Long-Term Effects of In Vitro Growth of Mouse Oocytes on Their Maturation and Development

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Abstract. Since very few oocytes grow completely in vivo, in vitro growth (IVG) of ovarian oocytes may provide a new source of functional oocytes. The long-term effects of in vitro maturation (IVM) of oocytes and in vitro culture of fertilized eggs have been reported; however, the effects of IVG of oocytes are unknown. Here in, we report the long-term effects of IVG of oocytes. Ovaries from 1-day-old mice containing non-growing oocytes were cultured for 10 days; the isolated follicles were then cultured for 11 days. Secondary follicles from 10-day-old mice were also cultured for 11 days. The nuclei of oocytes collected from the IVG and Graafian follicles of adult mice were transferred to enucleated oocytes grown in vivo, respectively. Developmental competence was examined following IVM of the reconstituted oocytes. Chronologically, oocytes of 1-day-old, 10-day-old and adult mice were cultured for 22, 12 and 1 day(s). The result showed that the reconstituted eggs developed into pups at high rates after nuclear transfer and in vitro fertilization (IVF) in all the experimental groups (29–45%). However, the pups from reconstituted eggs containing the nuclei of 22-day cultured oocytes were heavier than the control pups (P<0.05). We concluded that long-term culture of oocytes did not affect their nuclear ability to develop to term; however, fetal growth was affected by the culture duration or culture conditions during the initial phase of follicular growth.

Key words: Full-term development, In vitro culture, Long-term effect, Mouse, Nuclear transfer, Oocyte growth

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phologically develop into oocytes *in vitro* [12]. However, oocyte-like cells derived from ES cells in culture failed to arrest at metaphase in second meiosis (MII) and underwent parthenogenetic cleavage. Thus far, *in vitro* growth (IVG) and *in vitro* maturation (IVM) of primordial follicles are considered to be the most realistic models for producing competent oocytes and increasing the number of female gamete sources. Eppig et al. [13, 14], devised a 2-step culture method for production of produce oocytes from the primordial follicles of 1-day-old mice. First, the ovaries of 1-day-old mice are cultured intact for 8 days, and the obtained follicles are subsequently cultured for 14 days. These oocytes are sufficiently competent to undergo meiotic maturation, fertilization and development into live pups. We have also demonstrated successful development of pups from oocytes that were differentiated from the pre-meiotic female germ cells of mouse foetuses at 12.5 days post coitum (dpc) using a nuclear transfer technique combined with Eppig's 2-step culture method [15]. In this strategy, to overcome the cytoplasmic deficiency of IVG oocytes, the nuclei of oocytes that were differentiated after ovarian culture for 17 days and follicular culture for 11 days were transferred into ooplasm grown and matured *in vivo*. The key factor in this method is the provision of a large number of maternal genomes.

On the other hand, it has been suggested that *in vitro* culture of ruminant embryos causes a high frequency of multiple abnormalities, which are collectively referred to the large offspring syndrome [16, 17]. Several studies have reported that assisted reproductive technology (ART) is associated with an increased risk of low-birth-weight infants and birth defects in humans [18–20]. The issue regarding whether the abnormalities are associated with the duration of culture and the degree of complications encountered while manipulating the gametes is of particular interest.

In the present study, in order to investigate whether the period of *in vitro* culture during oocyte growth affects post-implantation and post-natal development, the following series of experiments were performed: (1) oocytes at various stages derived from 1-day-old, 10-day-old and adult mice were cultured for 22, 12 and 1 days, respectively; (2) the nuclei of oocytes cultured *in vitro* were transferred into ooplasm grown and matured *in vivo*; (3) the reconstituted M II oocytes were subjected to *in vitro* fertilization (IVF); and (4) the blastocysts obtained after 4-day *in vitro* culture were transferred to pseudopregnant female mice.

### Materials and Methods

#### Animals

All procedures described in this study were reviewed and approved by the Tokyo University of Agriculture Institutional Animal Care and Use Committee and were performed in accordance with the Guidelines for Proper Conduct of Animal Experiments as promulgated by the Science Council of Japan. BDF1 (C57BL/6N × DBA/2; Clea Japan, Tokyo, Japan) hybrid mice were used for all the experiments.

**IVG of non-growing oocytes from newborn mice (Experiment 1)**

The viability, growth, and maturation of oocytes after *in vitro* culture were assessed using alpha minimum essential medium (alpha-MEM; Invitrogen, Carlsbad, CA, USA) supplemented with 50 mg/l streptomycin sulphate and 75 mg/l penicillin G. Ovaries obtained from 1-day-old mice were cultured in medium containing 10% fetal bovine serum (FBS) on Coaster Transwell polycarbonate membranes (3.0 µm pore size, 24 mm diameter; Corning, Lowell, MA, USA) in 5% CO2 and 95% air at 37 C [13]. Approximately 1.5 ml of the medium was dispensed in the compartment, and 0.7–0.8 ml of the medium was added to the surface of the membrane. A total of 6 ovaries were placed on each membrane. On day 10, secondary follicles were mechanically isolated from the ovaries and cultured for 11 days in medium containing 10% fetal bovine serum (FBS) on Coaster Transwell polycarbonate membranes (3.0 µm pore size, 24 mm diameter; Corning, Lowell, MA, USA) in 5% CO2 and 95% air at 37 C [13]. Approximately 1.5 ml of the medium was dispensed in the compartment, and 0.7–0.8 ml of the medium was added to the surface of the membrane. A total of 6 ovaries were placed on each membrane. On day 10, secondary follicles were mechanically isolated from the ovaries and cultured for 11 days in medium containing 5% FBS, 0.1 IU/ml follicle-stimulating hormone (FSH; Sigma-Aldrich, St. Louis, MO, USA), 5 µg/ml insulin, 5 µg/ml transferrin and 5 ng/ml selenium (ITS; Roche, Basel, Switzerland) on Coaster Transwell-Col membranes (3.0 µm pore size, 12 mm diameter; Corning) in 5% CO2 and 95% air at 37 C. Approximately 1.5 ml of the medium was dispensed in the compartment, and 0.5 ml of the medium was added to the surface of the membrane. Half of the culture media was replaced every 3 days. An interference microscope (200 × magnification; Nikon, Tokyo, Japan) and a eyepiece micrometer (10 × A; Nikon) were used to measure the diameters of the oocytes grown *in vitro* (Fig.1).
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Secondary follicles isolated from the ovaries of 10-day-old mice (Experiment 2) were cultured as mentioned above. In short, secondary follicles were mechanically isolated and cultured for 11 days in medium containing 5% FBS, 0.1 IU/ml FSH and ITS on Coaster Transwell-COL membranes. Approximately 1.5 ml of the medium was dispensed in the compartment, and 0.5 ml of the medium was added to the surface of the membrane (Fig.1).

Fig. 1. Schematic diagram illustrating the experimental design and production of functional oocytes from immature oocytes. In vivo and in vitro derivatives are pink and yellow, respectively. With regard to the donor oocytes, primordial (experiment 1), secondary (experiment 2) and Graafian’s follicles (control 1) were cultured for 21, 11 and 0 days, respectively, in order to attain the same chronological age. Serial nuclear transfer to cytoplasm grown and matured in vivo was conducted at the GV and MII stages, respectively. Following IVF, the resultant blastocysts were transferred to pseudopregnant females, and the long-term effects were assessed.
Nuclear transfer and IVM

Adult female mice were injected with 5 IU equine chorionic gonadotropin (eCG) (Serotropin®; Teikoku Zoki, Tokyo, Japan), and 5 IU human chorionic gonadotropin (hCG) (Puberogen®; Sankyo Zoki, Tokyo, Japan) was injected 48 h after the eCG injection. Freshly ovulated MII oocytes were collected from the oviducts 14–16 h after the hCG injection. Fully grown oocytes were collected at the germinal vesicle (GV) stage from the ovaries 44–48 h after the eCG injection.

Serial nuclear transfers were performed as described previously [15, 21]. In order to prevent GV breakdown, the GV-stage oocytes were manipulated in M2 medium containing 240 µM dibutyryl cAMP (Sigma). Karyoplasts obtained from the oocytes, which were cultured for 21 days (Experiment 1) and for 11 days (Experiment 2), were fused with enucleated fully grown GV-stage oocytes using the inactivated Sendai virus (2700 hemagglutinating activity units/ml). As a control, karyoplasts obtained from fully grown GV-stage oocytes were fused with enucleated fully grown GV-stage oocytes using the inactivated Sendai virus (control 1). The reconstituted oocytes were cultured in alpha-MEM medium containing 5% FBS in 5% CO₂, 5% O₂ and 90% N₂ at 37°C. The resulting blastocysts were transferred to the uterine horns of pseudopregnant female mice at 2.5 dpc. As a control, freshly ovulated oocytes (control 2) were subjected to the same sequence of IVF, in vitro culture and embryo transfer as that performed for the reconstituted oocytes. The ability of these blastocysts to develop into pups was analysed at 19.5 dpc by performing a caesarean section (Fig. 1). All the experiments described above were repeated at least three times.

Statistical analysis

Data on the growth of the oocytes and weight of the pups were examined using the Student’s t-test. The rate of follicular viability, oocyte maturation and embryonic development were analyzed using the chi-square test.

Results

Growth of oocytes cultured in alpha-MEM medium

When the ovaries of 1-day-old mice were cultured for 10 days, the size (mean ± SD) of the oocytes enclosed within the secondary follicles was 51.7 ± 3.86 µm (n=179). Subsequently, the secondary follicles were cultured for 11 days. On day 21, 69% of the oocytes were enclosed within the follicles; they were now 68.6 ± 3.87 µm (n=124) in size. These oocytes underwent the first meiotic division (19/91) but were not successful with regard to IVF (1/19). The mean diameter of the oocytes enclosed within intact secondary follicles isolated from the 10-day-old mice was 53.8 ± 3.91 µm (n=250). Some of the oocytes ovulated by 11 days after follicular culture and 56% of the oocytes were enclosed

<table>
<thead>
<tr>
<th>Culture duration</th>
<th>No. of oocytes</th>
<th>No. of embryos developed to</th>
<th>2-cell</th>
<th>blastocyst</th>
</tr>
</thead>
<tbody>
<tr>
<td>Primodial follicles of 1-day-old mice</td>
<td>22 days</td>
<td>91 (57%)</td>
<td>19 (5%)</td>
<td>0 (0%)</td>
</tr>
<tr>
<td>Secondary follicles of 10-day-old mice</td>
<td>12 days</td>
<td>140 (75%)</td>
<td>51 (37%)</td>
<td>19 (42%)</td>
</tr>
</tbody>
</table>

Table 1. Development of oocytes after IVG

a vs. b: P<0.01. a vs. c: P<0.05.
within follicles. They had reached $69.2 \pm 3.57 \mu m$ (n=140) in size, and had undergone the first meiotic division (51/140). After IVF, 19 eggs extruded a second polar body and formed two pronuclei. No significant differences were observed in the sizes of oocytes of the same chronological age that were cultured for 21 and 11 days. However, the maturation and fertilization rates of the oocytes cultured for 11 days were significantly higher than for those cultured for 21 days (P<0.05). In both cases, the resultant eggs after IVF did not develop beyond the 4-cell stage (Table 1).

Post-implantation development of reconstituted eggs containing the nuclei of oocytes produced in vitro

We initially we observed that physically denuded oocytes at the GV stage had high potential to mature to the MII stage (487/528; 92%) similar to cumulus enclosed oocytes (291/307; 95%); however, denuded oocytes had poor ability to achieve fertilization (1/152; 0.6%) and pre-implantation development (0/1). When zonae pellucidae of denuded oocytes were cut, the fertilization rate improved (71/135; 52.6%) but the ability to develop to the blastocyst stage was quite low (3/70; 4%). These results and Table 1 indicate that IVG and IVM of oocytes and denudation of cumulus cells caused zona hardening and critical cytoplasmic deficiency. Therefore, we conducted serial nuclear transfer experiments to analyze the nuclear competence of oocytes grown and matured in vitro and to investigate whether the duration of culture affects post-implantation development (Fig. 1).

In order to attain the same chronological age for the donor oocytes, primordial (experiment 1), secondary (experiment 2) and Graafian follicles (control 1) were cultured for 21, 11 and 0 days, respectively (Fig. 1). After nuclear transfer, oocytes reconstituted with the nuclei of the oocytes cultured for 21, 11 and 0 days were highly competent to mature into MII-stage oocytes (95, 82 and 95%, respectively) and develop into blastocysts (96, 92 and 95%, respectively; Table 2). In the post-implantation stage, the competency of the reconstituted oocytes was unaffected by the duration of culture. When the maternal nuclei were donated from oocytes cultured for 22 or 12 days, approximately 30% of the reconstituted eggs developed into pups (14/49 and 25/84, respectively). This observation was made with regard to the developmental rate of the reconstituted eggs containing nuclei obtained from oocytes cultured for 1 day (control 1, 45%; Table 2).

Offspring from oocytes produced in vitro

No obvious abnormalities were observed in any of the pups or placentaes. The pups ($1.48 \pm 0.17$ g, mean $\pm$ SD) and placentaes ($135 \pm 35$ mg) from the reconstituted eggs, which contained the nuclei of oocytes cultured for 22 days after their isolation from 1-day-old mice, were heavier than those of control 2 (pups, $1.25 \pm 0.14$ g; placentaes, $89 \pm 23$ mg). In the other experimental group, no significant difference was observed between the weight of the pups and placentaes and those of the pups and placentae of control 2 (Fig. 2). In the present

<table>
<thead>
<tr>
<th>Culture duration</th>
<th>No. of oocytes</th>
<th>No. of embryos</th>
<th>No. of living pups</th>
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</thead>
<tbody>
<tr>
<td></td>
<td>NT fused matured into MII</td>
<td>NT fused normally fertilized developed to blastocyst</td>
<td>pups</td>
</tr>
<tr>
<td>Primodial follicles of 1-day-old mice (Experiment 1)</td>
<td>22 days</td>
<td>133 (94 (71%); 89 (95%))</td>
<td>79 (84%); 66 (74%); 49 (74%); 47 (96%); 14 (29%)</td>
</tr>
<tr>
<td>Secondary follicles of 10-day-old mice (Experiment 2)</td>
<td>12 days</td>
<td>187 (168 (90%); 138 (82%))</td>
<td>121 (106 (88%); 84 (79%); 77 (92%); 25 (30%))</td>
</tr>
<tr>
<td>Graafian's follicles of adult mice (Control 1)</td>
<td>1 day</td>
<td>114 (95 (83%); 90 (95%))</td>
<td>86 (73 (85%); 55 (75%); 52 (95%); 25 (45%))</td>
</tr>
<tr>
<td>MII oocytes of adult mice (Control 2)</td>
<td>0 day</td>
<td>— — — — — —</td>
<td>40 (39 (89%); 39 (98%); 31 (79%))</td>
</tr>
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</table>

NT: nuclear transferred.
experimental series, only 1 pup died at birth (1/64, 1.6%), and all the tested pups were fertile. The lifespan of the obtained pups was 818 days (experiment 1; n=4), 765 days (experiment 2; n=3), 720 days (control 1; n=3) and 810 days (control 2; n=4); this appeared to be normal despite the long culture period of the donated oocytes.

**Discussion**

In the present study, we analyzed whether the duration of culture affects pre- and post-implantation and post-natal development. No significant difference was observed between the size of the oocytes obtained from 1-day-old mice and cultured for a total of 21 days and those obtained from 10-day-old mice and cultured for 11 days. However, the maturation rate of the oocytes cultured for 11 days was significantly higher than that of oocytes cultured for 21 days (P<0.05). The same tendency was observed in the fertilization rate. There are several possibilities to explain the incompetency of the oocytes cultured for 21 days. Formation and maintenance of gap junctional communication, which is mediated by factors such as Connexin 37 [22] and kit ligand [23], between granulosa cells and the oocytes during the initial phase of follicular growth may be insufficient under these culture conditions. Furthermore, zona hardening might occur more severely or the zona structure might be defective in the oocytes cultured for 21 days. Since replacement of the cytoplasm and zona pellucida by nuclear transfer in both cases led to a high rate of successful maturation and fertilization, it is plausible that long-term IVG or *in vitro* culture during the initial phase of follicular growth induced these deficiencies. No significant differences were observed with regard to the rate at which the eggs reconstituted with the nuclei of the oocytes cultured for 22, 12 and 1 days developed into blastocysts and pups (Table 2). In contrast to use of complicated manipulation, application of the nuclear transfer technique may prevent critical long-term effects. However, a significant increase in weight was observed in oocytes from the primordial follicles of 1-day-old mice that were cultured *in vitro* for 22 days (Fig. 2). The overgrowth phenotype at birth exhibits many common features in ruminants and is caused by IVM and IVF [16, 17]. The expression of *Igf2r*, which is an imprinted gene that negatively regulates fetal growth, is decreased in the large offspring of sheep due to abnormal demethylation of *Igf2r* [24]. The *Igf2r* gene is meth-
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ylated in the early phase of oogenesis [25]. However, we previously reported the absence of abnormal methylation of the \( \text{Igf2r} \) gene in oocytes cultured for 28 days and the pups from these oocytes [15]. Although the precise mechanisms of overgrowth are not understood, the culture duration of oocytes or culture conditions during the initial phase of follicular growth might induce epimutations in some genes.

In this study, we used a nuclear transfer technique to successfully produce a high rate of live offspring (29%) from embryos that contained the nuclei of oocytes cultured for 22 days after isolation from 1-day-old mice. Many features are essential for formation of a functional oocyte; these include meiotic maturation, cytoplasmic maturation and the establishment of genomic imprinting [21, 26, 27]. In mice, oocyte-specific imprinting is naturally established during oocyte growth up to 60–65 \( \mu \)m in diameter [25, 28]. Therefore, even when the donor oocytes did not grow to their maximum size, as long as they attained a diameter of \( \geq 60 \mu \)m after IVG, our strategy produced functional oocytes. This is potentially applicable for preservation and increase of the number of wild animals, valuable livestock and transgenic/knockout animals. On the other hand, in the UK, USA, France and Australia, it has been reported that the frequency at which ART children are born with Beckwith-Wiedemann syndrome (BWS) is 4- to 15-fold higher than the population risk or that of non-ART children. In ART children with Angelman syndrome, the loss of methylation in the \( \text{Snrpn} \) gene is observed at a 6-fold higher frequency [29]. Analyses of a large number of IVG oocytes and pups from IVG oocytes in mice can help to elucidate the cause of epimutation and help to establish safe ART.

In conclusion, we demonstrated that (1) oocytes from the primordial follicles of 1-day-old mice and secondary follicles of 10-day-old mice acquired competence to develop into pups at a high rate (29% and 30%, respectively) after IVG, nuclear transfer, IVF and embryo transfer and that (2) the long-term culture of oocytes did not affect their nuclear ability to develop to term; however, fetal growth was affected by the duration of culture or culture conditions during the initial phase of follicular growth.

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