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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

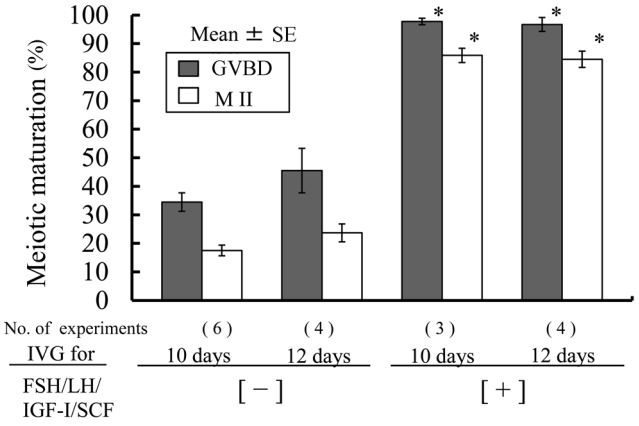

Fig. 1. Effects of supplementation of gonadotropins (FSH and LH) and cytokines (IGF-I and SCF) in IVG medium on *in vitro* maturation of oocytes following *in vitro* growth from oocyte–granulosa cell complexes in preantral follicles. * P<0.01 *vs.* groups without gonadotropins and cytokines.

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

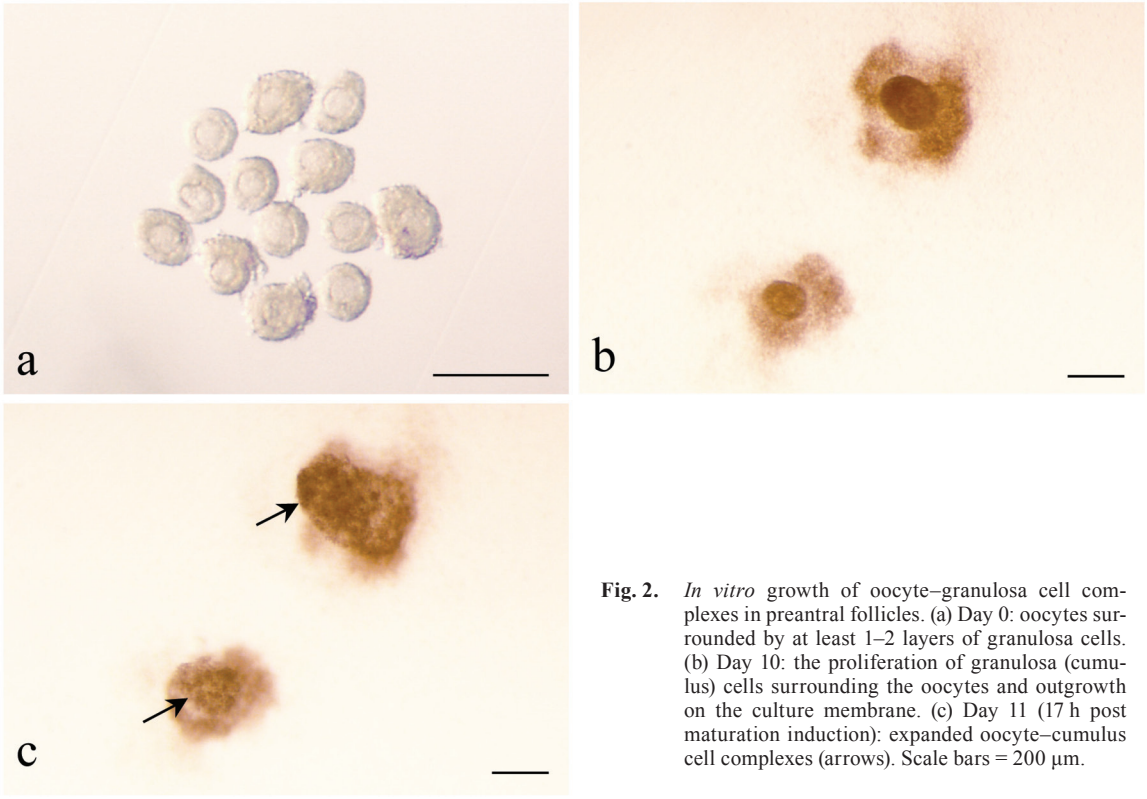

Fig. 2. *In vitro* growth of oocyte–granulosa cell complexes in preantral follicles. (a) Day 0: oocytes surrounded by at least 1–2 layers of granulosa cells. (b) Day 10: the proliferation of granulosa (cumulus) cells surrounding the oocytes and outgrowth on the culture membrane. (c) Day 11 (17 h post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

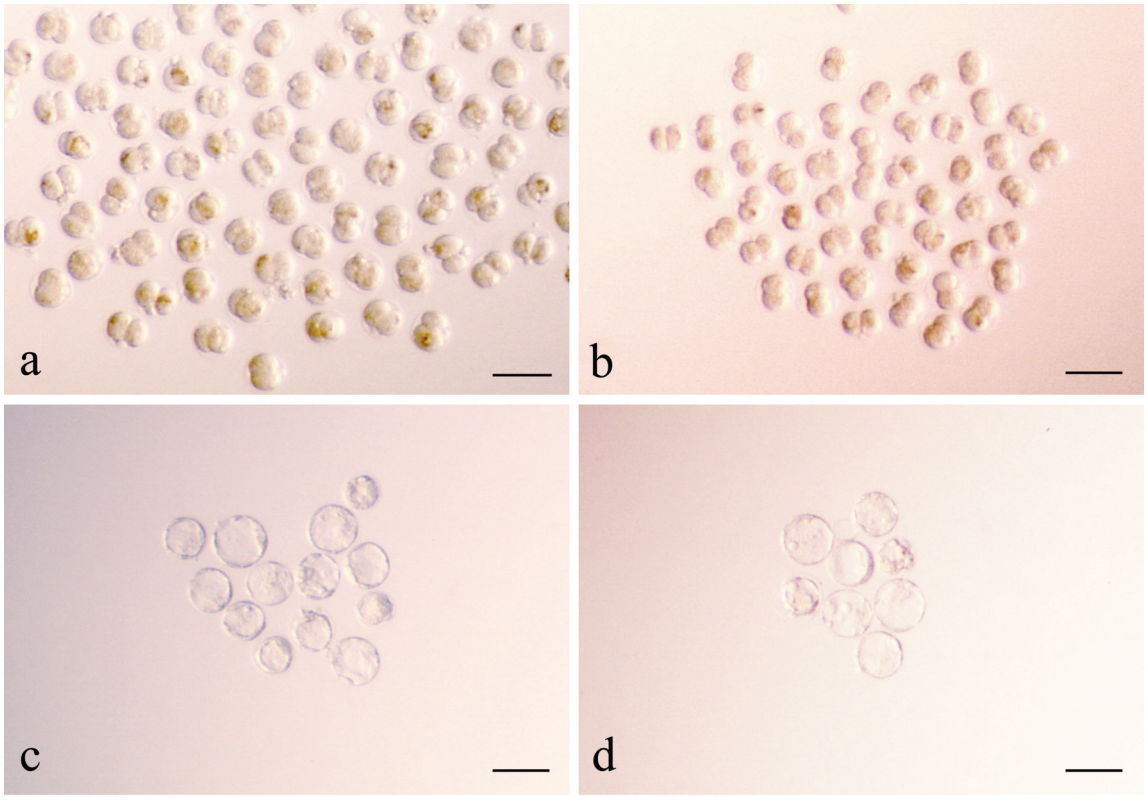

Fig. 4. Preimplantation development of (a) nonvitrified and (b) vitrified/warmed 2-cell embryos following *in vitro* growth, maturation and fertilization from oocyte–granulosa cell complexes in preantral follicles. Figs. 4 (c) and (d) were blastocysts from nonvitrified and vitrified/warmed, respectively.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

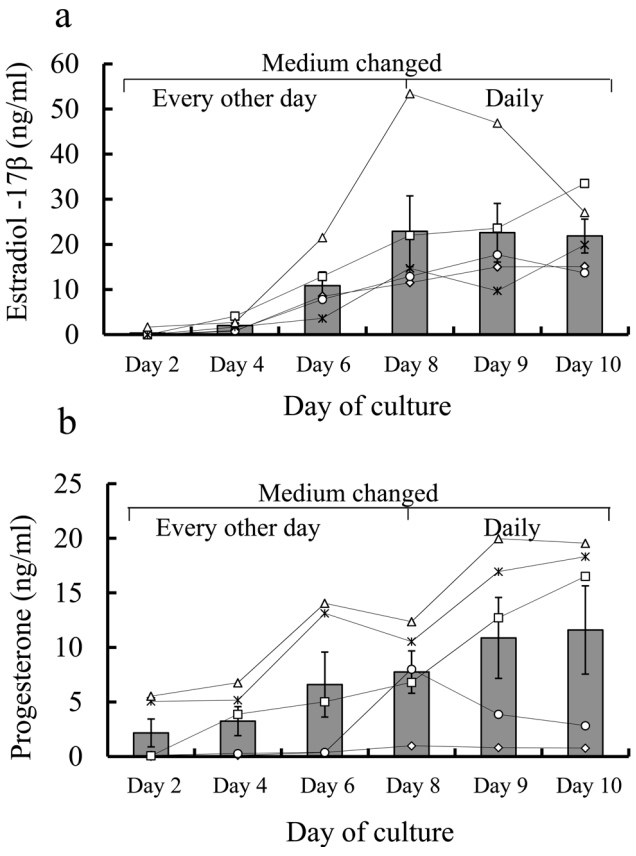

Fig. 3. Estradiol and progesterone production in conditioned medium during culture. Half of the fresh medium was changed every other day until day 8, and daily thereafter. (a) Estradiol-17β concentrations. (b) Progesterone concentrations. Values are means ± S.E. (n=5). Data for each sample are presented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

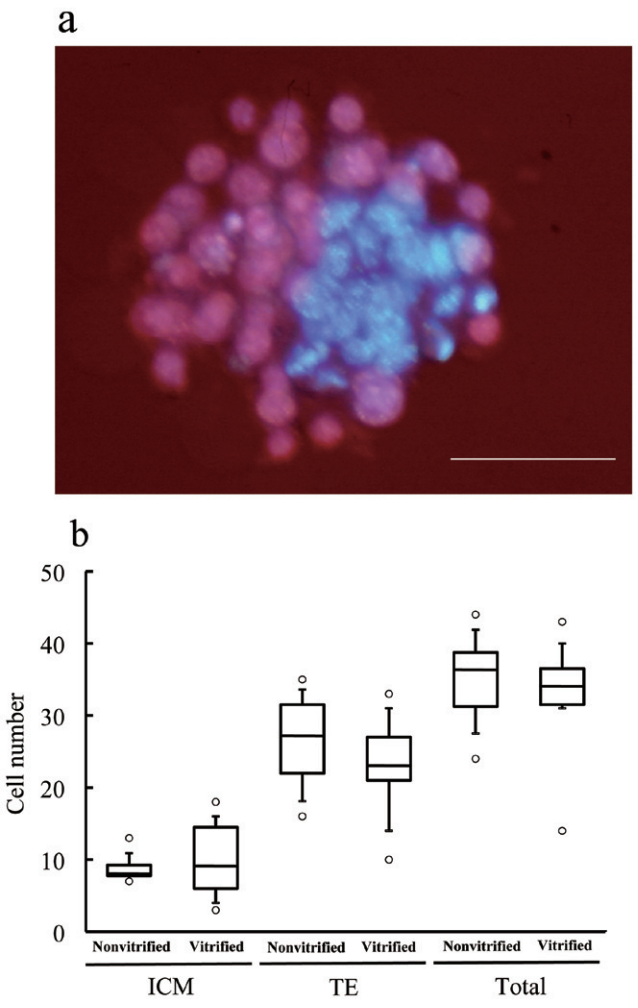

Fig. 5. Fluorescent double staining for differential inner cell mass (ICM) cell and trophectoderm (TE) cell counts in blastocysts that developed *in vitro* from nonvitrified and vitrified/warmed 2-cell embryos following *in vitro* growth, maturation and fertilization from oocyte–granulosa cell complexes 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.

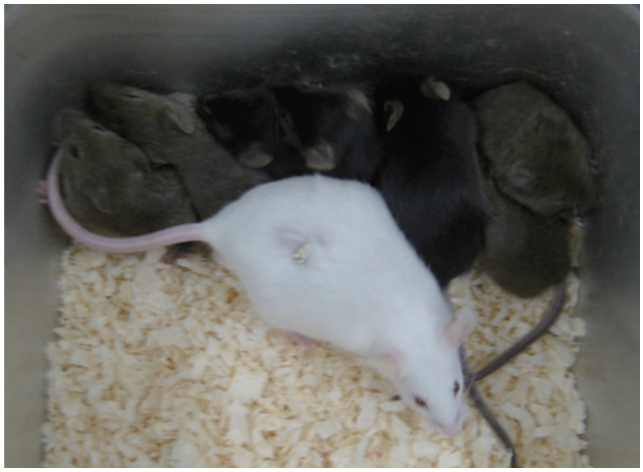

Fig. 6. Live offspring derived from vitrified/warmed 2-cell embryos following *in vitro* growth, maturation and fertilization from oocyte–granulosa cell complexes in preantral follicles: 21-day-old healthy offspring and a recipient female.

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 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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