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human reproduction ORIGINAL ARTICLE *Embryology*

Symmetrical division of mouse oocytes during meiotic maturation can lead to the development of twin embryos that amalgamate to form a chimeric hermaphrodite

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BACKGROUND: Gentle compression of mouse oocytes during meiosis-I prevented the usual extrusion of a small polar body and resulted in the symmetrical division of the ooplasm into two cells of similar size within the zona pellucida. The purpose of our study was to determine whether such cells, equivalent to two small oocytes, were capable of embryonic development and would result in birth following transfer to the uterus.

METHODS: IVF of the 2-celled oocytes was performed and the twin intra-zonal embryos were observed. In each case, the two embryos that originated from fertilized cells with two pronuclei were observed to amalgamate and form a single morula and subsequent blastocyst that was transferred to the uterus of a recipient of a different mouse strain. FISH analysis was performed on sectioned paraffin-embedded tissue of the offspring.

RESULTS: In symmetrically divided oocytes each cell contained a metaphase II spindle. Both cells were fertilizable and cleaved to form twin embryos within the same zona pellucida. Most twin embryos amalgamated to form a single compacted morula, which progressed to hatched blastocysts that contained a single inner cell mass. In total, 104 of these blastocysts were transferred to 19 mice, two of which became pregnant, resulting in the birth of three offspring. FISH analysis showed that one newborn contained both XX and XY cells.

CONCLUSIONS: We found that two small oocytes fertilized within the same zona pellucida to form twin embryos that amalgamate to establish a single chimeric embryo. This may be one mechanism that leads to the formation of a chimeric hermaphrodite when an embryo containing XX cells mixes with its intra-zonal twin containing XY cells.

Key words: 2-celled oocytes / meiotic spindle / embryo amalgamation / chimera / hermaphrodite origin

Introduction

Oocytes aspirated from their follicles are occasionally found to contain two cells, of similar size, within their zona pellucida. This condition has been described as 'immediate cleavage' in some papers (Kaufman, 1973; Van de Leur and Zeilmaker, 1990). Some investigators have reported that in mouse oocytes, immediate cleavage can be induced by exposing tubal ova to high or low temperatures, to hypertonic or hypotonic solutions or to stimulation with an electric current, or by ether anesthesia (Komar, 1982). In the human, a presumed 'embryo' consisting of two cells, each containing two pronuclei, was found in an IVF laboratory and the authors suggested that this resulted from parthenogenetic activation of the oocyte leading to immediate cleavage, followed by separate fertilization of each cell (Van de Leur and Zeilmaker, 1990). However, the mechanism that induces immediate cleavage has not been clearly explained for more than two decades. It should be noted that parthenogenetic activation of maturing oocytes does not always induce their cleavage into two equal cells. In most cases, parthenogenetically activated mature oocytes extrude second polar bodies and only

a small proportion of oocytes (Kaufman, 1973) cleave immediately into two equal cells.

In the present study, we found that the gentle compression of mouse oocytes during meiotic maturation prevented the extrusion of a small polar body, but instead resulted in the formation of two cells of similar size within the zona pellucida. One of these cells was considered to be an extruded large first polar body. In such 'double' oocytes each cell attained meiotic metaphase II (MII). Our aim was to determine whether each cell could be fertilized by a separate sperm, and whether the two intra-zonal embryos retained developmental competence. We therefore transferred such embryos to the uterus of recipient mice, to determine whether they were capable of developing to birth.

Materials and Methods

Mouse oocytes

Mice were exposed to a 12 h light/dark cycle with food and water provided ad libitum. Female BDF1 mice, aged 7–12 weeks and weighing 27–34 g, were injected with 7.5 IU of equine chorionic gonadotrophin (Teikoku-Zoki Pharmaceuticals, Tokyo, Japan) followed by an injection of 5 IU of hCG (Mochida Pharmaceutical, Japan). Oocytes undergoing meiotic maturation were retrieved from the ovaries 7–8 h after hCG injection. The cumulus cells were removed by gentle pipetting in HEPES-buffered human tubal fluid (HTF; *In Vitro* Care, Ferderick, MD, USA) medium containing 1 mg/ml human serum albumin (HSA; *In Vitro* Care). The cumulus-free oocytes containing a meiotic-1 (M1) spindle were washed three times in HTF medium containing 1 mg/ml HSA and they were then cultured at 37°C in a humidified atmosphere of 5% CO_2, 5% O_2 and 90% N_2. All experimental mice in this study were cared for using procedures approved by the Animal Care Committees of both the Nagai Clinic and the National Institute of Biomedical Innovation, Tsukuba Primate Research Center. Both institutions approved all animal handling and experimental procedures used in the present studies.

Time-lapse observations

Using a CO_2 chamber (SKHC-303, Sankei, Japan) equipped with a DIC inverted microscope (Nikon ECLIPSE TE2000-U), time-lapse recordings were performed on the cultured mouse oocytes. The inverted microscope was equipped with a digital camera (Nikon D200) connected to a computer and a display using Nikon Capture (Nikon, Japan) software. Digital images of the oocytes in culture were recorded every 1–2 min with an exposure time of 2 s. The chamber was maintained at 37°C with a humidified atmosphere of 5% CO_2, 5% O_2 and 90% N_2.

Compression of the ooplasm near the meiotic spindle

During the meiotic maturation in culture, mouse oocytes were pressed down to a thickness of 20–30 μm between a cover glass and the bottom of a glass dish using autoclaved silicon grease as support between them. This procedure was applied to compress the cortical cytoplasm when the M1 spindle was near the surface of the oocyte in order to impede the spindle's movement and promote symmetrical division of the oocyte.

Disruption of the zona pellucida by laser for IVF

After an oocyte divided into two cells of similar size, fertilization of each of the cells was achieved by *in vitro* insemination after partial disruption of the oocyte's zona pellucida using laser (OCTAX Laser Shot System for ART, from OCTAX Microscience). The use of the laser system for making an opening in the zona pellucida has been described in detail (Germond *et al.*, 1995). Briefly, a 1.48-μm diode laser aiming beam and a collimated 1.48 μm continuous-wave laser beam are passed into an inverted microscope (Olympus IX-70; Tokyo, Japan), redirected by several mirrors and focused on the microscopic field. The power routinely available at the image plane of the objective is 47 mW, corresponding to a maximum power density of 94 kW/cm^2. During the zona opening procedure, oocytes were suspended in 10 μl droplets of HTF medium containing 1 mg/ml HSA under mineral oil in a 35-mm culture dish.

IVF and embryo culture

Mouse spermatozoa were collected from the cauda epididymis and suspended in HTF medium that contained 1 mg/ml HSA. After pre-incubation for 1 h, an aliquot of sperm suspension was added to the medium containing oocytes to provide a final sperm concentration of 200 sperm/μl. After 3–4 h of insemination, oocytes were examined to confirm the presence of extruded second polar bodies. These fertilized oocytes were transferred into LGGG medium (Life Grobal, Japan) that contained 0.1 mg/ml rHSA (rHA; Vitrolife, Sweden). At 16 h after insemination, the oocytes were examined for the presence of two pronuclei.

Embryo culture experiments were performed using double small oocytes, each containing two pronuclei. Embryos arising from polyspermic fertilization were excluded. In addition, a study was performed to compare embryo development following fertilization of the two small oocytes in the one zona pellucida, with embryo development originating from the fertilization of a single large oocyte. The formation of blastocysts was the endpoint of this study, comparing embryo development from 104 double small oocytes with 106 single large oocytes. Statistical analysis was performed using Welch's *t*-test employing the statistical software StatMateIII (ATMS, Inc., Tokyo, Japan). A difference was considered to be significant when its *P*-value was <0.05.

Embryo transfer

The recipients for embryo transfer were pseudo-pregnant day 3 mice of an ICR strain. These recipients were selected after detecting the presence of copulatory plugs following mating with vasectomized ICR male mice. Day 4 cultured BDF1 mouse blastocysts were transferred to the recipients using a glass transfer pipette.

FISH analysis

Dual-color FISH analysis was performed on sections of paraffin-embedded tissues obtained from a newborn mouse, presumed to be a chimera. Briefly, 5 μm sections were washed in phosphate-buffered saline for 5 min, digested in pepsin solution (0.02% in 0.1 N HCl) at 37°C for 5 min and then dehydrated. A digoxigenin-labeled mouse X probe and a biotin-labeled mouse Y probe were applied to the pretreated sections, covered with cover slips and simultaneously denatured at 90°C for 10 min. Hybridization was carried out at 37°C overnight. Sections were then washed with 50% formamide/2 × saline sodium citrate (SSC) at 37°C for 20 min and 1 × SSC for 15 min at room temperature and blocking solution (5% skim milk, 0.1% non-idet P-40, 0.1 M phosphate buffer, pH7.5) was applied at 37°C for 30 min. The mouse X and Y probe signals were detected with Cy5-labeled anti-digoxigenin and avidin-Cy3, respectively. The sections were treated with antibodies at 37°C for

30 min, washed three times with 0.1% non-idet P-40/2 × SSC, counter stained with 4,6-diamidino-2-phenylindole and mounted on slides. The same procedures were applied to control tissues obtained from a normal male. The FISH images were captured with a CW4000 FISH system.

Results

Spindle behavior and oocyte division when the ooplasm was compressed

Meiotic maturation of mouse oocytes usually results in an asymmetric cell division. Polar body extrusion occurs after the spindle migrates to the oocyte's cortical region along its long axis and becomes positioned perpendicular to the surface. When gentle compression was applied to the oocyte, the MI spindle adopted a position in which its axis was observed to be parallel to the surface. A cleavage furrow was observed to form adjacent to the spindle and this surface indentation appeared to push its way toward the spindle's midzone. Then, as the entire midzone structure appeared to shrink, the spindle together with the cleavage furrow moved across the ooplasm and divided the oocyte into two approximately equal cells. Thus, instead of extruding a typical small polar body, the cytoplasm was bisected into two similar oval cells. Subsequently, a structure resembling a meiotic MII spindle formed in each of the daughter cells that now resembled two small oocytes within the same zona pellucida (Fig. 1).

In vitro embryonic development after fertilization of the 2-celled oocytes

After partial disruption of their zona with laser, *in vitro* insemination of the 2-celled oocytes was performed to evaluate the developmental potential of each daughter cell. When such 2-celled MII mouse oocytes were fertilized, in the presence of small openings in their zona pellucida, a typical second polar body was extruded from each cell and early male and female pronuclei were seen in each cell by 6 h after insemination (Fig. 2A). Division of each cell initially produced distinct twin 2-celled and then twin 4-celled embryos (Fig. 2B and C). However, beyond these stages, we could not determine whether embryos remained separated or whether mixing of their blastomeres was occurring. Moreover, compaction at about the 8-celled stage made it difficult to distinguish separate embryos (Fig. 2C). In all cases, amalgamation of the embryos produced a single morula (Fig. 2D and E). In these studies, we observed more than 100 blastocysts that originated from the amalgamated twin embryos, and each blastocyst was found to contain a single inner cell mass (Fig. 2F). Such blastocysts were transferred to recipient mice.

Comparison of embryo development after fertilization of small double oocytes and single large oocytes

After IVF, syngamy usually occurred in each small oocyte by 16–18 h after insemination. In these small double oocytes, the first cleavage division was observed in 72.1% of the zygotes by 24 h after insemination (Fig. 3). As a rule one cell divided before the other, and such asynchrony was seen regularly. Each fertilized cell divided into two regular blastomeres to form twin 2-celled embryos (Fig. 2B). The separate twin embryos, each containing two blastomeres, were quite distinct inside their zona. By 48 h after insemination, two definitive 4-cell embryos were observed in 44.1% of the fertilized double oocytes and in 88.5% of single normal oocytes ($P < 0.001$). At earlier times, 3-celled embryos were occasionally noticed in both groups. More advanced twin embryo stages, including approximately 8-cell embryos, could not be clearly distinguished, possibly due to the onset of compaction. However, by 60 h after insemination, it was clear that a significantly lower percentage of fertilized double oocytes attained this stage (Fig. 3). Similarly, a significantly lower percentage of fertilized double oocytes reached the morula and blastocyst stages by 72 and 96 h after insemination.

The birth of mouse pups after transfer of amalgamated embryos and FISH analysis

In total, 104 chimeric BDF1 mouse blastocysts were transferred to the uterus of 19 ICR mouse recipients. Two out of 19 mice became pregnant and three pups were born (Fig. 4). Two of these were partly eaten by the mother and one died on Day 5 after birth.

FISH analysis on the head and neck tissues of the partly eaten offspring showed that it contained both XX and XY cells (Fig. 5A). This indicated that an XX/XY chimera was established by the amalgamation of the twin embryos, inside the same zona pellucida, as a result of our experimental procedures.

Discussion

The human oocyte in the dominant follicle is arrested at the germinal vesicle stage until it is stimulated to resume meiotic maturation. After germinal vesicle breakdown, the first meiotic spindle is formed and homologous chromosomes become aligned along its midzone. The subsequent first meiotic division is asymmetrical and produces two cells: the secondary oocyte and the first polar body. Meiosis then remains arrested until the oocyte is fertilized or activated by another mechanism. Following activation meiosis resumes and the oocyte undergoes a second asymmetric division, again producing two cells: a zygote and the second polar body. Both first and second polar bodies are small and usually degenerate shortly after their extrusion. On rare occasions, the first meiotic division may be symmetrical and produces a large polar body. This large polar body can be regarded as a second intra-zonal oocyte, which can be fertilized by a second sperm. In the present study we have demonstrated that a controlled compression of mouse oocytes during meiosis-1, resulted in the formation of two fertilizable oocytes inside the same zona pellucida. Symmetric division, in which the oocyte and the released polar body are similar in size, can probably also occur during the extrusion of the second polar body.

In mouse oocytes, during meiosis-1, the established spindle migrates along its long axis, reaches the cortical region in a vertical position and remains in this position during its asymmetric meiotic division (Azoury *et al.*, 2008; Brunet and Verlhac, 2011; Schuh and Ellenberg, 2008) to produce a small first polar body. The question that needs to be answered from the present study is how, following gentle compression, oocytes undergo symmetrical meiotic division to yield two cells that are approximately equal in size. During this symmetrical division, the long axis of the meiotic

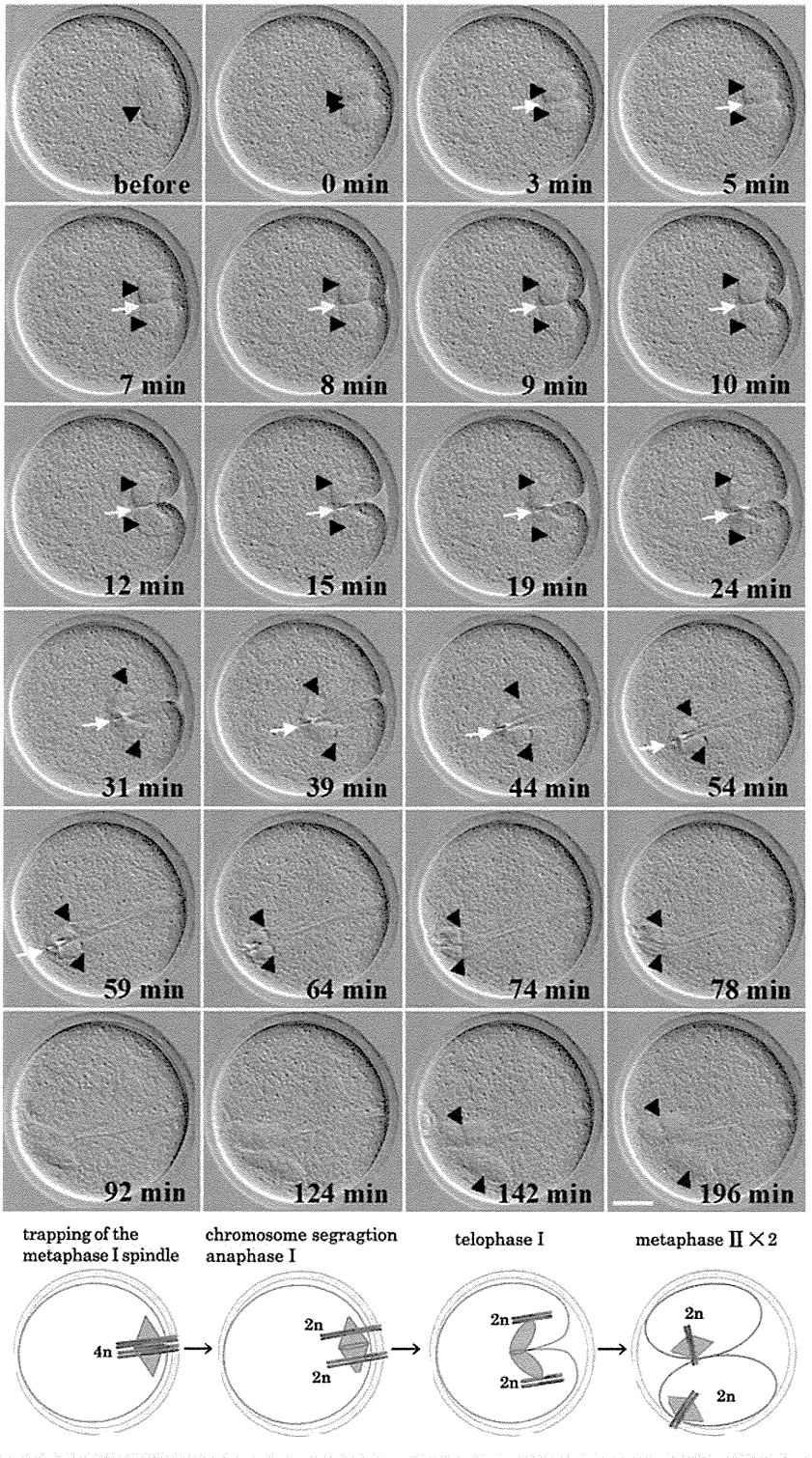

Figure 1 The time-course of symmetric oocyte division when the cortical ooplasm near the MI spindle was compressed. The sequence of oocyte changes, obtained from time-lapse photography, shows the time-course of a symmetrical meiotic division when the MI spindle was experimentally compressed in the cortical region of the oocyte. A cleavage furrow formed adjacent to the spindle and appeared to move toward the midzone (8–78 min). Subsequently, the spindle and cleavage furrow traversed the ooplasm, so that the oocyte became divided into two approximately equal cells (92–124 min), instead of extruding a typical small polar body. After the two cells were established, a MII spindle formed in each cell (142–196 min). An arrow shows the midzone. Scale bars = 20 μm.

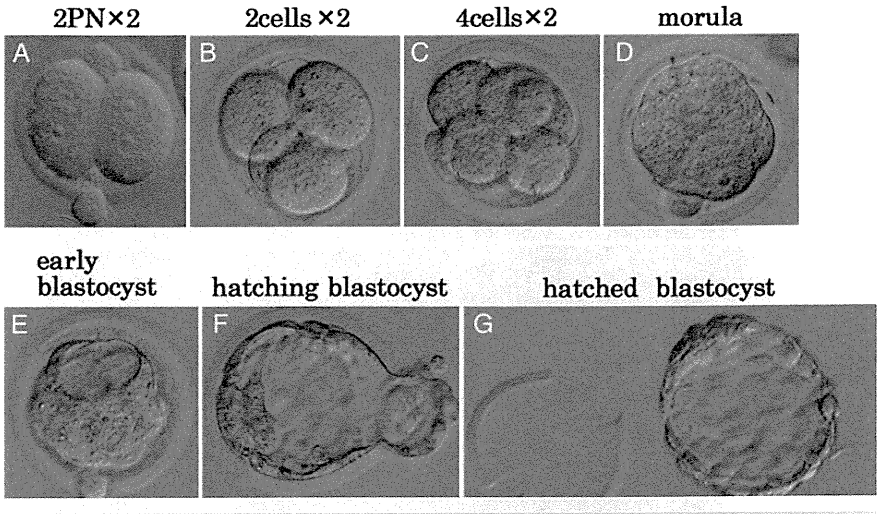

Figure 2 (**A**) Each of the two small oocytes was fertilized, revealed by the presence of female and male pronuclei and an extruded second polar body from each of the cells. (**B**) Each fertilized cell divided to form twin 2-celled embryos. (**C**) Two 4-celled embryos formed twin embryos within the same zona pellucida. (**D**) The twin embryos amalgamated to form a single compacted morula. (**E**) A single cavitating morula has formed. (**F**) A hatching blastocyst contained a single inner cell mass. (**G**) The chimeric blastocys is fully hatched.

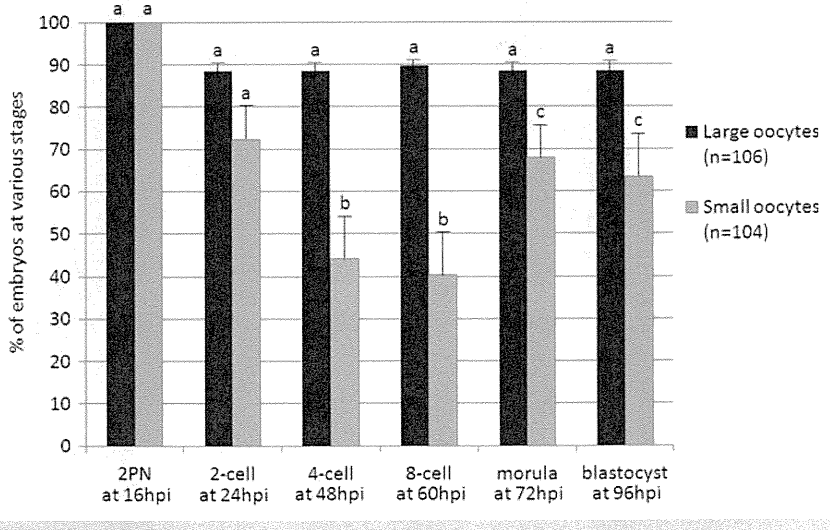

Figure 3 In the histogram, the timing of embryo development is compared after fertilization of small double oocytes and single normal large oocytes. At 24 h post insemination (hpi), 72.1% of small oocytes and 88.5% large oocytes formed 2-cell embryos. The formation of 4-cell embryos at 48 hpi in the two groups was 44.1 and 88.5%, respectively. The establishment of 8-cell embryos was more difficult to judge because of possible cell mixing and onset of compaction, but it was judged that 40.4% of the small oocytes and 89.6% of the large oocytes reached this stage. At 72 hpi, the establishment of a single morula was observed in 67.8% of the small oocyte group and 88.5% of the large oocyte group. By 96 hpi, blastocysts were observed in 63.3% of the small oocyte group, compared with 88.5% in the large oocyte group. The differences in embryo development were statistically significant at each time interval. a,a; NS, a,b; $P < 0.001$; a,c; $P < 0.05$.

spindle has been observed to be parallel to the surface of the oocyte. It would appear that the compression of meiotic oocytes may induce the spindle to take up a position that is parallel, instead of perpendicular, to the plasma membrane. It has been suggested that the compression of the oocytes may delay the onset of anaphase-I and this could lead to the spindle adopting a position parallel to the surface (Melina Schuh, personal communication, 2011). If a cleavage furrow begins while the spindle is parallel to the surface, symmetrical oocyte division will occur as illustrated in Fig. 1. However, the process that induces the spindle to become

Figure 4 A newborn chimera on Day 4 after its birth and an inset figure that shows the pup with its mother.

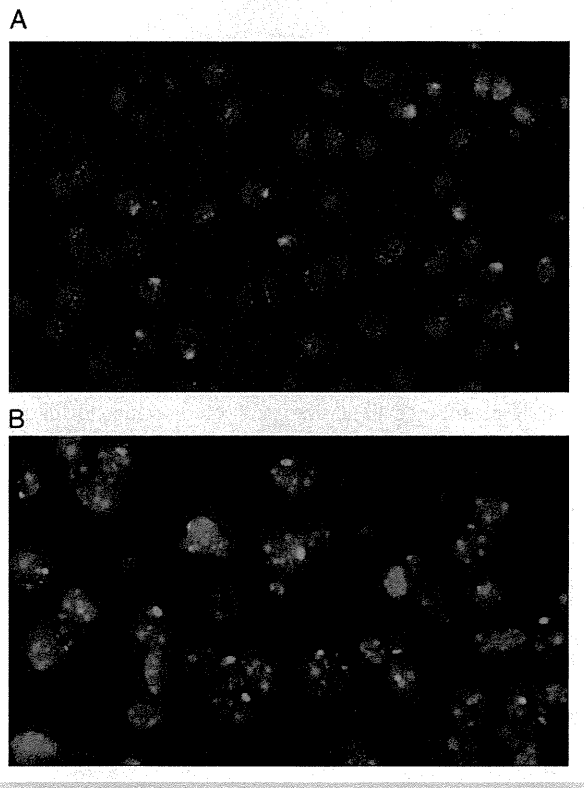

Figure 5 (**A**) Metaphase FISH analysis of the head and neck tissues of the chimera newborn mouse showing the X chromosomes stained in red and the Y chromosomes in green. (**B**) A control FISH analysis of head tissues of a normal male mouse showing the X chromosomes stained in red and the Y chromosomes in green.

parallel to the surface (Zhu *et al.*, 2003) and bring about symmetric oocyte division is not understood.

Our findings prompted us to compare 'immediate cleavage', described in human oocytes (Giltay *et al.*, 1998; Souter *et al.*, 2007) with the experimental model developed in the present studies. It has been proposed that 'immediate cleavage' involves the parthenogenetic activation of a mature oocyte, followed by symmetric division of the oocyte. One of the cells inside the zona would represent an activated small oocyte, while the other cell would represent a large second polar body. The female chromatin in such cells would form early pronuclei. If such cells can be fertilized, after the cortical granule reaction, the penetrated sperm would need to undergo decondensation to establish pronuclei that can undergo syngamy and further development. Currently there is no experimental information to support these proposals. The findings obtained in the present studies provide insight into the possibility that 'immediate cleavage' does not require parthenogenetic activation of an oocyte, but instead the 'cleavage' occurs in the course of meiotic maturation that involves symmetrical division of the oocyte. The semi-identical twins observed by Souter *et al.* (2007) could arise from the separate fertilization of the double oocytes. In such cases, the fertilization of each cell by separate sperm could produce non-identical twins when the inner cell mass of an amalgamated chimeric embryo becomes separated into two embryonic masses.

In this study, when an MII spindle formed in each daughter cell, each cell would have contained a different DNA pattern due to chromosome crossover during prophase-I of meiosis. Chromosomal crossover is an exchange of genetic material between homologous maternal and parternal chromosomes. At the first meiotic division, the paired homologs separate, but the chromatids remain attached to one another through their centromeres. Consequently, the secondary oocyte and the first polar body receive different versions of each homologous chromosome. In the present study, following symmetric division, each cell contained an MII spindle near the surface of each small oocyte. We found that each of these small oocytes could be fertilized and retained developmental competence. When a separate sperm fertilized each of the intra-zonal cells, two embryos were formed within the same zona pellucida, and these twin embryos eventually amalgamated to develop into a single chimeric blastocyst. In the present study, fertilization and development of the two small oocytes within the same zona pellucida was found to be one mechanism that could lead to the formation of a genetic intersex. It is likely that this occurred in our study when one embryo, developed after the entry of an X sperm, amalgamated with its twin embryo, formed by the entry of a Y sperm (Fig. 6). Our results suggest that a low incidence of other embryos of this kind could develop to term to give rise to chimeric organisms composed of a mixture of XX and XY cells.

A true chimera is an organism containing organs or body components consisting of two or more tissues containing cells of different genetic composition. Blood chimeras are completely different because they arise from the mingling of blood cells during multiple pregnancies conceived naturally or following IVF/ICSI treatment cycles (Miura and Niikawa, 2005; Walker *et al.*, 2007). Such blood chimeras are derived from two or more separately implanted embryos that originated from two or more different oocytes. In contrast to blood chimeras, the organisms that we produced in the present study are chimeras originating from a single oocyte that divided into two fertilizable cells within the same zona pellucida. We have demonstrated that each of these cells produced an embryo that amalgamated into a single chimeric embryo but only a small proportion of such chimeras were capable of implanting and of developing to term.

A number of factors may account for the low incidence of implantation and development to term of the amalgamated embryos following

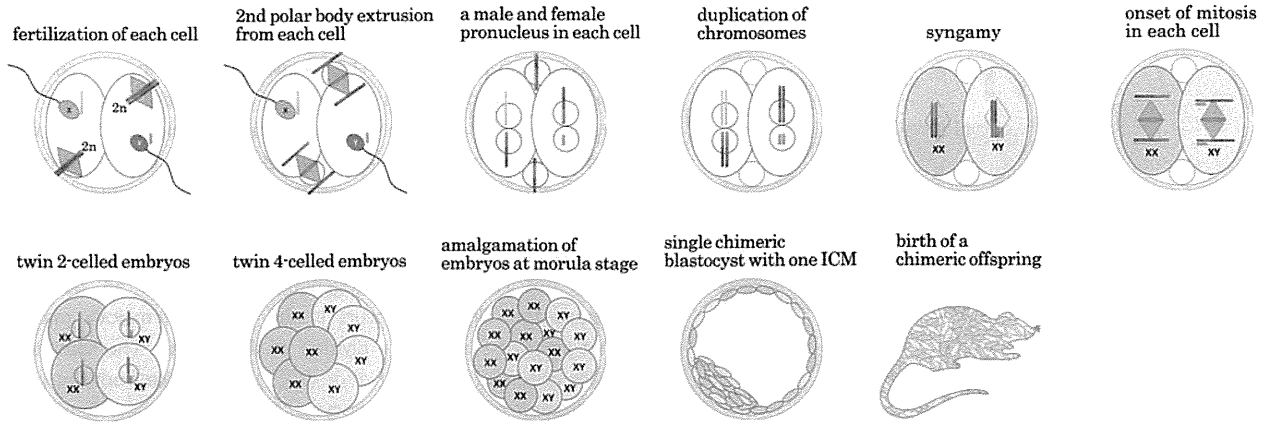

Figure 6 A diagram of fertilization and development of the two small oocytes showing initially the formation of twin embryos that eventually amalgamate within the same zona pellucida. If one embryo contains XX cells and the other XY cells, then this diagram depicts a proposed mechanism for the formation of a chimeric hermaphrodite organism.

their transfer to recipients. All of the oocytes used in the present studies were matured *in vitro*. Moreover, during their meiotic maturation in culture, the oocytes may have been stressed by the compression that was applied to induce symmetrical division. Also, the resulting two small oocytes acquired a markedly different nucleocytoplasmic ratio, compared with single large oocytes. It has been reported that such differences in the cytoplasmic to nuclear proportions could influence the cell cycle, cleavage rates, the onset of DNA and RNA synthesis as well as differentiation at more advanced embryonic stages (Satoh, 1985; Kominami and Takata, 2003).

In the present study, the FISH results indicated that the chimera contained both XX and XY cells in the newborn mouse head and neck tissues. We showed that such chimera could arise from the symmetrical division of oocytes during meiosis-I, followed by the fertilization of each cell by separate sperm inside the same zona pellucida. Although the size of each cell was about half that of a normal oocyte, each developed as a single embryo until mixing of their blastomeres followed by amalgamation of the twin embryos produced a single chimera. We propose that this mechanism could lead to the formation of a true hermaphrodite containing both male and female tissues.

Authors' roles

J.O. designed the main study and chimera embryo development and wrote the main part of the paper. She substantially contributed to the conception and design, acquisition of data, and the analysis and interpretation of data; she drafted the article and revised it critically for important intellectual content, and contributed to the final approval of the version to be published. Y.N. performed FISH analysis and assisted in plans for the experiments. He substantially contributed to analysis and interpretation of data. He contributed to drafting and revision of the paper for important intellectual content, and to the final approval of the version to be published. A.L. wrote parts of the paper and helped to interpret the results. He substantially contributed to analysis and interpretation of data. He contributed to drafting and revision of the paper for important intellectual content, and to the final approval of the version to be published. K.C. provided advice on experimental protocols and procedures. He substantially contributed to analysis and interpretation of data. He contributed to drafting and revision of the paper for important intellectual content, to the final approval of the version to be published. L.Y. assisted in the embryo transfer. She substantially contributed to analysis and interpretation of data. She contributed to drafting and revision of the paper for important intellectual content, and to the final approval of the version to be published. T.S. performed the embryo transfers, assisted in the experimental design and provided advice on experimental protocols. He substantially contributed to analysis and interpretation of data. He contributed to drafting and revision of the paper for important intellectual content, and to the final approval of the version to be published.

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Conflict of interest

None declared.

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Case Report

Triplet Pregnancy in a Cynomolgus Monkey (*Macaca fascicularis*) after Double Embryo Transfer

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At our research center, cynomolgus monkeys (*Macaca fascicularis*) are bred by mating or intracytoplasmic sperm injection (ICSI) and embryo transfer. We typically transfer 2 embryos, because the pregnancy rate is better than that for single embryo transfer. In the case we present here, 2 embryos that had been frozen and thawed after ICSI were transplanted into a recipient female macaque, and a multiple pregnancy (3 fetuses) was confirmed. All 3 fetuses were miscarried between days 81 and 85 of pregnancy. One fetus, which was wrapped in the amnion, was expelled along with its own placenta and one other. Because the other placenta had 2 umbilical arteries, 2 fetuses may have shared it. Therefore, we believe this pregnancy was a case of triplets, including a set of twins from an embryo that divided after transfer.

Abbreviation: ICSI, intracytoplasmic sperm injection.

The birth of twins to rhesus[2] and Japanese macaque[7] monkeys has been reported, but the probability of twins is extremely low (0.027% to 0.21%). At our institution, we carry out indoor artificial breeding of cynomolgus monkeys (*Macaca fascicularis*) by using a variety of techniques, including ovarian stimulation, oocyte collection, intracytoplasmic sperm injection (ICSI), and embryo transfer. Double embryo transfer typically is performed, because the pregnancy rate is better than that for single embryo transfer.

Here we describe a case of triplets generated from double embryo transfer. We believe that 1 of the 2 transplanted embryos divided after transfer.

Case Report

Experimental procedures were approved by the animal care and use committee of Shiga University of Medical Science (Shiga, Japan). Oocytes were collected from one sexually mature female cynomolgus monkey (age, 6 y; weight, 2.3 kg), and another (age, 5 y; weight, 2.5 kg) was the recipient of embryo transfer. The animal rooms were maintained at 25 ± 2 °C, with a relative humidity of 50% ± 5% and 12:12-h light:dark cycle (lights on, 0800 to 2000). Cynomolgus monkeys were housed individually (cage dimensions, 500 mm × 800 mm × 800 mm). In the morning, each animal was fed commercial pellet monkey chow (20 g per kg of body weight; CMK1, CLEA Japan, Tokyo), supplemented with 20 to 50 g of sweet potato in the afternoon. Water was given ad libitum by an automatic supplier.

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Oocytes were collected by laparoscopy (LA6500, Machida Endoscope, Tokyo, Japan) from adult female monkeys that had been treated with gonadotropin.[6] Insemination was performed by ICSI with oocytes and thawed sperm, and embryos were cultured in vitro.[8] Three days after fertilization, 12-cell and 8-cell embryos were vitrified (Cryotop and Vitrification Kit; Kitazato BioPharma, Shizuoka, Japan).[10] On day 547 after freezing, the embryos were thawed, and the 8-cell and 12-cell embryos were cultured for 16 h. The 8-cell embryo was confirmed to have a normal form (that is, showing neither death nor degeneration of the blastomere), and the 12-cell embryo had developed to the 16- to 32-cell stage (Figure 1). Both embryos were transplanted by laparoscopy through the fimbria into the oviduct of the recipient female macaque[8,9] at 0 to 4 d after ovulation. Pregnancy was determined by ultrasonography (SSD-620, Aloka, Tokyo, Japan) 37 d after embryo transfer. The presence of at least 2 heartbeats confirmed a multiple pregnancy (Figure 2).

At our research center, the number of days (mean ± 1 SD; $n = 14$) from ICSI to birth is 157 ± 8. The first miscarriage (fetus A; Figure 3) in the macaque we present here occurred on day 81 after ICSI (not counting the freezing time). Ultrasonography after miscarriage confirmed the presence of a heartbeat. Two days later (day 83), a second miscarriage (fetus B; Figure 3) occurred, with the third miscarriage (fetus C) on day 85. Fetus C was expelled with its placenta. Another placenta was expelled on the same day. Because this second placenta had 2 umbilical arteries (Figure 3), we believe that fetuses A and B had shared it, suggesting that they were monozygotic twins; fetus C, which had a separate placenta, likely originated from the second embryo. However, because we did not perform DNA analysis, we cannot exclude the possibility that the 3 fetuses were monozygotic triplets.

At our research center, double embryo transfer of cynomolgus macaques is performed to improve the pregnancy rate, and twin pregnancies do occur. Typically, however, one or both of the twins

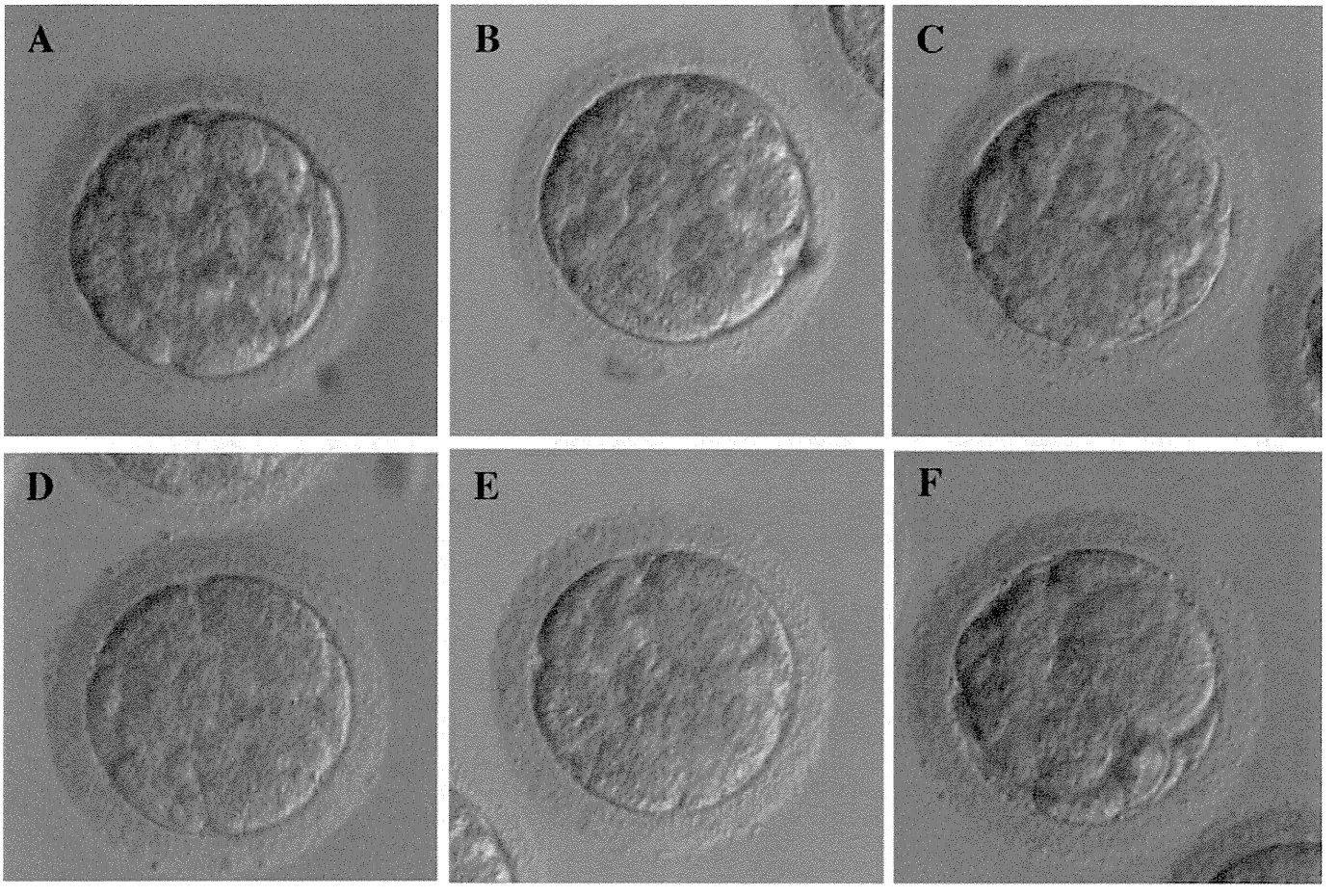

Figure 1. Cynomolgus monkey embryos (A, D) before freezing, (B, E) after thawing, and (C, F) at transfer. (A through C) Embryo that was frozen–thawed at 12 cells and transferred to the recipient at 16 to 32 cells after 16 h of culture. (D through F) Embryo frozen–thawed at 8 cells and transferred to the recipient at 8 cells after 16 h of culture. Bar, 50 µm.

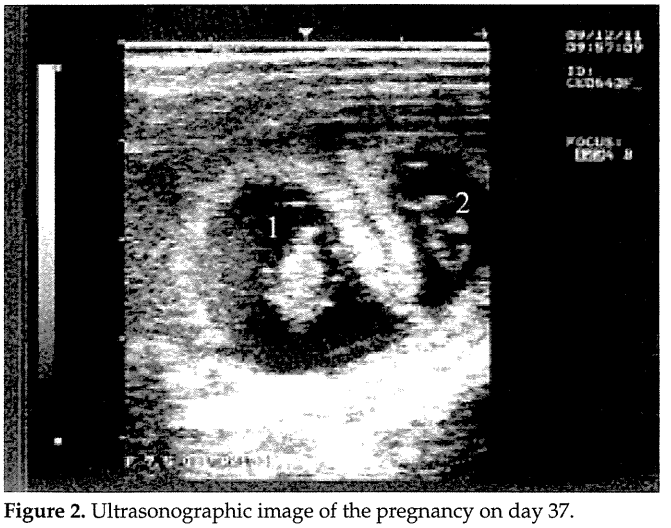

Figure 2. Ultrasonographic image of the pregnancy on day 37.

are miscarried. In addition, multiple pregnancies from single embryo transfer had not occurred previously at our institution, nor had triplets resulted from double embryo transfer. In the current case, natural separation after the 8-cell stage led to monozygotic multiple fetuses; to the best of our knowledge, we are the first to report such a result after assisted reproductive methods in cynomolgus monkeys. Embryo splitting similarly led to a twin pregnancy in a rhesus monkey, but one twin was miscarried.[5] Although the 2 heartbeats confirmed on day 37 led us to diagnose a twin pregnancy, we now believe that it actually was a triple pregnancy. We were unable to perform chromosomal and DNA analysis on any of the 3 fetuses described herein.

Monozygotic multiples and dizygotic quadruplets and quintuplets are well known to occur in humans.[3,4] The incidence of human monozygotic twins after assisted reproductive technologies varies depending on the number of days in embryo culture and the fertilization method, such as in vitro fertilization and ICSI. Ovary stimulation and expanded blastocyst transfer are other possible causes of monozygotic twins in humans.[1]

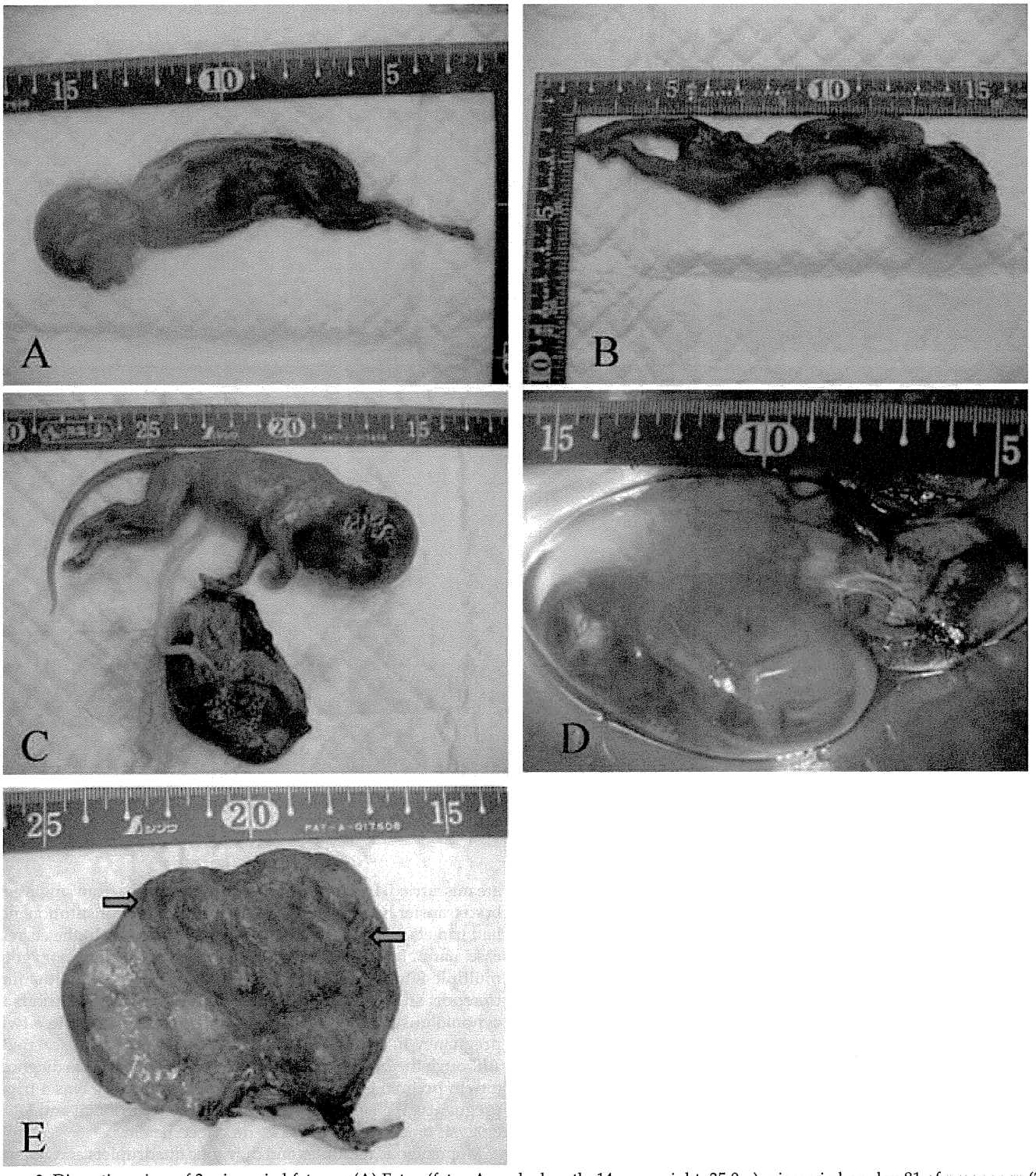

Figure 3. Dissection view of 3 miscarried fetuses. (A) Fetus (fetus A; male; length, 14 cm; weight, 35.0 g) miscarried on day 81 of pregnancy (25 January 2010). The tips of both feet were lost. The positions and shapes of the internal organs were normal, and the complexion was poor. (B) Fetus (fetus B; male; length, 15 cm; weight, 30.1 g) miscarried on day 83 of pregnancy (27 January 2010). Autolysis was pronounced, and portions of various internal organs had been resorbed. (C) Fetus (fetus C; male; length, 15 cm; weight, 51.1 g) miscarried on day 85 of pregnancy (29 January 2010). Fetus C was expelled while still wrapped in the amnion. The positions and shapes of the internal organs were normal, and it had its own placenta (weight, 19.2 g). Development of the internal organs was more advanced than in fetus A. (D) Appearance of fetus C when it was found. (E) Placenta (weight, 37.4 g) that was expelled with that of fetus C. We believe that fetuses A and B shared this placenta, because it had 2 umbilical arteries (arrows).

At our research center, we have encountered many twin pregnancies after double embryo transfer. However, except for 3 offspring raised by use of artificial nursing, both twins have never survived infancy. Maintaining multiple pregnancies is difficult in macaques, because they generally give birth to only one fetus.[2,7] Methods for maintaining multiple pregnancies in and managing the birth process of cynomolgus monkeys need to be improved. The current case is an unusual example of double embryo transfer leading to triplet pregnancy in cynomolgus monkeys.

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—Original Article—

Live Offspring from Cryopreserved Embryos Following *In Vitro* Growth, Maturation and Fertilization of Oocytes Derived from Preantral Follicles in Mice

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Abstract. This study was undertaken to examine pre- and postimplantation developmental potency of cryopreserved embryos that had undergone *in vitro* growth (IVG), maturation (IVM) and fertilization (IVF) of oocytes from the preantral follicle stage. An oocyte culture system for IVG and IVM was used in oocyte-granulosa cell complexes (OGCs) derived from preantral follicles in 12-day-old mice. The rate of oocyte maturation was improved by the addition of gonadotropins (FSH / LH) and cytokines (IGF-I / SCF) to culture medium for IVG. During culture for IVG, estradiol-17β and progesterone concentrations increased progressively to the latter period of culture. This culture system enabled IVG, IVM, IVF and pre- and postimplantation development. From 90 cryopreserved 2-cell stage embryos transferred into recipients after warming, 10 live pups were produced. Cryopreservation of embryos by vitrification at the 2-cell stage showed no harmful effect on development to the blastocyst stage or on the cell numbers of the inner cell mass (ICM) and trophectoderm (TE). This study demonstrated that embryos derived from oocytes grown *in vitro* have tolerance for vitrification and competence to develop to term after warming. This IVG-IVM-IVF technology combined with embryo cryopreservation might be useful for assisted reproduction in mice.

Key words: Embryo vitrification, Full-term development, Oocyte–granulosa cell complexes, Oocyte growth and maturation *in vitro*, Preantral follicle

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Mammalian ovaries contain numerous oocytes, far outnumbering those required for ovulation through life. Resources such as ovarian preantral follicles including growing oocytes are useful for basic research to produce healthy offspring through long-term culture of oocytes in laboratory animals, domestic animals and humans [1].

Generally, two methods have been devised for studies of oocyte development *in vitro* from preantral follicles in mice. One is the culture method for oocyte-granulosa cell complexes (OGCs) derived from preantral follicles harvested from ovaries using enzymatic digestion, and the other is for preantral follicles isolated using manual dissection [1].

The OGCs culture system was first developed mainly by Eppig's group [2–8] and has since been utilized or modified by other research groups [9–13]. The advantage of using this system is the ability to collect and culture almost all growing oocytes in the ovaries of prepubertal mice as OGCs. Furthermore, live offspring can be obtained [9, 11–13].

Attempts have been made to combine cryopreservation with a culture system for oocyte growth *in vitro*. For mice, some reports describe oocyte growth *in vitro* from cryopreserved ovaries [12, 14–17] and preantral follicles [9]. Because the total period of *in vitro* growth (IVG), *in vitro* maturation (IVM), *in vitro* fertilization (IVF) and *in vitro* (embryo) culture (IVC) of immature oocytes from preantral follicles is quite long, efficiency would be improved if cryopreservation were possible at the early embryo stage. However, no such study has been reported.

The objective of this study, using OGCs derived from preantral follicles of 12-day-old mouse ovaries, was to investigate preimplantation and postimplantation developmental competence of embryos that underwent IVG-IVM-IVF and cryopreservation/warming.

Materials and Methods

Animals

Mice used for this study were housed and bred under controlled lighting (14 h light: 10 h darkness), temperature and humidity, with food and water available *ad libitum*. Twelve-day-old female mice [C57BL/6J × CBA/JN] (Clea Japan, Tokyo, Japan; Charles River Laboratories Japan, Kanagawa, Japan) were used for collection to ovaries. Sperm from 8–12-week-old male mice [C57BL/6N × C3H/HeN] (Clea Japan, Tokyo, Japan) was used for IVF. As recipients for embryo transfer, 8–16-week-old CD-1 (ICR) females (Charles River Laboratories Japan, Kanagawa, Japan) were used.

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Culture of OGCs

Isolation of OGCs from 12-day-old mouse ovaries was performed by enzymatic digestion [2]. The ovaries were immersed in Leibovitz's L-15 medium (Life Technologies, Carlsbad, CA, USA) containing 5% heat-inactivated FBS (Japan • Bio serum, Hiroshima, Japan), 1–1.3 mg/ml collagenase type I (Worthington Biochemical, Freehold, NJ, USA) and 0.02% DNase I (Sigma-Aldrich, St. Louis, MO, USA) for 15 min at 37 C, and the OGCs were isolated by repeatedly pipetting up and down 50 times, with incubation for 5 min. Collected OGCs were washed twice and cultured on 6-well dishes equipped with Transwell-COL collagen-coated membrane inserts (3.0 μm pore size, 24 mm diameter; Corning, Corning, NY, USA) under 5% CO_2 in air at 37 C for 10 days. Each well was filled with 4 ml of MEM alpha (GlutaMAX; Life Technologies, Carlsbad, CA, USA) supplemented with 5% FBS, 100 mIU/ml recombinant human FSH (Follistim; Merck & Co, Kenilworth, NJ, USA), 10 mIU/ml human LH (AbD Serotec, MorphoSys UK, Oxford, UK), 50 ng/ml recombinant human insulin-like growth factor I (IGF-I; Sigma-Aldrich), and 50 ng/ml recombinant murine stem cell factor (SCF; PeproTech, Rocky Hill, NJ, USA). During culture, the medium was overlaid with 1.5 ml of paraffin oil (Nacalai Tesque, Kyoto, Japan) on each insert membrane and the outer area in the well. Half of the medium was changed every other day until day 8 and daily thereafter.

On day 10 of culture, the OGCs were allowed to mature for 17 h by the addition of 1.5 IU/ml hCG (ASKA Pharmaceutical, Tokyo, Japan) and 5 ng/ml recombinant human epidermal growth factor (EGF; Sigma-Aldrich). For assessment of nuclear maturity, expanded oocyte–cumulus complexes (OCCs) were collected and denuded. The oocyte stage was evaluated as GV when the germinal vesicle was recognizable, as GVBD when the GV was absent or as metaphase II when the first polar body was extruded.

IVF and embryo culture

Spermatozoa collected from the cauda epididymis of male mice were incubated for 1.5 h in 200 μl droplets of TYH medium under paraffin oil for capacitation. Expanded OCCs produced by maturation induction for 17 h were transferred to 100 μl droplets of TYH medium after washing several times. Insemination was conducted at a sperm concentration of 2–3 × 10^7/ml, with subsequent incubation for 4 h. After insemination, oocytes were washed using a narrow pipette to remove the remaining unexpanded cumulus cells and the attached sperm; then they were cultured in 50 μl droplets (15–25 embryos/droplet) of KSOM medium supplemented with amino acids (KSOM/AA) [18] for additional embryo development. When the number of embryos was less than 15, they were cultured in 10 μl droplets (35 embryos/droplet). Observation of development to the 2-cell stage and blastocyst stage was carried out at 24 h and 96 h post insemination, respectively.

Hormone measurements

The conditioned media were collected at the time of medium exchange and stored at −30 C. For estradiol determination, the conditioned media were diluted 1/100. Estradiol-17β and progesterone concentrations were measured using an AxSYM Analyzer (Abbott Japan, Tokyo, Japan) using enzyme immunoassay kits (AxSYM-Estradiol-Dinapack-I and AxSYM-Progesterone-Dinapack, Abbott Japan).

Embryo cryopreservation

We conducted simple vitrification using DAP213 (2 M dimethyl sulfoxide, 1 M acetamide and 3 M propylene glycol) solution [19]. Briefly, 2-cell stage embryos that had undergone IVG, IVM and IVF were first pretreated with 1 M DMSO in PB1 medium at room temperature. Of the medium containing the embryos, 5 μl was transferred into a 1-ml cryotube (Nalge Nunc International KK, Tokyo, Japan) in ice water and kept for 5 min. Then 95 μl of DAP 213 solution was added and kept at 0 C for 5 min, and the cryotubes were then plunged directly into liquid nitrogen and stored for 2–3 weeks.

For warming, the samples were removed from the liquid nitrogen and allowed to stand at room temperature for 30–60 sec. Then they were diluted with 0.25 M sucrose in PB1 medium at 37 C. The recovered embryos were kept for 10 min in microdroplets of KSOM/AA medium and then washed several times.

Blastocyst cell counts

Determination of cell numbers of the inner cell mass (ICM) and trophectoderm (TE) in the blastocyst stage (96 h post insemination) was performed by immunosurgery and using fluorescent double staining [20]. Briefly, blastocysts after removal of the zona pellucida using acidic Tyrode's solution (Sigma-Aldrich) were incubated in KSOM/AA medium containing 1% rabbit anti-mouse serum for 30 min. Then they were incubated in M16 containing 10 μg/ml Hoechst 33342, 20 μg/ml propidium iodide (Sigma-Aldrich) and 5% standard guinea pig complement (Cedarlane Laboratories, Burlington, ON, Canada) for 30–60 min. Finally, the blastocysts were washed gently, placed on a glass slide and sealed under a coverslip. The cell numbers of the ICM and TE were examined under a fluorescence microscope (Olympus, Tokyo, Japan).

Embryo transfer

The 2-cell stage embryos that had undergone IVG, IVM and IVF were transferred to the oviducts of pseudopregnant females (0.5 days post coitus) mated with vasectomized males whose sterility was confirmed.

Statistical analysis

The variations are presented as the standard error of the mean. The data of *in-vitro* maturation and post-implantation development were subjected to arcsine transformation, followed by ANOVA. The comparison of preimplantation developmental competence was carried out using χ^2 analysis with Yates' correction. The number of cells per blastocyst was presented as a statistical summary using a box plot, and the statistical analysis was carried out using the Student's *t*-test. In this study, statistical significance was inferred for $P<0.05$.

Results

IVG and IVM

First, an experiment for development of the IVG system was

Table 1. *In vitro* maturation of oocytes following *in vitro* growth from oocyte–granulosa cell complexes in preantral follicles

No. of experiments	No. of oocytes examined	% (mean ± S.E.) of oocytes progressed to		Oocyte diameter (μm, mean ± S.E.)
		GVBD	Metaphase II	
6	100	91.1 ± 4.4	79.8 ± 3.5	73.1 ± 0.4

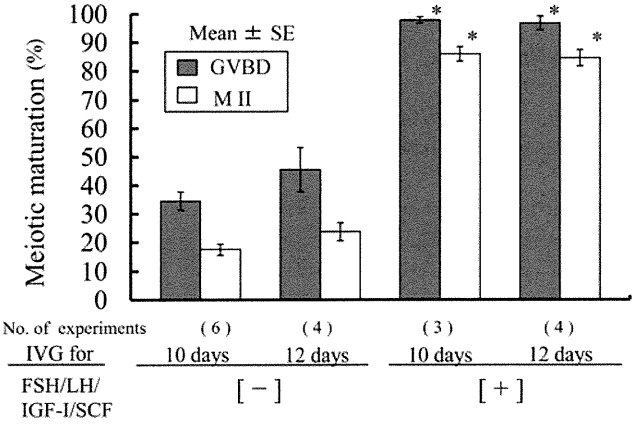

Fig. 1. Effects of supplementation of gonadotropins (FSH and LH) and cytokines (IGF-I and SCF) in IVG medium on *in vitro* maturation of oocytes following *in vitro* growth from oocyte–granulosa cell complexes in preantral follicles. * P<0.01 *vs.* groups without gonadotropins and cytokines.

carried out. Addition of gonadotropins (FSH / LH) and cytokines (IGF-I / SCF) to IVG culture medium dramatically increased the maturation rate of oocytes (Fig. 1). During IVG culture, the outer granulosa cell layers spread out on the insert membrane, but the oocyte-surrounding granulosa (cumulus) cells proliferated without spreading, maintaining a sphere shape (Figs. 2a and 2b). Of the cultured OGCs, 88.0 ± 4.0% (n=6) had maintained that condition without denudation. The OCCs expanded (Fig. 2c), and the oocytes progressed to metaphase II (79.8 ± 3.5%, Table 1) as a result of induction of maturation for 17 h. The IVG oocytes reached 73.1 ± 0.4 μm in diameter (Table 1).

Hormone production

The concentration profiles (n=5) of estradiol-17β and progesterone in conditioned media are presented in Fig. 3. The estradiol concentration increased progressively to day 8, after which the concentration in media that were changed daily tended to level out (Fig. 3a). The progesterone concentration increased gradually to the end of IVG culture (Fig. 3b). The maximum concentration was 11.6 ± 4.0 ng/ml on day 10.

Preimplantation embryo development

On day 11, the oocytes (expanded OCCs) that had undergone IVG-IVM were used for IVF and preimplantation development *in vitro*. Table 2 shows that 65.9 ± 5.8% (n=9) of the inseminated oocytes achieved fertilization and first cleavage; 64.0 ± 8.5% (n=7) of the cultured 2-cell embryos developed to the blastocyst stage

Table 2. *In vitro* fertilization of oocytes following *in vitro* growth and maturation from oocyte–granulosa cell complexes in preantral follicles

No. of experiments	No. of oocytes inseminated	% (mean ± S.E.) of oocytes developed to 2-cells
9	734	65.9 ± 5.8

(Table 3, Figs. 4a and 4c).

The survival rate of vitrified/warmed 2-cell embryos derived from IVG-IVM-IVF oocytes was 91.0%; the rates of development to the blastocyst stage were 50.0 and 60.0% for nonvitrified and vitrified/warmed 2-cell embryos, respectively (Table 4, Figs. 4b and 4d).

Fig. 5 depicts the results of differential cell counts in blastocysts. The ICM and TE cells were stained as blue and pink, respectively (Fig. 5a). The ICM, TE and total cell numbers were 8.8 ± 0.7, 26.4 ± 2.4 and 35.1 ± 0.3 (n=8) in the nonvitrified group and 9.9 ± 1.6, 23.3 ± 2.1 and 33.2 ± 2.2 (n=11) in the vitrified/warmed group, respectively. No significant difference was found between these two groups. In Fig. 5b, all data are presented as a statistical summary using a box plot.

Postimplantation embryo development

The results of *in vivo* development after embryo transfer are presented in Table 5 and Fig. 6. In the nonvitrified group, each oviduct of four recipients received 80 embryos at the 2-cell stage produced by IVG-IVM-IVF. From them, 3 live pups (4.4 ± 1.7%) were born through natural delivery (one male and two females). In the vitrified/warmed group, 90 of the 2-cell embryos were also transferred into four recipients. From them, 10 live pups (11.2 ± 7.5%) were born (six males and four females). All live offspring grew normally.

Discussion

The present study clarified that the early embryos derived from oocytes grown *in vitro* had freezing tolerance. Embryo cryopreservation at the 2-cell stage had no harmful effects on pre- and postimplantation development. We adopted vitrification as a cryopreservation method. Vitrification is a simple method for low-temperature storage of early embryos, oocytes and recently, ovarian tissues. Several studies have been made on ovarian vitrification followed by IVG-IVM [9, 12, 15–17, 21]. However, ovarian vitrification is not necessarily efficient. Ovarian tissue and preantral follicle cryopreservation cause follicular loss by immediate cell death after warming [9] and temporary suppression of the granulosa cell proliferation through cell cycle regulators [22] and delay of follicular growth and maturation *in vitro* [12, 15]. If the IVG-IVM

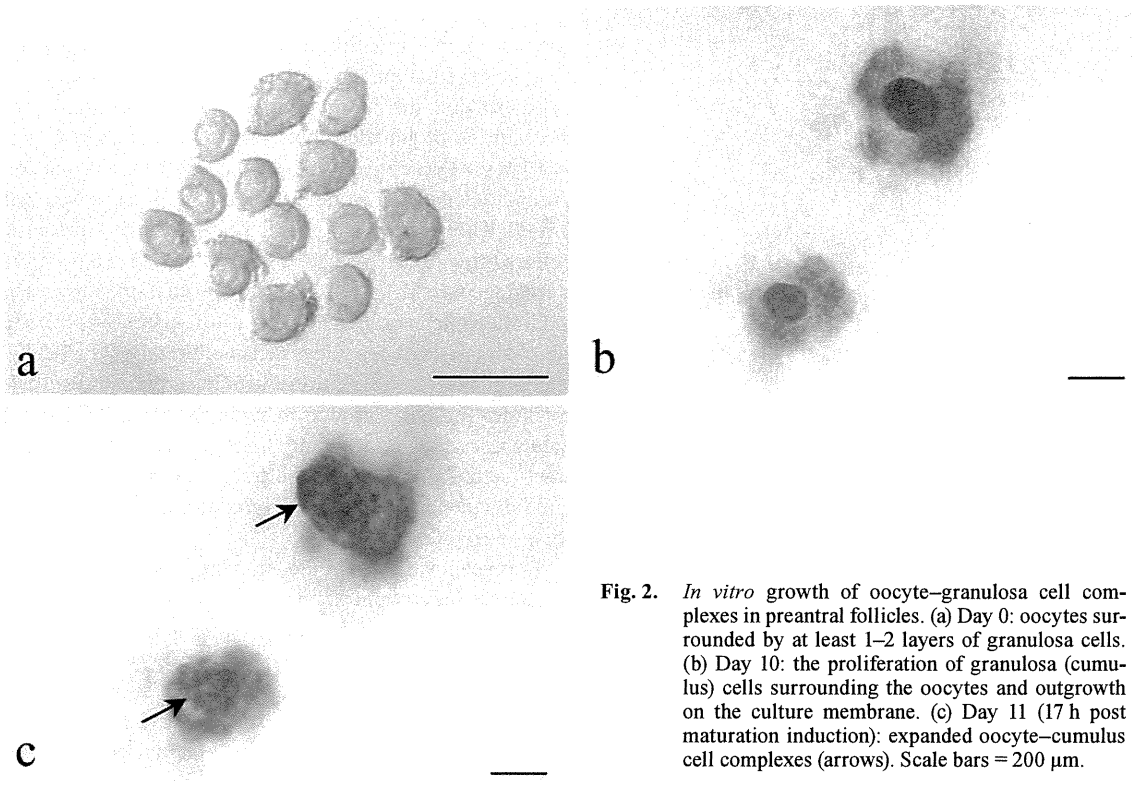

Fig. 2. *In vitro* growth of oocyte–granulosa cell complexes in preantral follicles. (a) Day 0: oocytes surrounded by at least 1–2 layers of granulosa cells. (b) Day 10: the proliferation of granulosa (cumulus) cells surrounding the oocytes and outgrowth on the culture membrane. (c) Day 11 (17 h post maturation induction): expanded oocyte–cumulus cell complexes (arrows). Scale bars = 200 μm.

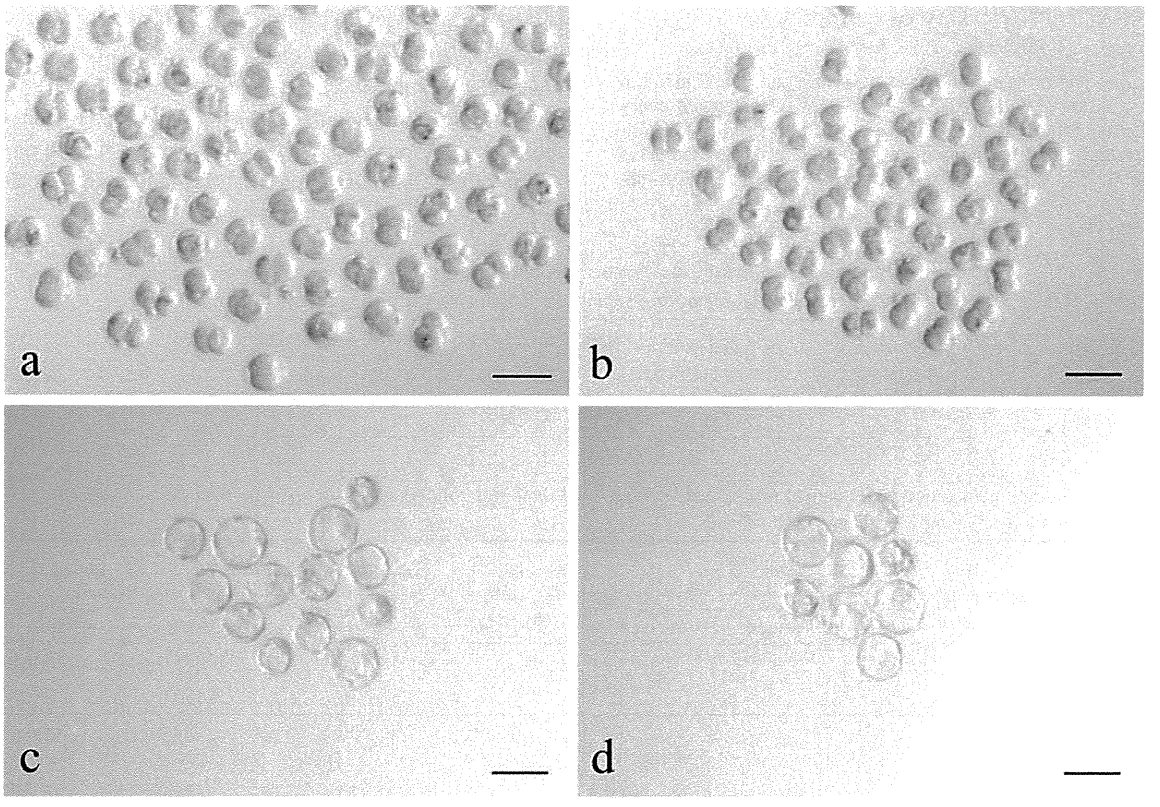

Fig. 4. Preimplantation development of (a) nonvitrified and (b) vitrified/warmed 2-cell embryos following *in vitro* growth, maturation and fertilization from oocyte–granulosa cell complexes in preantral follicles. Figs. 4 (c) and (d) were blastocysts from nonvitrified and vitrified/warmed, respectively. Scale bars = 100 μm.

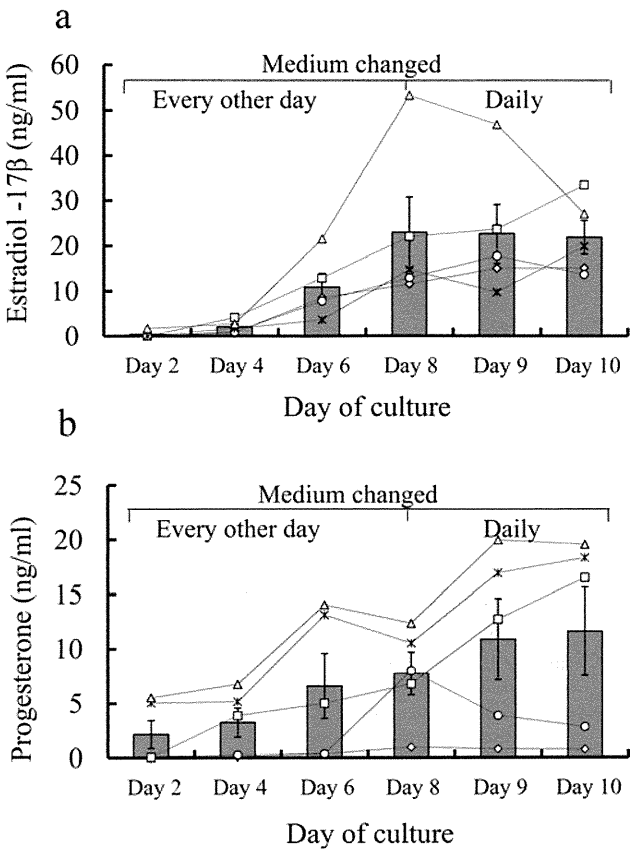

Fig. 3. Estradiol and progesterone production in conditioned medium during culture. Half of the fresh medium was changed every other day until day 8, and daily thereafter. (a) Estradiol-17β concentrations. (b) Progesterone concentrations. Values are means ± S.E. (n=5). Data for each sample are presented as a line graph.

system were used as one of the technologies for strain maintenance in mice, cryopreservation at the early embryo stage would probably be more efficient than ovary or follicle cryopreservation.

To establish the culture system, we first examined the effect of the addition of gonadotropins (FSH / LH) and cytokines (IGF-I / SCF) to the IVG culture medium on oocyte maturation. As a result, the oocyte maturation rate was dramatically improved by these additives. The addition of gonadotropins in intact preantral follicle culture provides a beneficial effect on ovarian cell proliferation and steroidogenesis [23, 24]. IGF-I is involved in the regulation of ovarian folliculogenesis in mammals; it stimulates the proliferation and steroidogenesis of granulosa cells cultured *in vitro* [25–27]. When added during *in vitro* culture of mouse preantral follicles, IGF-I promotes follicular growth in synergy with FSH [28]. SCF, alternately known as c-kit ligand, mast cell growth factor (MGF) and steel-factor (SLF), plays important roles in mammalian oogenesis and folliculogenesis [29]. SCF promotes oocyte growth and viability *in vitro* [30, 31].

To reveal ovarian steroidogenic profiles on our IVG system, we measured the concentrations of estradiol-17β and progesterone in conditioned media. In preantral follicle culture, estrogen and progesterone secretion increases progressively up to the end of culture [15, 23, 24, 32]. A similar tendency was also observed in the present study. This result suggests that ovarian steroidogenesis is at least functioning in our IVG system and indirectly supports oocyte growth *in vitro*. According to the two cell-two gonadotropin theory, estrogen synthesis under gonadotropin is dependent on the presence of a critical mass of theca cells. It is therefore surmised that ovarian steroidogenesis in OGC culture is imperfect. In rodents, it has been well known for a long time that theca cells provide the substrates for steroids in the ovarian follicle and provide the conditions for higher estradiol and progesterone production rates via the cultured units. The OGCs of preantral follicles are isolated using

Table 3. Preimplantation development of oocytes following *in vitro* growth, maturation and fertilization from oocyte–granulosa cell complexes in preantral follicles

No. of experiments	No. of 2-cell embryos cultured	% (mean ± S.E.) of embryos developed to	
		Morula	Blastocyst
7	194	81.6 ± 6.1	64.0 ± 8.5

Table 4. Viability and preimplantation development of vitrified/warmed 2-cell embryos following *in vitro* growth, maturation and fertilization from oocyte–granulosa cell complexes in preantral follicles

Treatment	No. of vitrification experiments	No. (%) of 2-cell embryos		No. of culture experiments	No. (%) of embryos		
		Vitrified/Warmed	Survived		Cultured	Developed to	
						Morula	Blastocyst
Nonvitrified	-	-	-	2	20	17 (85.0)	13 (65.0)
					10	5 (50.0)	2 (20.0)
				Total	30	22 (73.3)	15 (50.0)
Vitrified/Warmed	3	40	38 (95.0)				
		21	21 (100)	2	10	7 (70.0)	5 (50.0)
		84	73 (86.9)		15	11 (73.3)	10 (66.7)
	Total	145	132 (91.0)	Total	25	18 (72.0)^{NS}	15 (60.0)^{NS}

NS, no significant differences (P>0.05) *vs.* the nonvitrified group.

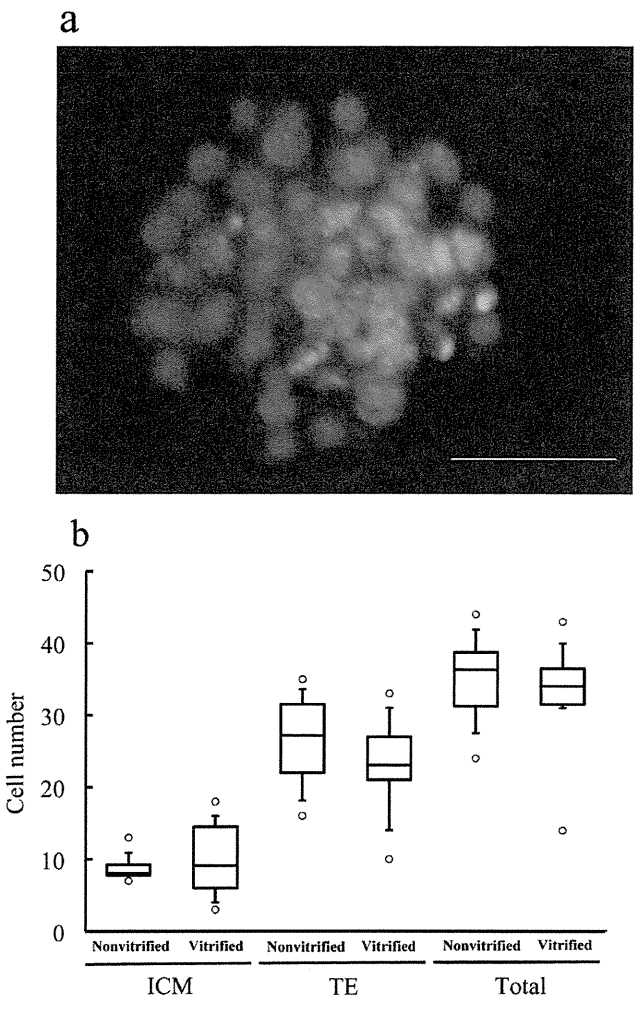

Fig. 5. Fluorescent double staining for differential inner cell mass (ICM) cell and trophectoderm (TE) cell counts in blastocysts that developed *in vitro* from nonvitrified and vitrified/warmed 2-cell embryos following *in vitro* growth, maturation and fertilization from oocyte–granulosa cell complexes in preantral follicles. (a) A fluorescence image of a blastocyst from the vitrified/warmed 2-cell embryo. ICM and TE cells were stained blue and pink, respectively. Scale bar=50 μm. (b) The cell numbers of ICM cells and TE cells in blastocysts. Data are represented as a box plot. The lines of the boxes show the 25th, 50th and 75th percentiles; the whiskers represent the 10th and 90th percentiles. Open circles show the minimum and maximum. There were no significant differences ($P>0.05$) between the nonvitrified and vitrified/warmed groups.

collagenase and DNase I digestion. Eppig *et al.* [33] reported that collagenase digestion removes most, if not all, of the primitive thecal-like cells of the preantral follicles and degrades the basement membrane. In other words, contamination of the primitive thecal-like cells in OGC culture cannot be denied. In our IVG system, LH was added to the culture medium. The LH could have promoted growth and differentiation of the primitive thecal-like cells *in vitro*. This possibility, if true, may be one of the causes of the unevenness in the steroid concentration in the present study. The steroid

Fig. 6. Live offspring derived from vitrified/warmed 2-cell embryos following *in vitro* growth, maturation and fertilization from oocyte–granulosa cell complexes in preantral follicles: 21-day-old healthy offspring and a recipient female.

production profiles seem to be divided into two tendencies. The progesterone profile is particularly remarkable. Interestingly, no differences in oocyte maturation, fertilization and developmental competence of preimplantation embryos were found between the two tendencies. From these findings, we inferred that (i) even if the primitive thecal-like cells became mixed with our OGC culture, subsequent oocyte/embryo development was not affected and that (ii) the progesterone profile is not suitable as a monitor of normal oocyte development, at least in our OGC culture; rather, monitoring of the estradiol-17β level is more important.

In our culture system, the medium is overlaid with paraffin oil, allowing daily observation and medium changes to be conducted conveniently. The culture medium became more hypoxic than that without oil [34]. The oxygen diffusion rate in paraffin oil at 37 C is about two-thirds of that in water at the same temperature [35]. Preantral follicle culture requires the use of normal oxygen (about 20% O_2) tension [36], but OGC culture demonstrates high preimplantation developmental competence under reduced oxygen (5% O_2) tension [4]. The oil overlay during IVM reduces progesterone levels in the medium and delays the timing of nuclear maturation [37, 38], but in long-term follicle culture, the oil overlay did not affect follicle survival, the MII rate or the oocyte diameter and did not alter the methylation status of differentially methylated regions of imprinted genes [39].

Of the nonvitrified 2-cell embryos derived from IVG-IVM-IVF, 3.8% (3/80) developed to live offspring. This result is in no way inferior to results described in other reports (5.1 and 3.3%) of studies using OGCs from 12-day-old mice [2, 11]. Additionally, 11.1% (10/90) of the vitrified/warmed 2-cell embryos developed to term. These results suggest that cryopreservation by vitrification does not affect postimplantation development. Generally, a low birth rate is a feature of oocytes grown *in vitro* [2, 5, 8, 11–14, 40]. Although further studies might be necessary to improve the culture system, our system demonstrated that full-term development can

Table 5. Postimplantation development of vitrified/warmed 2-cell embryos following *in vitro* growth, maturation and fertilization from oocyte–granulosa cell complexes in preantral follicles

Treatment	No. recipients	No. of embryos transferred	No. (%) of live offspring
Nonvitrified	1	20	1 (5.0)
	2	12	1 (8.3)
	3	24	1 (4.2)
	4	24	0 (0)
	Total	80	3 (3.8) (4.4 ± 1.7)*
Vitrified/Warmed	1	23	0 (0)
	2	22	7 (31.8)
	3	22	0 (0)
	4	23	3 (13.0)
	Total	90	10 (11.1) (11.2 ± 7.5)*NS

*, mean percent ± S.E. NS, no significant differences (P>0.05) *vs.* the nonvitrified group.

be achieved in mice after IVG-IVM-IVF combined with embryo cryopreservation.

In conclusion, the present study produced live offspring from vitrified/warmed 2-cell embryos that had undergone IVG-IVM-IVF from OGCs derived from preantral follicles. This is the first report of the production of pups from preimplantation embryos that had been cryopreserved following IVG-IVM-IVF. This technology combined with embryo cryopreservation might be useful for assisted reproduction such as strain maintenance in laboratory animals.

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