

Effects of reaggregated granulosa cells and oocytes derived from early antral follicles on the properties of oocytes grown *in vitro*

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Abstract. In this study, we examined the effects of reconstructed oocyte–granulosa cell complexes (OGCs) on the development of porcine oocytes derived from early antral follicles (EAFs; 0.5–0.7 mm in diameter). When denuded oocytes were cocultured with granulosa cells derived from other EAFs, the oocytes and granulosa cells aggregated to form OGCs after 2 days of culture. After 14 days of culture, we compared cell number, oocyte diameter, and oocyte chromatin configuration in unmanipulated (natural) OGCs, reconstructed OGCs, and OGCs collected from antral follicles (AFs, 3.0–6.0 mm in diameter). The diameters of oocytes from reconstructed OGCs grown *in vitro* were not different from those of oocytes from natural OGCs, although they were significantly smaller than those of oocytes from antral follicle (AF) OGCs. Oocyte chromatin configuration did not differ among the 3 OGC groups, but the oocyte nuclear maturation rate was lower in the reconstructed OGCs and higher in the AF OGCs. However, when the *in vitro* culture period for the reconstructed OGCs was extended by 2 days, the nuclear maturation rate of oocytes from reconstructed OGCs was similar to that of oocytes from natural OGCs. In addition, blastocysts were successfully obtained from oocytes from reconstructed OGCs. In conclusion, we established an innovative culture method that allows oocytes and granulosa cells from EAFs to reaggregate as reconstructed OGCs, which yield oocytes with the ability to develop to the blastocyst stage.

Key words: Cell exchange, Early antral follicle, Granulosa cells, Oocyte, Pigs

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On the ovary surface, visible antral follicles (AFs) 3–6 mm in diameter contain developmentally competent oocytes. Accordingly, these oocytes have been used for embryo production in many domestic animals, including pigs. Unlike AFs, which are present in a limited number, follicles at an early developmental stage, termed early antral follicles (EAFs), are present in large amounts in the ovaries. However, EAFs have not been used for embryo production because of the low developmental competence of the immature oocytes within these follicles and the lack of suitable culture conditions for immature oocytes.

When oocytes from EAFs are cultured together with their surrounding granulosa cells for approximately 2 weeks, the granulosa cells form pseudoantrum-like follicle structures [1]. It has been shown that well-orchestrated interactions between oocytes and granulosa cells are necessary for oocyte growth [2, 3]. Although a close relationship between the conditions of granulosa cells and the developmental abilities of oocytes has been reported in humans [4–7], the effect of granulosa cell conditions on oocyte development has been difficult to assess because of the limited number of developmentally competent

oocytes available from EAFs and the lack of a suitable culture system in which the effects of granulosa cells with different properties on oocyte development could be examined. However, Veitch *et al.* [8] reported that when they cocultured granulosa cells with denuded mouse oocytes, the oocytes restored gap-junction communication with granulosa cells.

Guided by this pioneering research, in this study, we tested whether denuded EAF oocytes cocultured with granulosa cells derived from EAFs could reconstruct oocyte–granulosa cell complexes (OGCs) and whether these reconstructed OGCs could form pseudofollicle structures in which the oocytes could develop to a size sufficient to acquire the capacity for meiotic maturation. Oocytes from EAF-OGCs were denuded from their surrounding granulosa cells and then cocultured with granulosa cells derived from EAF-OGCs from other gilts. We found that the recombined oocytes and granulosa cells were able to reconstruct OGCs. We then examined the effect of the reconstructed OGCs on the growth of oocytes *in vitro*. In addition, we determined the length of time required for reconstructed OGCs to undergo development in culture and also examined whether oocytes in the reconstructed OGCs were able to develop to the blastocyst stage.

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Materials and Methods

Media and chemicals

All reagents were purchased from Nacalai Tesque (Kyoto, Japan), unless otherwise stated. For OGC collection, we used minimum essential medium (MEM; Sigma-Aldrich, St. Louis, MO, USA) supplemented with 5 mM taurine, 5 mM mannitol, 0.68 mM L-glutamine, 1

mg/ml BSA (Fraction V) and antibiotics. For OGC culture, we used α -MEM (Sigma-Aldrich) supplemented with 10 mM L-taurine, 0.1 mAU/ml follicle-stimulating hormone (Kawasaki Mitaka, Tokyo, Japan), 2% polyvinylpyrrolidone-360 (Sigma-Aldrich), 2 mM hypoxanthine (Sigma-Aldrich), 1% Insulin-Transferrin-Selenium (Gibco BRL, Paisley, UK), 1 μ g/ml 17 β -estradiol, 3 mg/ml BSA and antibiotics.

For *in vitro* oocyte maturation (IVM), we used North Carolina State University Medium-23 supplemented with porcine follicular fluid (pFF, 10% v/v) for 44 h. For the first 20 h of the maturation period, the oocytes were cultured in maturation medium that contained 1 mM dibutyryl cAMP (dbcAMP, Sigma-Aldrich), 10 IU/ml equine chorionic gonadotropin (ASKA Pharmaceutical, Tokyo, Japan) and 10 IU/ml human chorionic gonadotropin (Fuji Pharma, Tokyo, Japan). The oocytes were then transferred to the maturation medium without both dbcAMP and hormones and were cultured for 24 h. The pFF aspirated from AFs (3–6 mm in diameter) was centrifuged for 20 min. The resulting supernatants were collected, sterilized, and stored at -20 C until use.

The medium for oocyte activation and *in vitro* culture of the embryos was PZM3 [9]. OGC culture and oocyte IVM were conducted at 38.5 C in an atmosphere containing 5% CO₂. *In vitro* culture of activated embryos was conducted at 38.5 C in an atmosphere containing 5% CO₂, 5% O₂ and 90% N₂, with maximum humidity.

Ovaries and oocytes

The ovaries were collected from prepubertal gilts at a local abattoir and transported to the laboratory (at approximately 35 C, in PBS containing antibiotics) within 1 h. The ovarian cortical tissues were excised from the ovarian surface under a stereomicroscope, and OGCs were collected from EAFs (0.5–0.7 mm in diameter). OGCs containing oocytes with diameters ranging from 90–100 μ m were then selected under a digital microscope (BZ-8000; Keyence, Osaka, Japan). Fully grown oocytes were collected from AFs (3–6 mm in diameter) as *in vivo* grown oocytes.

Culture of OGCs *in vitro* and oocyte IVM

OGCs were individually cultured (day 0) in 200 μ l of the culture medium in 96-well plates (Falcon 3072; Becton, Dickinson and Company, Franklin Lakes, NJ, USA) for 14 days. The culture medium contained BSA instead of serum such as FCS or FBS, and the culture conditions suppressed the spread of granulosa cells from OGCs during the culture period. Every 2 days, the number of OGCs that had formed an antrum was counted, and at 4-day intervals, half of the medium was replaced with fresh medium. At the end of the culture period, OGCs that had formed an antrum were selected, and the oocytes were allowed to mature for 44 h (OGCs/10 μ l).

Reconstructed OGCs

Oocytes in OGCs collected from EAFs were denuded from the surrounding granulosa cells. Granulosa cells were retrieved from another OGC that was selected at random and cocultured with the denuded oocytes in a 96-well plate for 14 days (1 oocyte/well; Fig. 1). OGCs that were collected from EAFs but were otherwise unmanipulated were termed “natural OGCs” and used as controls. OGCs that formed from the reaggregation of oocytes and granulosa

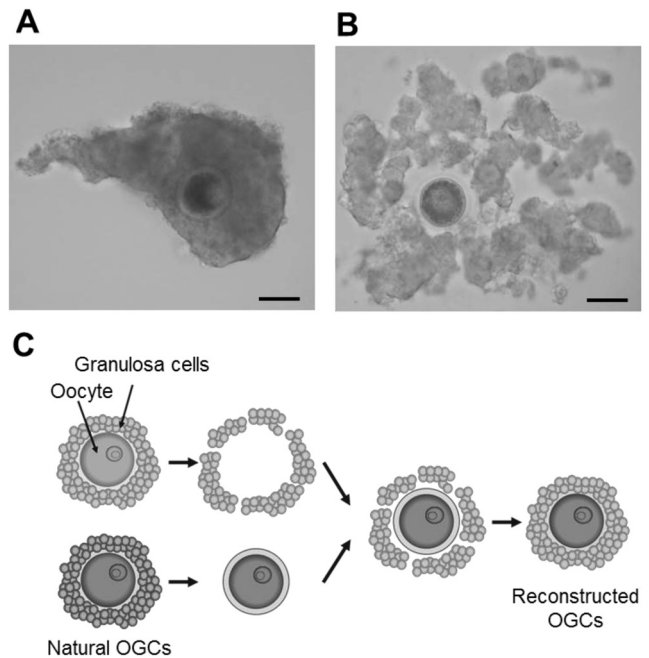


Fig. 1. Oocyte–granulosa cell complexes (OGCs) (A) and coculture of denuded oocytes with granulosa cells derived from EAFs (B). Denuded oocytes and granulosa cells derived from OGCs at the same stage were cocultured to produce reconstructed OGCs (C). Scale bar = 100 μ m.

cells in coculture were termed “reconstructed OGCs.”

Oocyte diameter and chromatin configuration of oocytes grown *in vitro*

The diameter of the ooplasm was measured under a digital microscope (Keyence, Tokyo, Japan). To determine the number of granulosa cells in OGCs, the granulosa cells were dispersed by vigorous pipetting in Accumax (Innovative Cell Technologies, San Diego, CA, USA), and cellular density was measured by using a hemocytometer; the total granulosa cell number was then calculated. To determine the number of granulosa cells in AFs, AFs were flushed with PBS by using a 24-gauge needle connected to a 1-ml syringe. After centrifugation of the follicular content, the cellular pellets were dispersed in Accumax, as described above. To assess the chromatin configuration, oocytes were fixed in aceto-alcohol and stained with 2% aceto-orcein. According to a previous report [11], chromatin configuration can be divided into five categories, and an oocyte at the germinal vesicle stage (i.e., the oocyte has a nuclear membrane and intact, but condensed, chromatin forming a ring or horseshoe around the nucleolus) is categorized as GV1. In the present study, the percentage of oocytes that were GV1 was determined under a digital microscope (Keyence). To examine the characteristics of *in vivo* grown oocytes, OGCs were collected from EAFs and AFs (3–6 mm) from the same batch of ovaries.

Experimental design

Development of reconstructed OGCs and properties of oocytes

within reconstructed OGCs: We examined the antrum formation rate of OGCs in culture, the diameter and chromatin configuration of oocytes that developed *in vitro* and the number of granulosa cells in the OGCs. Ten natural OGCs and 10 reconstructed OGCs were cultured for 14 days. The experiment was performed 14 times. OGCs from the first seven trials were used for determining oocyte diameter, and OGCs from the remaining seven trials were used to determine granulosa cell number and oocyte chromatin configuration.

Comparison of the meiotic competence of oocytes from control and reconstructed OGCs: Ten natural and 10 reconstructed OGCs were cultured for 14 days. At the end of the culture period, OGCs that had formed an antrum were subjected to IVM, and then nuclear maturation was examined. OGCs collected from AFs (3–6 mm in diameter) were defined as *in vivo* grown oocytes. The experiment was repeated 7 times.

Determination of optimal culture periods for OGCs

Based on the timing of antrum formation and the percentage of oocytes showing nuclear maturation, it was deduced that the low nuclear maturation rate observed in reconstructed OGCs was due to the culture period being too short. To test this hypothesis, OGCs were cultured for 14, 16 and 18 days. Ten natural and 10 reconstructed OGCs were used for each period. At the end of each culture period, OGCs that had formed an antrum were used for IVM, and the states of nuclear maturation of the oocytes obtained from three culture periods were compared. The experiment was repeated 9 times.

Developmental ability of oocytes from cultured OGCs

Using the optimal culture periods for natural and reconstructed OGCs as determined by the rate of nuclear maturation, natural and reconstructed OGCs ($n = 15$ per group) were cultured for 14 days and 16 days, respectively. After IVG, OGCs presenting an antrum were matured for 44 h, and the oocytes were then denuded from the surrounding cells. Oocyte activation and subsequent culture were performed as described previously [10]. In brief, oocytes were activated in PZM3 containing 10 $\mu\text{g/ml}$ ionomycin for 5 min. These oocytes were further cultured for 6 h in medium containing 10 $\mu\text{g/ml}$ cytochalasin B and 10 $\mu\text{g/ml}$ cycloheximide, and the presumptive embryos were then cultured in PZM3 for 8 days. The rate of blastulation and the cell number of the resulting blastocysts were determined and compared between the two groups. The experiment was repeated 5 times.

Data analysis

Comparison of the data between control and reconstructed OGCs was performed using the Student's *t*-test. Comparison of the data from the three culture periods was performed using analysis of variance followed by Tukey's post hoc test. The rate of antrum formation, chromatin configuration, maturation and development were arcsine transformed prior to the analysis. A *P* value less than 0.05 was considered to denote statistical significance.

Results

Reconstruction of OGCs with denuded oocytes cocultured with granulosa cells

When granulosa cells were cocultured with denuded oocytes, the cells and oocytes reaggregated to form "reconstructed OGCs" by day 2 of culture (Fig. 2B). These OGCs were morphologically similar to "natural OGCs", which were OGCs that had been collected from EAFs, but were otherwise unmanipulated (Fig. 2A). The rate of reconstruction was 79.2%. Both natural and reconstructed OGCs maintained relative morphological integrity (i.e., the oocytes were surrounded by multilayered granulosa cells) during the culture period (91% for reconstructed OGCs and 87.9% for natural OGCs) and formed antrum-like spherical structures (Fig. 3A). Almost all OGCs from which oocytes were extruded degenerated during *in vitro* culture (Fig. 3B). Natural OGCs formed antra starting on day 4 of culture. By the end of the 14-day culture period, 80% of the natural OGCs had formed an antrum (Fig. 4). In contrast, reconstructed OGCs did not start to form antra until day 6. By the end of the culture period, 70% of the reconstructed OGCs had formed an antrum. However, the difference between the OGCs was not significant.

Similar diameters and chromatin configurations, but lower meiotic maturation ability, in oocytes from reconstructed OGCs compared with those from natural OGCs

The diameters of oocytes derived from the two types of OGCs were similar ($113.2 \pm 1.3 \mu\text{m}$ and $113.4 \pm 1.3 \mu\text{m}$ for natural and reconstructed OGCs, respectively; Table 1) but were smaller than those of oocytes collected from AFs ($121.2 \pm 5.5 \mu\text{m}$; Table 1). When first collected from EAFs, OGCs contained $7,977 \pm 776$ granulosa cells. By the end of the culture period, natural and reconstructed OGCs contained $161,931 \pm 6,796$ and $151,582 \pm 9,342$ granulosa cells, respectively. The difference between these two values was not significant (Fig. 5). However, the number of granulosa cells in OGCs that had developed *in vitro* was substantially lower than the number of granulosa cells collected from AFs ($1,391,000 \pm 15,400$; Fig. 5). Although none of the oocytes in either the natural OGCs or the reconstructed OGCs were categorized as GV1 at the start of culture, by the end of the culture period, the percentage of GV1 oocytes had increased to similar levels for both natural and reconstructed OGCs (60.8 and 68.4%, respectively). Moreover, these values were not significantly different from that for OGCs derived from AFs (62.1%). However, when oocytes grown *in vitro* were subjected to IVM, 47.1% of the oocytes from natural OGCs reached the MII stage, whereas a significantly smaller percentage of oocytes from reconstructed OGCs (25.2%) reached the MII stage ($P < 0.05$, Table 2). In addition, the maturation rate of oocytes grown *in vitro* was significantly lower than that of oocytes grown *in vivo* (67.7%; $P < 0.05$).

Improvement of ability to undergo meiotic maturation by extending the culture period for reconstructed OGCs by 2 days

As shown in Fig. 6A, the rate of antrum formation in natural OGCs reached a plateau on days 8–10 of culture. The rate of antrum formation in reconstructed OGCs reached a plateau on days 10–12 of culture (Fig. 6B), and the antra were maintained for up to 18 days. In natural

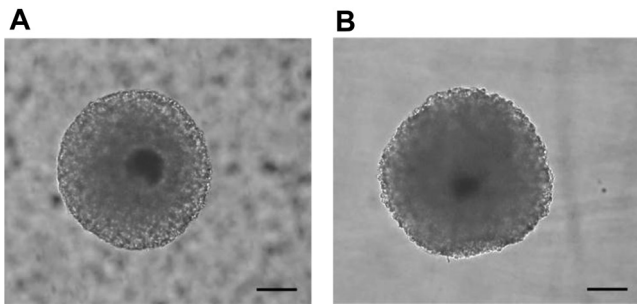


Fig. 2. Representative pictures of natural (unmanipulated) oocyte-granulosa cell complexes (OGCs) (A) and reconstructed OGCs (B). Scale bar = 100 μm.

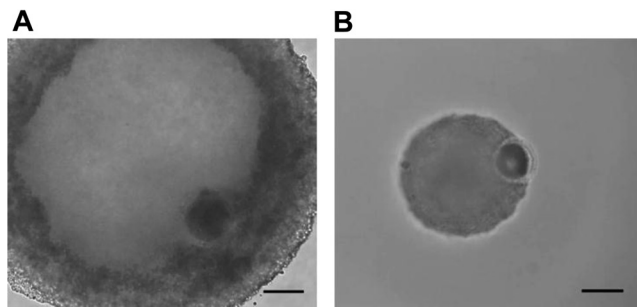


Fig. 3. Representative pictures of oocyte-granulosa cell complexes (OGCs). OGC with an antrum-like structure (A). OGC with an extruded oocytes (B). Scale bar = 100 μm.

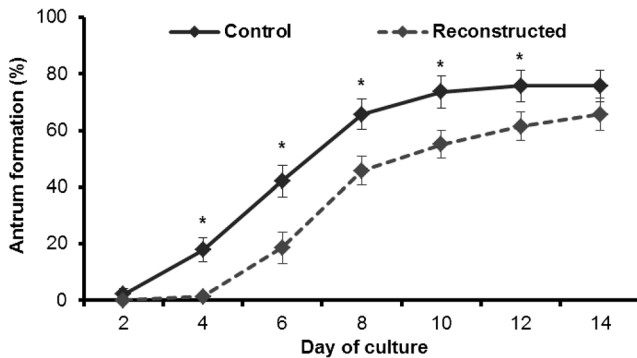


Fig. 4. Percentage of oocyte-granulosa cell complexes with antrum formation during the *in vitro* culture period. *Significant differences.

Table 1. Diameter of oocytes grown *in vitro*

	No. of oocytes cultured	No. of oocytes examined	Mean diameter of surviving oocytes (μm)
Control	70	41	113.2 ± 1.2 ^a
Reconstructed	70	38	113.4 ± 1.3 ^a
AFs	–	99	121.2 ± 5.5 ^b

Data are presented as the mean ± SEM. ^{a-b} P < 0.01. AFs, oocytes were collected from antral follicles (3–6 mm in diameter) of the same ovary series, and the diameters of the oocytes were measured immediately after collection.

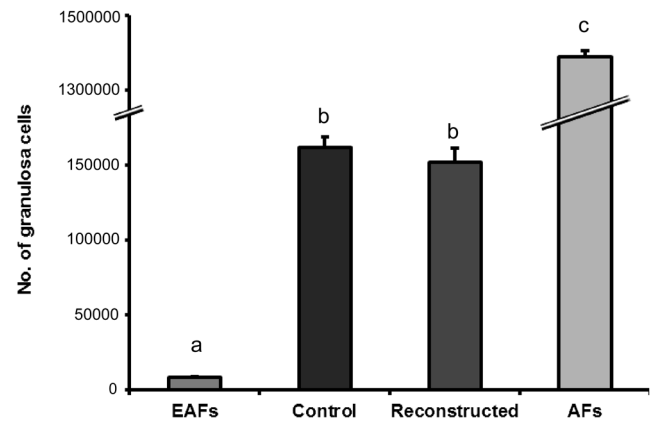


Fig. 5. Number of granulosa cells in oocyte-granulosa cell complexes (OGCs) grown *in vitro*. Antral follicles (AFs) and early antral follicles (EAFs) were collected from the same ovary series. All values are presented as the mean ± SEM (%) (n = 35–60/group). ^{a-c} P < 0.01.

OGCs, the percentage of oocytes that reached the MII stage was the highest on day 14 (40.9%), declining to 31.1% on days 16 and 18 of the culture period, concomitantly with an increasing percentage of degenerated or activated oocytes. In reconstructed OGCs, the percentage of MII oocytes was the highest on day 16 (42.8%) and significantly declined on day 18 (16.4%, P < 0.05), concomitantly with an increasing percentage of degenerated or activated oocytes (Table 3). In the final experiment, we examined the developmental ability of the oocytes from natural and reconstructed OGCs. As shown in Table 4, oocytes from reconstructed OGCs successfully developed to the blastocyst stage, although their developmental competence was slightly lower than that of natural OGCs.

Table 2. Nuclear maturation of oocytes grown *in vitro*

	No. of oocytes	No. of oocytes examined	Nuclear morphology of oocytes (%)			
			GV	GVBD immature	MI	DE
Control	70	61	6 (10.3) ^{ab}	24 (37.4)	28 (47.1) ^a	3 (5.2)
Reconstructed	70	50	11 (20.6) ^a	22 (45.4)	13 (25.2) ^b	4 (8.7)
AFs	70	56	2 (3.6) ^b	16 (28.6)	38 (67.7) ^c	0 (0)

^{a-c} P < 0.05. Oocytes were categorized into 4 groups: oocytes at germinal vesicle (GV) stage, oocyte underwent germinal vesicle breakdown (GVBD) but did not reach the metaphase II (MI) stage, oocytes at the MI stage, and degenerated (DE) oocytes. AFs, oocytes collected from antral follicles (3–6 mm in diameter).

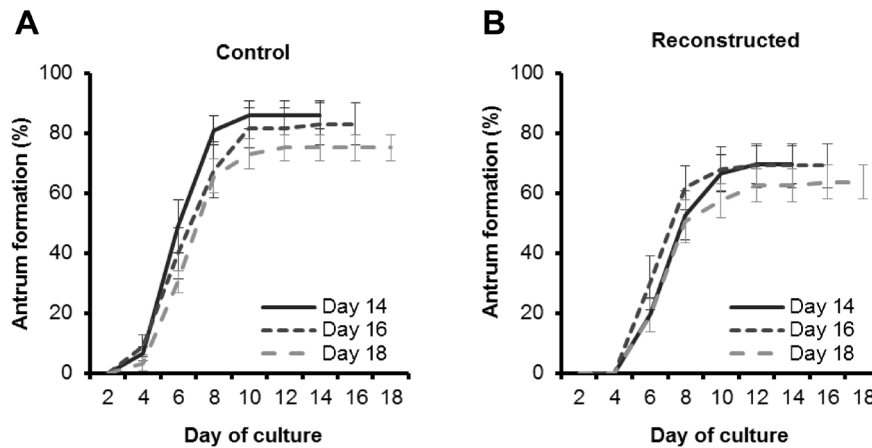


Fig. 6. Percentage of natural (unmanipulated) (A) and reconstructed (B) OGCs that formed an antrum in culture. OGCs were cultured for 14, 16, or 18 days, during which antrum formation was monitored.

Table 3. Effect of culture period (days) on rate of nuclear maturation of oocytes grown *in vitro*

	No. of OGCs	Culture length	No. of oocytes examined	Nuclear morphology of oocytes (%)				
				GV	GVBD immature	MII	DE	PA
Control	61	14	47	3 (7.1)	23 (49.1)	20 (40.9)	1 (2.9)	0 (0)
	62	16	44	5 (11.3)	21 (49.8)	15 (31.1)	3 (7.7)	0 (0)
	66	18	50	9 (18.4)	21 (42.3)	16 (31.5)	2 (4.2)	2 (3.6)
Reconstruct	60	14	41	8 (18.9)	20 (49.6)	11 (27.5) ^{ab}	0 (0)	2 (4.1)
	62	16	37	5 (17.9)	13 (33.4)	17 (42.8) ^a	0 (0)	2 (6.0)
	64	18	34	10 (31.9)	10 (32.9)	5 (16.4) ^b	3 (6.9)	6 (12.9)

^{a-b} $P < 0.05$. Oocytes are categorized into 5 groups: oocytes at the germinal vesicle (GV) stage, oocyte underwent germinal vesicle breakdown (GVBD) but did not reach the metaphase II (MII) stage, oocytes at the MII stage, degenerated (DE) oocytes, and parthenogenetic (PA) embryos.

Table 4. Development of oocytes grown *in vitro*

	No. of OGCs	No. of oocytes activated	No. of blastocysts (%)	Mean number of cells in blastocysts
Control	75	59	3 (4.0)	36.6 ± 6.6
Reconstructed	75	49	1 (1.3)	23
AFs	-	120	28 (23.3)	52.6 ± 4.0

Data are presented as the mean ± SEM. AFs, oocytes collected from antral follicles (3–6 mm in diameter).

Discussion

This study demonstrates that coculturing denuded oocytes with granulosa cells leads to the reconstruction of OGCs and that the oocytes grown in the reconstructed OGCs were able to complete meiotic maturation and develop to the blastocyst stage.

Interactions between oocytes and granulosa cells are crucial for oocyte development, and many factors secreted from oocytes and granulosa cells have been defined [12]. In our preliminary experiment, culturing denuded oocytes alone did not support oocyte growth. In the present experiment, once oocytes were extruded from granulosa cells, they degenerated within a few days. From these, we deemed that the presence of granulosa cells was essential for proper oocyte

growth. However, the effects of granulosa cells on oocyte growth *in vitro* have been difficult to study. Here, we present an innovative culture system in which granulosa cells collected from EAF OGCs are combined with denuded oocytes from other EAFs to reconstruct OGCs in culture. This technique may help not only to increase knowledge about the role of granulosa cells in oocyte development but also to expand the number of available oocytes from donors who have dysfunctional granulosa cells.

When OGCs derived from porcine EAFs are cultured, the granulosa cells form an antrum cavity-like structure, and antrum formation is considered an indicator of successful *in vitro* growth in OGCs [1, 13]. In the present study, natural OGCs started to form an antrum on day 4, whereas reconstructed OGCs did not form antra until day

6. This observation indicates that the reconstruction process takes approximately two days. To compare the quality of oocytes grown in natural OGCs and reconstructed OGCs, the OGCs were tested for the following characteristics: granulosa cell number, oocyte diameter, chromatin configuration, and meiotic maturation. In pigs, a diameter of 115 μm is required for oocytes to acquire complete developmental competence [14]. In the present study, the average diameters of oocytes grown *in vitro* were 113.4 ± 1.3 and 113.2 ± 1.3 μm for reconstructed and natural OGCs, respectively. These values are less than the mean diameter of oocytes derived from AFs (121.2 ± 5.5 μm). In addition, the percentage of oocytes with diameters >115 μm was 50% for natural OGCs and 43.6% for reconstructed OGCs.

The results indicate that the *in vitro* culture system is still not perfect. Similarly, the nuclear maturation rate of oocytes grown *in vitro* was less than that of oocytes grown *in vivo*, irrespective of the culture conditions, although the nuclear maturation rate was higher for control OGCs than for reconstructed OGCs. Chromatin configuration in oocytes changes as the follicle and oocytes grow [15], and the percentage of oocytes in which the chromatin condenses around the nucleolus (i.e., GV1 oocytes) is high for fully grown oocytes as compared with immature oocytes [16]. In the present study, when the chromatin configuration of oocytes was examined immediately after they were collected from EAFs, almost all the oocytes were categorized as GV0 (i.e., clearly visible nucleolus and nuclear membrane and a diffuse, filamentous pattern of chromatin in the whole nuclear area). However, during the culture period, the number of GV0 oocytes diminished. Segers *et al.* [17] reported that, in mice, oocyte chromatin configuration in immature follicles changed with the duration of culture. Once the maximum percentage of GV1 oocytes was attained, extension of the culture period no longer had an effect on oocyte developmental competence. In the present study, the percentages of oocytes at GV1 in natural OGCs, reconstructed OGCs and *in vivo* developed oocytes were similar.

When first collected from EAFs, OGCs contain 7,977 granulosa cells. This number increases by 200 fold during oocyte development *in vivo*. In contrast, while *in vitro* developed control and reconstructed OGCs contained similar numbers of granulosa cells, the number was substantially lower than that observed in *in vivo* developed OGCs. This observation suggests that the small number of granulosa cells in the cultured OGCs may be one possible reason for the reduced developmental competence of the oocytes grown *in vitro*. Thus, improving culture conditions to support granulosa cell proliferation may improve the quality of oocytes grown *in vitro*. In addition, the results indicate that the chromatin configuration does not reflect intrinsic differences in the developmental competence of oocytes in natural OGCs, reconstructed OGCs, and *in vivo* grown oocytes. Furthermore, oocyte diameter and granulosa cell number only differed between *in vivo* and *in vitro* grown OGCs, whereas the maturation rate differed among the three groups. Thus, the maturation rate was used to examine oocyte quality in the subsequent experiments.

After considering the low maturation rate and later timing of antrum formation in the reconstructed OGCs, we deduced that the original duration of the culture period was likely to be insufficient for reconstructed OGCs. Therefore, in the subsequent experiment, we addressed the effect of culture duration on the nuclear maturation ability of *in vitro* grown oocytes by extending the culture duration

by 2 or 4 days. As observed in Fig. 6, the results confirmed that reconstructed OGCs started to form an antrum 2 days after control OGCs did and that the antrum was maintained during the culture period extended by 4 days. For natural OGCs, the highest percentage of MII oocytes was obtained when the OGCs were cultured for 14 days (40.9%). As the duration of culture was extended, the percentage of oocytes remaining at the GV stage and that of activated oocytes increased.

In line with these results, previous studies reported that, as the duration of culture increases, the developmental competence of mouse oocytes decreases, with a reduced percentage of oocytes showing nuclear maturation and smaller amounts of transcripts being expressed [18, 19]. In reconstructed OGCs, the highest percentage of cells at the MII stage was observed on day 16 (42.8%), which was 2 days later as compared with natural OGCs. However, like the natural OGCs when the culture period was extended to 18 days, the percentage of GV oocytes and activated oocytes in reconstructed OGCs increased (31.9 and 12.9%, respectively). These results indicate that the optimal duration of culture is 14 days for natural OGCs and 16 days for reconstructed OGCs.

When reconstructed OGCs were cultured for 16 days, the oocytes derived from the OGCs reached the blastocyst stage with a success rate that was similar to that of oocytes derived from natural OGCs. This finding indicates that this culture system allows denuded oocytes to develop with granulosa cells collected from other EAFs or other donors. Note that, in the present study, we only used granulosa cells collected EAFs at one stage. Thus, determining the optimal number of granulosa cells and the optimal follicle stages of follicles from which to derive granulosa cells will be the target of future experiments. In conclusion, denuded oocytes derived from EAF OGCs can reconstruct a follicle-like structure with granulosa cells from other EAFs and develop to a size at which they are as developmentally competent as oocytes from natural OGCs.

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