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Original Article

In vitro production of viable eggs from isolated mouse primary follicles by successive culture

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Abstract. To produce viable eggs from single primary follicles *in vitro*, primary follicles containing oocytes (average 39.0 ± 0.2 µm in diameter) were isolated from the ovaries of 1-week-old mice, and cultured in combination with culture membranes for the first 8 days up to the secondary follicle stage, followed by the next 12 days to the later stages. After culture with a combination of first and second culture membranes using high and low adhesion characteristics, the average oocyte diameters of the surviving follicles increased by almost two-fold in all four groups. Further, the oocyte maturation rate was the highest (74.1%) in the culture group with low adhesion with collagenase and high adhesion. In this culture group, when the O_2 concentration was changed from 20% in the first culture to 5% in the second culture, the cleavage rate increased to 47.5%, which was comparable to the level of the *in vivo* control (34.6%). Finally, 39 embryos at the 2- to 8-cell stages were transferred into the oviducts of three pseudopregnant females, and eight live pups (20.5%) were obtained. Of the eight pups, six survived for at least six months and were fertile. The present study shows successive *in vitro* cultures of single isolated primary follicles for the production of viable eggs. We believe that this culture system, with a combination of culture membranes under controlled O_2 conditions, is applicable to other mammalian species, including humans.
Key words: Follicle growth, *In vitro* culture, Viable eggs

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In most mammals, including humans, mature eggs with developmental capacity are produced during follicle development in the ovary, where numerous primary oocytes exist within follicles. However, the number of viable eggs produced is known to be limited and dependent on the species; only less than 1% of primary oocytes can mature and be ovulated, whereas over 99% are degenerated throughout follicle development [1–3]. Alternatively, since the birth of the first baby, Louise J Brown, by *in vitro* fertilization (IVF), assisted reproductive technology (ART) has substantially developed and progressed for humans, and the number of newborn babies born owing to ART has steadily increased [4].

Based on the above biological limitations and the requirement for more viable eggs in ART, many studies have sought to establish feasible protocols for the *in vitro* production of viable eggs from either whole or pieces of the ovary. In earlier reports, Eppig's group cultured whole ovaries of mouse neonates and were the first to succeed in the production of matured eggs from primordial follicles, followed by live birth of pups after IVF and embryo transfer (ET) [5, 6]. In contrast, based on the size of the ovary for *in vitro* culture, a culture method of single isolated follicles seems to be more feasible and applicable, irrespective of the species. During follicle growth, the oocyte and its surrounding granulosa cells within a single follicle communicate with each other [7–9]. Therefore, when the follicle, especially at earlier follicle stages, is cultured *in vitro*, the follicle 3-dimensional (3D) structure must be maintained during culture. Accordingly, some 3D culture trials have been performed using scaffold materials, such as alginate, gelatin, and extracellular matrix (ECM). For example, from the viewpoint of clinical use for human ART, Rios *et al*. demonstrated *in vitro* culture of mouse primordial, primary, and secondary follicles by embedding in 0.5% alginate gels, which were transplanted into subcutaneous and bursa sites [10]. Kniazeva *et al*. also showed that mouse primordial to secondary follicles embedded in VEGF-fibrin beads can be grown by bead allografting [11]. Mochida *et al*. also cultured isolated primary to secondary follicles with a diameter of 60–100 µm using collagen gels, but without transplantation. To date, an *in vitro* culture of single primary follicles has been suggested to have a lower competence in the production of matured eggs and subsequent live birth pups than secondary follicles [12, 13]. Additionally, the *in vitro* growth of follicles in cryopreserved ovarian tissue is considered an essential method for women received cancer treatment instead of re-implantation of the cryopreserved tissue [14–16]. Thus, a feasible and applicable protocol for *in vitro* culture of single follicles, especially at the primary stage, which produces viable eggs, needs to be developed. Unfortunately, few studies have reported a reliable culture method for the isolated primary follicles. In a previous study [17], we demonstrated the *in vitro* culture of neonate ovaries using culture membranes. Although the culture membrane has been developed to culture single cells *in vitro* to support their proliferation, the use of this culture membrane was found to have a positive effect on the *in vitro* growth of the ovaries, with follicle development from primordial to secondary follicle stages. These findings motivated us to demonstrate that the use of culture membranes might be beneficial for the *in vitro* culture of isolated primary follicles. Thus, as the collapse of the follicle 3D structure can occur in long *in vitro* cultures due to the proliferation of theca cells, we examined two types of culture membranes with

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high and low adhesion characteristics, both of which are certified to help cell proliferation *in vitro*. Reactive oxygen species (ROS) are generally known to induce the apoptosis of granulosa cells, triggering follicle atresia both *in vivo* and *in vitro* [18, 19]. Further, low oxygen concentration is reported to improve oocyte maturation rates in bovine granulosa cell-oocyte complexes (GOCs) derived from antral follicles and mouse GOCs from preantral follicles [20–22].

In the present study, we aimed to develop an *in vitro* culture system for single primary follicles to produce viable eggs that can develop into live pups after IVF and ET. Accordingly, we investigated whether viable oocytes could be produced even with isolated primary follicles using our modified method for organ culture of the ovaries, to enable the application of the same culture conditions (e.g., culture membrane inserts, medium, and gas phase conditions) [17]. To the best of our knowledge, the present study is the first to report the successful production of viable eggs in mice by *in vitro* culture of isolated primary follicles with consequent production of live pups.

Materials and Methods

Animals

BDF1 female mice were used in this study. Mice were generated by mating C57BL/6N females with DBA/2 males (Japan SLC, Hamamatsu, Japan). All animals were maintained in accordance with national and institutional regulations and guidelines. All experiments were performed according to the protocol approved by the Committee for Animal Experiments of Shinshu University, Japan (No.019014).

Isolation of primary follicles

Ovaries of 1-week-old BDF1 mice were collected using fine forceps. After the removal of extra tissues with 26G needles (Terumo, Tokyo, Japan) in warmed Leibovitz's L-15 medium (Merck, Darmstadt, Germany) containing 5% (v/v) fetal bovine serum (FBS; Thermo Fisher Scientific, Waltham MA, USA) and a mixture of 5 units penicillin-5 µg streptomycin/ml (Merck, Darmstadt, Germany), the ovaries were mechanically minced with 30G needles (NIPRO, Osaka, Japan) in fresh L-15 medium. Under a dissection microscope (Olympus, Tokyo, Japan), only primary follicles of 50–70 µm in diameter with an oocyte enclosed by a layer of cuboidal granulosa cells [23], were collected (Fig. 1A). These follicles were washed three times with Minimum Essential Medium Alpha (αMEM; Thermo Fisher Scientific, Waltham MA, USA) containing 2% polyvinylpyrrolidone (PVP), 5% FBS, and a penicillin-streptomycin mixture using a 100 µm glass capillary (Drummond, Broomall PA, USA). The diameter of each follicle was estimated using CellSense software (Olympus, Tokyo, Japan) under an inverted microscope IX73 (Olympus, Tokyo, Japan).

In vitro culture of primary follicles

In our previous report [17], we cultured ovaries of mouse neonates on culture membranes up to the secondary follicle stage; thus, the same culture conditions (e.g., culture membrane inserts, medium, gas phase conditions, and collagenase treatment) were used in this study.

In experiment 1, to examine the effects of the maintenance of the architecture of isolated primary follicles during *in vitro* culture on the subsequent survival and maturation of the oocytes, we used two types of culture membranes and enzymatic treatment: a high adhesion type of culture membrane (High), Transwell-COL membrane (Corning, Corning NY, USA) comprising a PTFE membrane coated with collagen types I and III; and a low adhesion type of culture membrane (Low), MilliCell membrane (Merck, Darmstadt, Germany) comprising hydrophilized PTFE membrane. Follicles were treated with 0.1% (w/v) collagenase type I in αMEM (Worthington Biochemicals, Freehold NJ, USA) for 15 min at 37°C after the first culture period with low. Thereafter, follicles were pipetted with a fine glass capillary, resulting in the removal of theca cells and basement membrane.

Based on follicle development from primary to tertiary stages and the results of previous studies [5, 6], we designed two culture periods: first period lasting 8 days, which helped to develop isolated follicles from primary to secondary stages, and a second period that lasted 12 days from the secondary to tertiary stages. The isolated primary follicles were randomly divided into four groups depending on the combination of culture membranes during the first and second periods: high and high, low and low, low and high, and low with collagenase and high. The culture membranes were inserted into each well of a 6-well plate or 35 mm dish. Thereafter, the inside and outside of the membrane inserts were filled with 1 and 2 ml of the culture medium, respectively. Alpha-MEM supplemented with 2% PVP, 5% FBS, and 0.1 IU/ml follicular stimulating hormone (FSH: Gonal-f 75, Merck Serono, Geneve, Switzerland) was used as the culture medium. Fifteen to thirty primary follicles were cultured on each membrane in an incubator (PHC, Tokyo, Japan) under 5% CO_2, 95% air, and 37°C with high humidity. Throughout the culture period, half of the medium was replaced with fresh medium every other day. To evaluate the survival and growth of primary oocytes, two perpendicular diameters of each oocyte within a single follicle were measured using CellSense software. The oocytes that showed a decrease in their diameter, uneven surface with darkness, or were denuded from the follicle were classified as degenerated.

In experiment 2, we demonstrated the O_2 conditions required during follicle culture to improve the quality of matured eggs. In earlier studies with cultured mouse preantral follicles [20, 22], 5% O_2 in the early preantral follicle stage increased the follicle growth rate and cleavage rate after IVF; however, 20% O_2 was required in the later follicle stages. In this study, we assessed the effects of a combination of 5% and 20% O_2. Based on the results of experiment 1, isolated primary follicles were cultured under the same culture periods and conditions as those in the low with collagenase and high under either of the following four treatments: 20% O_2 (5% CO_2/95% air) to 20% O_2 (20% O_2 constant), 20% O_2 to 5% O_2 (5% CO_2/5% O_2/90% N_2), 5% O_2 to 20% O_2, or 5% O_2 to 5% O_2 (5% O_2 constant). The survival and growth of primary oocytes were evaluated using the method employed in experiment 1.

In experiments 1 and 2, cumulus-oocyte complexes (COCs) were collected from the surviving follicles and subjected to *in vitro* maturation (IVM). As a control (designated as *in vivo* oocytes), COCs were collected from the ovaries of 27 days old females at 48 h after injection of equine CG (Serotropin, ASKA Pharmaceutical, Tokyo, Japan), and subjected to IVM.

Based on the results of experiments 1 and 2, we cultured single early primary follicles with more restricted sizes between 50 and 60 µm in diameter in the low with collagenase and high group under 20% to 5% O_2; however, we extended the first culture period for 2 days to allow the follicles to reach the size of secondary follicles (approximately 120 µm in diameter). After 22 days of *in vitro* culture, IVM, IVF, *in vitro* culture of embryos (IVC), and ET were performed using the grown oocytes.

IVM, IVF, IVC, and ET

The protocols used for IVM, IVF, IVC, and ET were previously described [24]. The collected COCs were matured in αMEM supplemented with 5% (v/v) FBS, 0.1 IU/ml FSH, 1.2 IU/ml human chorionic gonadotropin (hCG; Gonatropin, ASKA Pharmaceutical,

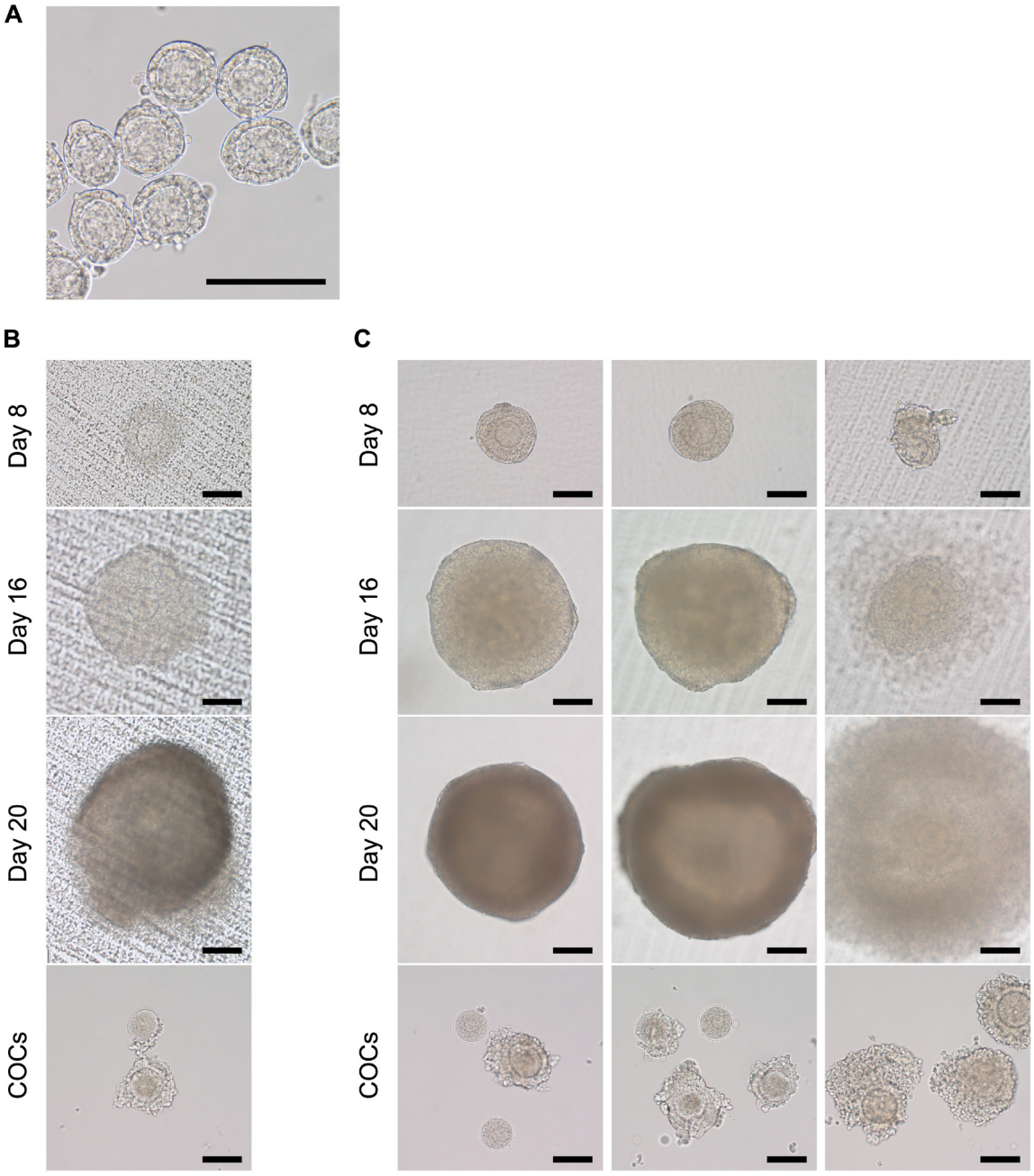

Fig. 1. Typical morphologies of primary follicles and COCs collected Day 20 after culture *. (A) follicles isolated and collected before culture, (B) in the High and High group, (C) in the Low and Low (left), Low and High (middle), and Low with collagenase and High (right) groups. Each bar indicates 100 µm. * See details in Materials and Methods.

Tokyo, Japan), and 4 mg/ml epidermal growth factor (EGF; Thermo Fisher Scientific). After 17 h of culture, the oocytes were denuded and assessed for meiotic maturation, the stage at which germinal vesicle breakdown (GVBD) or metaphase II (MII: presence of the first polar body) occurs. For IVF, spermatozoa from fertile BDF1 males were released by epididymal puncture into TYH medium (LSI Medience, Tokyo, Japan), and preincubated at 37°C in 5% CO_2 and 95% air for 1.5 h to allow capacitation. Approximately 20 matured oocytes were cultured in 100 µl of TYH medium with approximately 2×10^5 cells/ml of preincubated motile sperm. After 6 h of IVF, the oocytes were washed and cultured in KSOM medium (EmbryoMax KSOM Mouse Embryo Media, Merck, Darmstadt, Germany). The oocytes showing cleavage at 24 h after IVF were determined to be fertilized. The 2- and 8-cell stage embryos obtained were transferred into the oviducts of pseudopregnant ICR females (Japan SLC, Hamamatsu, Japan) at 0.5 days postcoitum.

Statistical analysis

Numeric differences in oocyte diameters and blastocyst development rates were compared using the Steel-Dwass test. Data on the survival rates of oocytes and 2-cell embryonic development rates were compared using the Bonferroni multiple comparison test, after a normality test. Differences were considered significant at $P < 0.05$.

Results

Status of the follicle architecture affects in vitro growth and subsequent maturation of isolated primary oocytes

As follicle growth is dependent on the interaction between an oocyte and its surrounding granulosa cells during the progression from primary to tertiary follicle stages, in experiment 1, we sought to determine the effects of culture membrane and enzymatic treatment on the growth and subsequent maturation of primary oocytes to culture the isolated primary follicles using the combined successive *in vitro* culture. After the first culture for 8 days up to the secondary follicle stage, although follicles in both groups of high and low culture membranes showed obvious growth compared to that before culture, the morphologies of primary follicles were quite different between the high and low culture groups (Fig. 1B and C). In the High group, the follicles seemed to be attached to the membrane, and moderately spread with a 2-dimensional (2D) structure, indicating a possible loss of their base membrane. In contrast, in the Low group, the follicles displayed a morphology similar to that of the entire secondary follicles.

After the second culture for 12 days up to the tertiary follicle stage, over half of the oocytes survived in high and high, low and low, and low and high (Table 1), with survival rates ranging from 56.5% to 66.9%, showing no significance among groups. When the surviving oocytes were subjected to IVM, the maturation rates in high and high, low and low, and low and high were 14.9%, 27.1%, and 42.1%, respectively, showing higher maturation rates in the low and high groups, with less significance than that in the control group (89.3%). In the low with collagenase and high group, the follicles formed an antral-like cavity with a dorm-like 3D structure that is characteristic of tertiary follicles, in contrast to those of low and low, and low and high groups (Fig. 1C). The maturation rate in the low with collagenase and high group (74.1%) was significantly higher than that in the groups without collagenase. The average diameters of the oocytes that survived in high and high, low and low, low and high, and low with collagenase and high were 76.5 ± 0.5, 81.7 ± 0.6, 76.1 ± 0.9, and 79.0 ± 0.8 µm (mean ± S.E.), respectively. Such findings indicate the apparent growth of oocytes compared to that at the start of culture (39.0 ± 0.2 µm), but are significantly smaller sizes than those of *in vivo* oocytes (89.8 ± 0.8 µm).

To summarize experiment 1, when culture membrane and enzymatic treatment were combined in the successive *in vitro* cultures of single isolated primary follicles, the architecture of follicles dependent on follicular stages may affect *in vitro* follicle growth and the resultant oocyte growth, and may be important for further maturation.

Concentration of O_2 in the successive in vitro culture affects the quality of oocytes, especially the viability of eggs after IVM

In experiment 1, we showed that almost fully grown oocytes can be produced by a new protocol of successive *in vitro* culture of single isolated primary follicles; however, after IVM, the maturation rate in any group was not necessarily comparable to that of *in vivo* oocytes. Thus, to improve oocyte quality after IVM, we determined the effect of a combination of 5% and 20% O_2.

Based on the results of experiment 1, isolated primary follicles were cultured in a combination of low with collagenase and high, and under either of the four culture conditions during the first and second culture periods: 20% O_2 constant, 20%–5% O_2, 5%–20% O_2, or 5% constant (Table 2). As a result, the survival rates at day 8 and day 20 of culture were over 75% and approximately 60% in each of the four groups, indicating no significance among groups. After culture, when IVM was performed, the maturation rates in groups with 20% O_2 constant, 20%–5% O_2, 5%–20% O_2, and 5% constant were 66.4%, 87.9%, 82.1%, and 75.9%, respectively. Although there was no significant difference among the groups, the highest rate was observed in the group with 20%–5% O_2, which was comparable to

Table 1. Effects of culture membranes on the survival and maturation of single primary oocytes in successive *in vitro* culture *

	Experimental groups **		No. of follicles cultured	No. of oocytes survived (% ± S.E.)	Average diameters of oocytes survived (µm ± S.E.)	No. of oocytes matured (% ± S.E.)
	1st term	2nd term				
1	High	High	68	39 (56.5 ± 7.7)	76.5 ± 0.5 ^{c}	6 (14.9 ± 2.0) ^{b}
2	Low	Low	98	67 (66.9 ± 5.1)	81.7 ± 0.6 ^{b}	20 (27.1 ± 4.9) ^{b}
3	Low	High	53	34 (65.0 ± 11.5)	76.1 ± 0.9 ^{c}	13 (42.1 ± 10.5) ^{b}
4	Low with collagenase	High	81	55 (66.5 ± 10.0)	79.0 ± 0.8 ^{c}	41 (74.1 ± 5.7) ^{a}
5	*in vivo*	-	-	93	89.8 ± 0.8 ^{a}	83 (89.3 ± 1.9) ^{a}

* Data were collected from at least three replicates. ** See details in Materials and Methods in the main text. $^{a–c}$ Different superscripts in each column indicate significant differences ($P < 0.05$).

Table 2. Survival and maturation of single primary oocytes after *in vitro* culture under different O_2 conditions and the subsequent developmental ability of matured eggs *

Oxygen concentrations	No. of follicles cultured	No. of follicles survived at day 8 (% ± S.E.)	No. of GOCs survived at day 20 (% ± S.E.)	No. of COCs for IVM	No. of oocytes matured (% ± S.E.)	No. of matured eggs for IVF	No. of 2-cell embryos (% ± S.E.)	No. of blastocysts (% ± S.E.)
20% constant	133	102 (76.9 ± 2.7)	76 (57.6 ± 5.8)	76	53 (66.4 ± 7.3)	35	1 (3.3 ± 3.3) ^{c}	0 (0)
20%–5%	163	128 (78.0 ± 2.5)	94 (57.6 ± 3.2)	94	84 (87.9 ± 4.5)	35	16 (47.5 ± 4.8) ^{a}	5 (36.7 ± 18.6)
5%–20%	167	125 (76.0 ± 4.4)	98 (60.1 ± 4.4)	96	79 (82.1 ± 4.3)	44	5 (10.8 ± 5.5) ^{bc}	2 (27.8 ± 14.7)
5% constant	158	124 (78.5 ± 3.5)	97 (61.5 ± 4.2)	97	76 (75.9 ± 7.2)	45	7 (13.3 ± 6.7) ^{bc}	1 (8.3 ± 8.3)
in vivo	-	-	-	122	111 (91.2 ± 0.7)	111	38 (34.6 ± 2.2) ^{ab}	31 (85.2 ± 7.8)

* Data were collected from at least three replicates. $^{a–c}$ Different superscripts in each column indicate significant differences ($P < 0.05$).

that of the control (91.2%).

When matured eggs after IVM were subjected to IVF and IVC, the cleavage rates varied among the groups. In the groups with 20% O_2 constant, 20%–5% O_2, 5%–20% O_2, and 5% constant, the cleavage rates were 3.3%, 47.5%, 10.8%, and 13.3%, respectively, indicating significant differences among the groups. The highest rate was observed in the 20%–5% O_2 group, comparable to that of the control (34.6%), although the developmental rate of blastocysts in all groups was less than that of the control group.

Based on the results of experiments 1 and 2, we cultured single isolated early primary follicles with more restricted sizes between 50 and 60 µm in diameter using low with collagenase and high under 20%–5% O_2, with the exception of a 2-day extension to the first culture. When 86 isolated early primary follicles were cultured, the average diameter of the oocytes that survived after culture was 85.7 ± 0.5 µm (mean ± S.E.), showing distinct growth compared to that before culture (35.5 ± 0.3 µm) and almost the same size as that of full-grown oocytes. After IVM, IVF, and IVC using the oocytes that survived, the maturation rate was 85.4%, whereas the cleavage rate to the 2-cell stage was 34.3%, 75% of which developed into blastocysts (Fig. 2). After ET of 39 embryos at the 2- to 8-cell stages to oviducts of three pseudopregnant females, we obtained eight live pups with an average body weight of 1.7 ± 0.1 g (mean ± S.E.), indicating a birth rate of 20.5%. An analysis of the DNA sequence encoding tyrosinase was performed to identify the origin of the pups. The sequence showed that none of the pups had transversion of G to C, which causes an amino acid change from cysteine to serine in the albino allele of ICR (Fig. 3B). Of the 8 pups live-birthed, 2 died 2 weeks after birth, while the other 6 pups survived for at least six months (Fig. 3A). All six mice that survived were fertile, and produced the next generation.

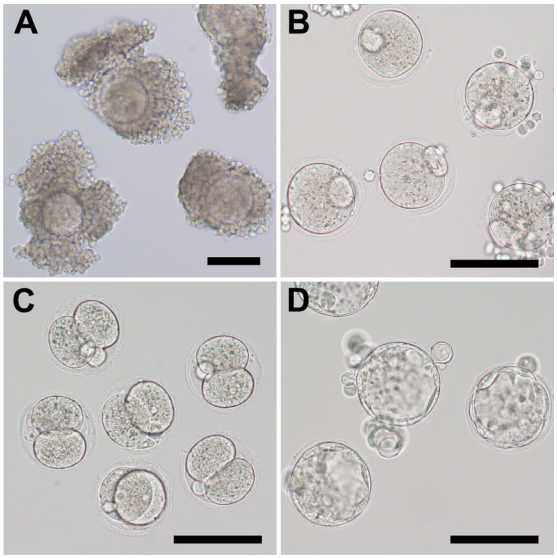

Fig. 2. Developmental competence of the oocytes produced by successive culture of single isolated primary follicles with diameters of 50–60 µm. (A) COCs collected after culture, (B) matured eggs after IVM, (C) 2-cell embryos after IVF, and (D) blastocysts after IVC. Each bar indicates 100 µm.

Discussion

Although the *in vitro* growth of mammalian primary follicles to produce matured eggs has been assessed in various species over the last 30 years, previous studies have shown lower competence in the production of matured eggs with a resultant birth of pups, than that with secondary follicles [13]. In the current study, we found that for the *in vitro* growth of isolated primary follicles, the maintenance of follicle architecture might be a key detail, especially in the process from primary to secondary stages. Moreover, the O_2 concentration may be another key factor for improving the quality of primary oocytes grown *in vitro*. These findings offer, for the first time, a new and valuable approach for the *in vitro* production of viable eggs by culturing isolated primary follicles.

In the ovary, single primary oocytes are stocked within single follicles. Consequently, each follicle consists of a single oocyte and its surrounding granulosa cells. Thus, during follicle growth, the oocyte communicates with granulosa cells and grows via paracrine systems, and at the later follicle stage, with gap junctions [7–9]. Considering this biological angle, previous studies have attempted to maintain and improve oocyte-granulosa cell communication during *in vitro* culture. In earlier reports by O'Brien & Eppig and Morohaku *et al*., *in vitro* organ culture of mouse neonate ovaries was performed up to the secondary follicle stage [6, 17]. In contrast, Shea *et al*. cultured isolated preantral follicles in alginate acid capsules for clinical use in humans [25]. They found that the capsules can maintain the follicle structure during *in vitro* culture, and succeeded in obtaining matured eggs in mice and humans [16]. Their findings suggest that cross-linked biomaterials can retain the growth factors around the oocyte [22]. However, although the capsule can maintain follicle structure, the speed of follicle growth, especially oocyte growth, is slower than that of 2D culture on plastic dishes or culture

B

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C57BL/6  GCCAGTGCTCAGGCAACTTCATGGGTTTCAACTGCGGAAACTGTAAGTTTGGATTTGGGG
#1       GCCAGTGCTCAGGCAACTTCATGGGTTTCAACTGCGGAAACTGTAAGTTTGGATTTGGGG
#2       GCCAGTGCTCAGGCAACTTCATGGGTTTCAACTGCGGAAACTGTAAGTTTGGATTTGGGG
#3       GCCAGTGCTCAGGCAACTTCATGGGTTTCAACTGCGGAAACTGTAAGTTTGGATTTGGGG
#4       GCCAGTGCTCAGGCAACTTCATGGGTTTCAACTGCGGAAACTGTAAGTTTGGATTTGGGG
#5       GCCAGTGCTCAGGCAACTTCATGGGTTTCAACTGCGGAAACTGTAAGTTTGGATTTGGGG
#6       GCCAGTGCTCAGGCAACTTCATGGGTTTCAACTGCGGAAACTGTAAGTTTGGATTTGGGG
#7       GCCAGTGCTCAGGCAACTTCATGGGTTTCAACTGCGGAAACTGTAAGTTTGGATTTGGGG
#8       GCCAGTGCTCAGGCAACTTCATGGGTTTCAACTGCGGAAACTGTAAGTTTGGATTTGGGG
ICR      GCCAGTGCTCAGGCAACTTCATGGGTTTCAACTGCGGAAACTCTAAGTTTGGATTTGGGG
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Fig. 3. Offspring obtained after IVM, IVF, IVC, and ET of the oocytes produced by successive culture of single isolated primary follicles with diameters of 50–60 µm. (A) a photograph of offspring at six months after birth. (B) DNA sequence analysis of the 8 pups obtained, which indicates that all pups were live-birthed from the oocytes produced.

membranes [26, 27]. This slower growth may be due to the hardness of gels acting as a restricting factor in follicle growth, especially in the proliferation of granulosa cells. Mochida *et al*. [22] used collagen gel to embed primary to secondary follicles of 60–100 µm in diameter in the first culture for 9 days, and performed the second culture for 8 days without the gel, which resulted in grown oocytes with the ability to cleave after IVM and IVF. Unfortunately, when any method that requires embedding of isolated follicles is applied for *in vitro* culture, the follicles must be removed from the materials for embedding, indicating that the handling of the follicles seems to be a complicated and laborious work, not only in the field of animal production, but also for human clinical use.

In the present study, instead of embedding follicles, we determined whether viable oocytes could be produced even in the case of isolated primary follicles using our modified culture method for organ culture of ovaries using culture membranes. Within the ovary, follicles are present in interstitial tissues that consist of cellular matrices, including collagen. Accordingly, in our previous reports, we used culture membranes to culture the ovaries before and after birth and succeeded in follicle growth from primordial germ cells and primordial follicles [17, 24]. In this study, two types of culture membranes were used, namely membranes with high and low adhesion characteristics. Both culture membranes were found to support the *in vitro* growth of isolated primary follicles. Further, after the first culture for 8 days, the morphologies of the surviving follicles were quite different between culture membranes. In the high-adhesion group, the follicle structure was almost flattened, while in the low-adhesion group, this structure was well maintained. As both culture membranes were developed to support the *in vitro* proliferation of mammalian cells by their attachment to the membrane [28, 29], the proliferation of cells in the follicles was expected to be supported. The morphological differences observed in this study may be due to the adhesion characteristics of the culture membranes to the base membrane cells of the primary follicle. The high-adhesion culture membrane was coated with collagen types I and III, where the extracellular matrix can be easily used for *in vitro* cell growth to ensure that the cells may spread and proliferate more rapidly, inducing the resultant appearance of the follicle-like 2D structure. In contrast, the low-adhesion culture membrane, MilliCell membranes, consisted of hydrophilized PTFE membrane, which resulted in fewer follicle cells attached to the membrane during culture. This finding may explain the maintenance of the morphology of primary follicles during the first culture period. Before the start of the second culture, the follicles were treated with collagenase because they were approximately 120 µm in diameter, suggesting that they may reach the secondary follicle stage. In our previous report [24], secondary follicles treated with collagenase showed a well-developed and higher maturation rate to metaphase II after IVM. In addition, in *in vitro* cultures, secondary follicles can easily accelerate the proliferation of granulosa cells by expressing genes related to follicle growth. Granulosa cells-oocyte complexes can transport molecules directly from and to the medium by removal of theca cells and basement membranes [24]. The present study revealed that the follicles in the group cultured with low adhesion followed by collagenase treatment, and subsequently cultured with high-adhesion membranes developed well and showed higher maturation after IVM, consistent with the findings of previous studies [24, 30]. Furthermore, as the secreted cytokines and hormones could remain around the follicles [31], the medium was supplemented with 2% PVP, which may also affect follicle growth by making the culture medium more viscous. PVP may thus function as a scaffold material.

To improve the maturation ratio, many studies have tested low oxygen conditions during *in vitro* culture of follicles or GOCs by reducing ROS [18, 19]. In the second experiment of the present study, 5% O_2 instead of 20% increased both the rates of maturation and embryo production, which were comparable to those in the control (*in vivo* oocytes). These results are consistent with those of previous reports on bovine GOCs derived from antral follicles and mouse GOCs from preantral follicles [20–22]. As bovine and swine antral follicles have been reported to have lower O_2 concentrations in situ as measured with oxygen sensing tools [32, 33], and this is also the same in human follicular fluids [34], we speculate that 5% O_2 in the second period of the present culture might represent a similar condition to the inside of antral follicles in the later follicle stage. Hypoxia-inducible factor 1 (HIF1) is a known regulator of cellular response to low oxygen levels [35], and HIF1 is expressed in granulosa cells (but not in theca cells) in growing follicles in some species, including mice [36]. The experiment involving bovine granulosa cell culture under low oxygen levels indicated that HIF1 regulates steroidogenesis and cell proliferation [37]. Another study that examined dynamic oxygen tension by increasing oxygen concentration by 1% every 24 h from 4% during a 7-day culture of mouse preantral follicles showed that the survival rate of the oocytes increased; however, the maturation rate was low [38]. Shiratsuki *et al*. [39] also showed that bovine granulosa cells derived from antral follicles proliferate more efficiently under low oxygen concentrations owing to the reduction in ROS. Thus, considering the follicle stage at the beginning of *in vitro* culture, oxygen concentration is an important factor for the production of viable eggs.

In conclusion, in the current study, we revealed a new approach for the successive *in vitro* culture of single isolated primary follicles, by which viable eggs can be produced with the subsequent birth of pups. In previous reports (Eppig's group and our earlier studies) [6, 17], *in vitro* organ culture of neonate ovaries was performed to grow primordial follicles up to the secondary follicle stage. Therefore, no information on the number of primary follicles cultured at the start of ovarian organ culture was available. Accordingly, only direct comparisons were possible among the results after culture of secondary follicles. For example, when comparing birth rates, 5.7% was reported by Eppig's group [6], whereas in the present study, the rate was 20.5%. Mochida *et al*. [22] reported a birth rate of 1.6% in a study of isolated primary follicles; however, they transferred embryos derived from both primary and other follicle stages. We believe that this culture system is applicable to other mammalian species, including humans, for which whole and pieces of ovaries cannot be cultured due to scarce exchange of gases and nutrients during *in vitro* culture because of the size of the ovary and the presence of thick and hard cortical tissues.

Conflicts of interests: The authors declare that there are no conflicts of interest regarding the contents of this article.

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