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 (MI) 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% CO₂, 5% O₂ and 90% N₂. 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 CO₂ 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% CO₂, 5% O₂ and 90% N₂. After 3–4 h of incubation, oocytes were examined to confirm the presence of extruded second polar bodies. These fertilized oocytes were transferred into LGGG medium (Life Global, Japan) that contained 0.1 mg/ml rHSA (rHSA; 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 StatMatell (ATMS, Inc., Tokyo, Japan). A difference was considered to be significant when its P-value was <0.05.

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 x saline sodium citrate (SSC) at 37°C for 20 min and 1 x SSC for 15 min at room temperature and blocking solution (5% skim milk, 0.1% non-iod fant-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

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/cm². 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.
30 min, washed three times with 0.1% non-ident P-40/2 x 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 MI 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 MI 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-I, 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-I, 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 spindle cell and early male and female pronuclei were seen in each cell by 6 h after insemination.
The time-course of symmetric oocyte division when the cortical ooplasm near the M1 spindle was compressed. The sequence of oocyte changes, obtained from time-lapse photography, shows the time-course of a symmetrical meiotic division when the M1 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.
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-1 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 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 blastocyst 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.
**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-1 of meiosis. Chromosomal crossover is an exchange of genetic material between homologous maternal and paternal 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 chimaera 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 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

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.
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 contributed to the final approval of the version to be published. A.L. wrote parts of the paper and contributed to drafting and revision of the paper for important intellectual content, and 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. 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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