (mechanical fusion)	ICSI	Removed at oocyte	84	32	26	23 (88)	23	1 (4)[a]
Double (electrofusion)	ICSI	Removed at oocyte	313	198	147	71 (48)	71	11 (15)[a]
Single (control A)	ICSI	Removed before ICSI	40	–	31	30 (97)	30	2 (6)[a]
Single (control B)	ICSI	Removed after ICSI	70	–	59	52 (88)	52	5 (10)[a]
Single (control C)	ICSI	Intact	37	–	33	32 (97)	32	25 (78)[b,c]
Single (control D)	IVF	Removed after IVF	60	–	–	58 (97)	58	2 (3)[a]
Single (control E)	*In vivo* F	Removed at zygote	74	–	–	73 (99)	73	27 (37)[b,d]
Single (control F)	*In vivo* F	Intact	43	–	–	42 (98)	42	29 (69)[b,c]
Double (control G: aggregation chimera)	*In vivo* F	Removed at 8-cell stage	60	30	–	30 (100)	30	25 (83)[b,c]

achieved was nine times larger than a normal oocyte. None of these mouse oocytes was spontaneously activated by either electrofusion or mechanical fusion, in contrast to the human oocytes studied by Cohen *et al.* (1998).

About 2 h after fusion, which was required for recovery, the oocytes was activated parthenogenetically using strontium treatment. Almost all the electrofused AG oocytes were activated, irrespective of size. However, about 10% of the mechanically fused AG oocytes failed to activate. All activated AG oocytes formed pronuclei and extruded the expected number of second polar bodies. For example, constructs produced by fusing seven oocytes extruded seven second polar bodies within 2 h and formed seven female pronuclei (Fig. 3*c*).

When we examined their *in vitro* development 96 h after activation, most AG oocytes (88%–100%) had reached the morula or blastocyst stage, except for the double-sized AG oocytes produced by electrofusion (77%; Table 2). Interestingly, two of the nine-times normal-sized AG oocytes had divided into more than five cells at 24 h after activation, but they continued to grow and formed normal-looking blastocysts, except that they were much larger than normal 96 h after activation.

In vivo developmental potential after ICSI

We examined the full-term developmental potential of double-sized AG oocytes after ICSI (Table 3). These AG oocytes were formed by fusing non-enucleated and enucleated oocytes, so they had only one metaphase spindle. After mechanical fusion, 26 of the AG oocytes survived after ICSI; 23 (88%) of the embryos developed to the morula or blastocyst stage *in vitro* (Fig. 3*d*, *e*) and after embryo transfer, one of these (4%) reached full term (Fig. 3*g*). After electrofusion, although the rate of development to morula or blastocyst stage was significantly lower (71/148; 48%) than after mechanical fusion, 11 of these embryos produced live offspring (15%; not significantly different from that of embryos derived from mechanical fusion). All offspring were born by Caesarean section and six

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Table 4 Full-term development of twice large oocyte after NT and embryo transfer.

Oocytes (type)	Donor cell (type)	Oocytes used (*n*)	Fused oocytes[a] (*n*)	Oocytes survived after NT (*n*)	Activated oocytes (*n*)	Embryos examined 76 h after nuclear transfer (% from activated) (*n*)				Embryo transfer (*n*)	Offspring (*n*)
						1-cell	2-cell	4- to 8-cell	Morula/ blastocyst		
Giant	Cumulus	300	82	66	16 (24)	5 (31)	5	3	3 (19)	3	0
	ES	150	39	28	14 (50)	11 (79)	2	1	0	0	0
Control	Cumulus	120	–	94	52 (55)	0	2	0	50 (96)	50	1 (2)
	ES	60	–	47	37 (78)	21 (57)	3	2	11 (30)	11	1 (5)

[a]Fusion rate of enucleated oocytes were decreased than intact oocytes.

survived to become healthy adults (three females, three males; the remaining six were cannibalized by their foster mothers). One mouse derived from mechanical fusion and five from electrofused oocytes were mated and all demonstrated normal fertility.

Despite these encouraging results, the overall success rate for full-term development in embryos produced from AG oocytes was much lower than for usual ICSI (Yanagimachi, 1998). As controls, we also examined the effects of zona pellucida removal, the ICSI and *in vitro* fertilization (IVF) procedures and the size of the embryos on full-term development (Table 3). When we injected sperm heads into zona-free single oocytes or removed the zona after ICSI, most of the embryos developed to blastocysts *in vitro*, but only two (6%) and five (10%) offspring, respectively, were born at full term (Control A and B). In contrast, most zona-intact oocytes (78%) developed into full-term pups after ICSI. We also found that ICSI and IVF themselves had detrimental effects on zona-free embryo development, because the zona-free *in-vivo* fertilized zygotes showed little better development (37%) than zona-free ICSI or IVF embryos (Control D and E). Conversely, the rate of full-term development of aggregation chimeras (double-sized embryos) was almost the same as that of intact embryos (83%), which suggests that the increased mass of an embryo *per se* does not impair development (Control G).

Developmental potential of cloned embryos

Finally, we examined the reprogramming potential of AG oocytes by injecting either somatic (cumulus cell) or ES cell nuclei into AG oocytes produced by electrically fusing two enucleated oocytes. The fusion rate for such pairs of enucleated oocytes was lower than that observed in the other experiments; most nonfused oocytes lysed. The oocytes may have been damaged by enucleation, for example during cytochalasin B treatment.

Using cumulus cell NT, 66 oocytes survived injection, but only 16 AG oocytes (24%) contained pseudopronuclei after activation. From those, 11 embryos started first cell division and three cloned embryos (19%) developed to the morula or blastocyst stage, but none of them developed to full term after embryo transfer. In the ES cell experiments, 28 AG oocytes survived after nuclear injection and 14 (50%) contained pseudopronuclei. However, only three embryos started first cell division and none of them developed to the morula or blastocyst stage. In the control experiments, mice cloned from both somatic and ES cells were obtained when donor nuclei were injected into zona-intact enucleated oocytes.

https://doi.org/10.1017/S0967199408004620 Published online by Cambridge University Press

Discussion

Giant oocytes provide interesting material for basic biological research. In our laboratory, the frequency of spontaneously ovulated giant oocytes has not been recorded, but at most, only one is seen every year (Fig. 3*a*), about 0.01% to 0.001% of all the oocytes we study. Therefore, in this study, we generated giant oocytes artificially using both mechanical and electrofusion methods. We succeeded in obtaining normal offspring from those AG oocytes after ICSI and first report that doubling the volume of the cytoplasm in the mouse oocyte allows normal developmental potential to full term after fertilization.

About 9% of the AG oocytes generated mechanically by piezo-activated fusion failed to be activated. This finding suggests that the mechanical approach produced considerable damage in the oocytes, from which some did not recover by the time of activation. However, once the AG oocytes were activated, all of them extruded second polar bodies and formed female pronuclei in numbers that corresponded to the number of oocytes used for fusion. The MII spindles probably did not mix after oocyte fusion. Most of those parthenogenetically activated oocytes developed to the morula or blastocyst stage, irrespective of their size and chromosome numbers. This situation clearly demonstrates that the amount of cytoplasm of an oocyte *per se* does not impair *in vitro* preimplantation development. This finding is consistent with previous reports of double-sized AG oocytes produced with Sendai virus or PEG-mediated fusion (Soupart, 1980; Gulyas *et al.*, 1984; Karnikova *et al.*, 2000) (Table 2).

We examined the developmental potential of embryos produced from AG oocytes *in vivo.* When the oocytes were fertilized by ICSI and the giant morulae or blastocysts were transferred into recipient females, we obtained normal healthy and fertile offspring. To the best of our knowledge, this outcome is the first demonstration that such AG oocytes possess normal developmental potential to term after fertilization. The rate of development of giant embryos to the morula or blastocyst stage was similar to the rate observed for control single embryos. However, the full-term development of giant embryos was very low: only one pup (4% of embryos) resulted from mechanical fusion and 11 pups (15%) from electrofusion. This was significantly lower than the success rate following intact embryo transfer (78%). In the control experiments, normal-sized *in vivo*-fertilized zygotes cultured without the zona pellucida (Control E) developed to blastocysts at similar rates as intact oocytes (Control F), but their full-term developmental rate was significantly decreased, as in previous studies (Naito *et al.*, 1992; Suzuki *et al.*, 1995). Moreover, ICSI and IVF itself reduced the success rate of offspring production, whether the zona was removed before or after ICSI/IVF (Control A, B and D). Then, in these AG oocytes, both zona removal and ICSI had negative effects on full-term development. We also examined the developmental potential of SCNT-cloned embryos using AG oocytes. Before starting the experiments, we hypothesized that AG oocytes may possess extra nuclear reprogramming potential by dint of their increased cytoplasmic mass or increased reprogramming factor(s), so that the cloning success rate would be improved. However, none of the cloned embryos developed to full term. Recently, cloned cattle and mice have been born from zona-free oocytes fused with somatic or ES cells (Vajta *et al.*, 2003; Tecirlioglu *et al.*, 2004; Ribas *et al.*, 2005). Moreover, double-sized or larger chimeric cloned embryos produced by aggregation developed to term more efficiently than control embryos (Peura *et al.*, 1998; Boiani *et al.*, 2003; Vajta *et al.*, 2003). These reports suggest that neither zona-free nor large-sized embryos are disadvantaged. Why did the AG oocytes we studied here have a reduced success rate in terms of the survival of cloned embryos to term? Previously, we have reported that cloned mouse embryos often fail to generate normal spindles (Van Thuan *et al.*, 2006) and form asymmetric 2-cell embryos (Wakayama *et al.*, 1998). One possibility is that the AG oocytes have twice as much cytoplasm as normal. Then, the diameter is theoretically 1.26 times greater than that of normal oocytes (volume of double size AG oocyte $= 2(4/3\pi r^3) = 4/3\pi(r + X)^3$; increased half diameter $X = 0.26$), which may impair the internal cell machinery of the first cell division of the giant cloned embryos. if so, even if the AG oocytes have twice the amount of nuclear reprogramming factor(s) and are able to reprogram the somatic cell nuclei more efficiently, the subsequent cloned embryos may stop at the 1-cell stage or become aneuploid. In fact, 31% of somatic cloned embryos derived from AG oocytes failed first cell division, whereas in control oocytes, none of cloned embryo failed at this stage. It is known that abnormal karyotypes, such as triploidy (Sherard *et al.*, 1986; Henery & Kaufman, 1993), tetraploidy (Snow, 1975), or damaged chromosomes (Kusakabe *et al.*, 2001), do not prevent preimplantation development, but most of these embryos die during foetal development. Therefore, even if giant cloned embryos could develop to morulae or blastocysts, they might have no hope of developing to full term.

In this experiment, although we did not obtain any cloned mice, 12 healthy offspring were obtained after ICSI, which is the first demonstration that AG oocytes can develop to term when one chromosome set is removed before fertilization. Although further analysis is required, AG oocytes should provide us with interesting tools for basic biological research, including providing new material for the study of

nuclear reprogramming and genomic imprinting and may be important in widening the treatment options in human assisted reproductive technology.

Acknowledgements

We are grateful to the Laboratory for Animal Resources and Genetic Engineering for housing the mice. This work was supported by Scientific Research in Priority Areas (grant no. 15080211) and by a project for the realization of regenerative medicine (research toward technical developments in stem cell manipulation) to T. W. from the Ministry of Education, Culture, Sports, Science and Technology of Japan.

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