

# Addition of granulosa cells collected from differential follicle stages supports development of oocytes derived from porcine early antral follicles

Ai Ishiguro | Yasuhisa Munakata | Koumei Shirasuna | Takehito Kuwayama |  
Hisataka Iwata 

Department of Animal Science, Tokyo University of Agriculture, Atsugi, Japan

## Correspondence

Hisataka Iwata, Department of Animal Science, Tokyo University of Agriculture, Atsugi, Japan.  
Email: h1iwata@nodai.ac.jp

## Funding information

Grant-in-Aid for Scientific Research C, Grant/Award Number: 16K07996; Grant-in-Aid for JSPS Fellows, Grant/Award Number: JP25450400; Japan Society for the Promotion of Science; Science Research Promotion Fund from the Promotion; Mutual Aid Corporation for Private Schools of Japan

## Abstract

**Purpose:** Improvement of in vitro oocyte growth by addition of granulosa cells derived from differential developmental stages of follicles.

**Methods:** Granulosa cells (GCs) collected from either early antral follicles (EAFs) or antral follicles (AFs) were added to oocyte-granulosa cell complexes (OGCs) derived from EAFs, and the in vitro growth of the oocytes was evaluated.

**Results:** Granulosa cells were incorporated into OGCs to form new OGCs within 2 days of culture. After 14 days of culture, the number of GCs surrounding oocytes was similar among the three OGCs conditions (unmanipulated “natural OGCs,” “EAF-GCs add OGCs,” and “AF-GCs add OGCs”), whereas the survival rate of the GCs and diameter of oocytes grown in vitro were the greatest for “AF-GCs added OGCs.” After parthenogenetic activation, developmental rate till the blastocyst stage tended to be higher for “AF-GCs add OGCs” compared with other groups. Addition of AF-GCs significantly increased a hypoxic marker (pimonidazole staining) and increased the lipid content in oocytes grown in vitro compared with unmanipulated OGCs.

**Conclusion:** Addition of GCs derived from more advanced stages of follicles to the OGCs changes the metabolism of oocytes and is beneficial for in vitro growth of oocytes derived from EAFs.

## KEYWORDS

early antral follicles, granulosa cells, in vitro growth, oocyte, pigs

## 1 | INTRODUCTION

It is generally accepted that only full-grown oocytes in large antral follicles have high developmental ability to term.<sup>1</sup> The number of the large antral follicles is limiting in ovaries, and a large number of oocytes are present in small-sized immature follicles. However, oocytes of the small-sized follicles are not available for embryo production

due to their low developmental ability and/or lack of ability to complete meiotic division.

Oocytes develop under close interaction with their surrounding granulosa cells (GCs),<sup>2,3</sup> and the quality and quantity of GCs surrounding an oocyte profoundly affects developmental competence and growth in vitro.<sup>4,5</sup> Furthermore, it is demonstrated in pigs that follicle with a greater number of GCs contained high-quality oocytes

compared to those with less GCs.<sup>6</sup> Moreover, physiological changes profoundly affect granulosa cell number and oocyte quality; for example, oocytes of age cows, compared to that of younger counterparts, have low developmental competence and lower number of surrounding GCs.<sup>7</sup> From these studies, it seems conceivable that manipulation of granulosa cell number could improve oocyte development.

It has been reported that GCs and denuded oocytes derived from the porcine early antral follicle (EAF) can reconstruct a new oocyte-granulosa cell complex (OGC), and this structure helps the oocyte to acquire the developmental ability to the blastocyst stage.<sup>8</sup> In addition, the addition of GCs derived from EAFs to OGCs supports the growth of bovine oocytes derived from EAFs.<sup>9</sup> However, the collection of GCs from EAFs is laborious work, while collection from antral follicles (AFs) is easy. Moreover, characteristics of the GCs change during follicle development,<sup>10</sup> yet whether GCs from more advanced stage follicles supports the development of oocytes derived from EAFs is unclear.

In the present study, we collected OGCs from EAFs of porcine ovaries, and cultured the OGCs with or without additional granulosa cells masses, which were collected from EAFs or AFs, and examined the properties and parthenogenetic developmental abilities of the oocytes grown in vitro.

## 2 | MATERIALS AND METHODS

### 2.1 | Media and chemicals

All reagents were purchased from Nacalai Tesque (Kyoto, Japan), unless otherwise stated. For the collection of OGCs from EAFs, we used minimum essential medium (MEM; Sigma-Aldrich, St. Louis, MO, USA) supplemented with 5 mmol/L taurine, 5 mmol/L mannitol, 0.68 mmol/L L-glutamine, 1 mg/mL BSA (Fraction V), and antibiotics. For culture of OGCs, we used  $\alpha$ -MEM (Sigma-Aldrich) supplemented with 10 mmol/L taurine, 0.1 mAU/mL follicle-stimulating hormone (Kawasaki Mitaka, Tokyo, Japan), 2% polyvinylpyrrolidone-360 (Sigma-Aldrich), 2 mmol/L hypoxanthine (Sigma-Aldrich), 1% insulin transferrin selenium (Gibco BRL, Paisley, UK), 1  $\mu$ g/mL 17 $\beta$ -estradiol, 3 mg/mL BSA, and antibiotics. The in vitro maturation medium consisted of Medium 199 (Gibco)<sup>11</sup> supplemented with 10% porcine follicular fluid (pFF), 0.5 mmol/L L-cysteine, 0.9 mmol/L sodium pyruvate, 1 mmol/L L-glutamine, 10 ng/mL epidermal growth factor, 5% fetal calf serum, 10 IU/mL equine chorionic gonadotropin (ASKA Pharma Co. Ltd, Tokyo, Japan), and 10 IU/mL human chorionic gonadotropin (Fuji Pharma Co. Ltd, Tokyo, Japan). The pFF supplemented to the maturation medium was aspirated from AFs (3-5 mm in diameter) and centrifuged for 20 minutes. The resulting supernatants were collected, sterilized, and stored at -20°C until use. In vitro culture of embryos and oocyte activation was conducted in porcine zygote medium 3 (PZM3).<sup>12</sup> In vitro culture of OGCs and oocyte maturation was performed at 38.5°C in an atmosphere containing 5% CO<sub>2</sub> and 95% air, whereas in vitro embryo culture was performed at 38.5°C in an atmosphere containing 5% O<sub>2</sub>, 5% CO<sub>2</sub>, and 90% N<sub>2</sub>.

### 2.2 | Collection of ovaries and OGCs

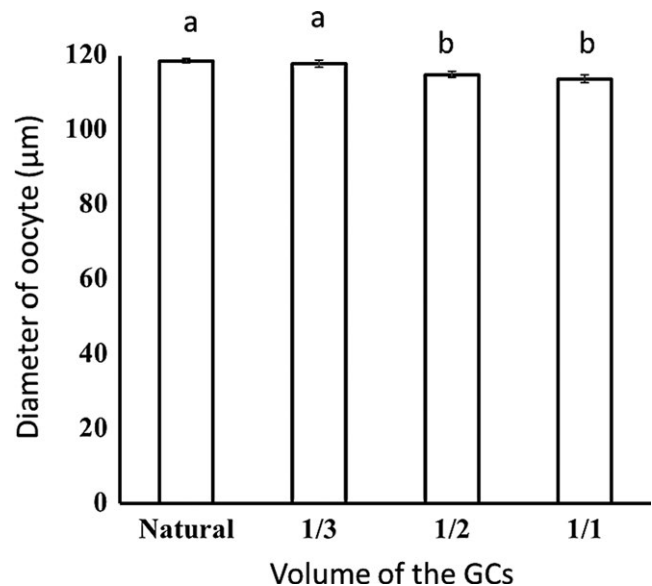
Ovaries were collected from prepubertal gilts at a local slaughterhouse and transported to the laboratory (at approximately 35°C, in PBS containing antibiotics) within 1 hour. The ovarian cortical tissues were excised from the ovarian surface under a stereomicroscope, and OGCs were collected from EAFs (0.5-0.7 mm in diameter). OGCs containing oocytes with diameters ranging from 90 to 100  $\mu$ 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.

### 2.3 | Culture of OGCs in vitro and oocyte in vitro maturation (IVM)

Oocyte-granulosa cells were individually cultured (day 0) in 200  $\mu$ L of culture medium in 96-well plates (Falcon 353072; Becton Dickinson, Franklin Lakes, NJ, USA) for 14 days. 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 formed an antrum were selected and the oocytes were used for maturation for 48 hours (OGCs/10  $\mu$ L).

### 2.4 | Collection of granulosa cells (GCs) and their addition to OGCs

Granulosa cells were collected from EAFs and AFs of gilts ovaries. GCs of EAFs were retrieved from randomly selected OGCs using narrow-pulled glass pipettes. GCs of AFs were collected from large



**FIGURE 1** Diameter of oocytes grown in vitro. Granulosa cells (Gcs) masses were collected from early antral follicles (EAFs) and the volume of the GC masses was processed to 1/3, 1/2, and 1/1 levels of the oocyte-GCs (OGCs). GCs and OGCs were cocultured for 14 d. Diameters of 24 oocytes grown in vitro were measured. a-b,  $P < 0.05$

antral follicles (3-5 mm in diameter); antral follicles were carefully ruptured in a share and granulosa cell masses were collected under stereomicroscope. The volume of the GCs was processed depending on experimental design under stereomicroscope. Figure 1 shows representative pictures of OGC cultures where GCs were added to the OGCs. From here on in, we use the term “natural OGCs” to represent OGCs collected from EAFs but were otherwise unmanipulated. In addition, we use “EAF-GCs add OGCs” and “AF-OGCs add OGCs” for OGCs that were formed from the addition of GCs derived from EAFs and AFs, respectively.

## 2.5 | Measurement of oocyte diameter and number of GCs

The diameter of the ooplasm was measured under a digital microscope (Keyence). To determine the number of GCs in OGCs, the GCs were dispersed by vigorous pipetting in Accumax (Innovative Cell Technology, San Diego, CA, USA), and total cell number was calculated using a hemo-cytometer to obtain an average granulosa cell number per OGC.

## 2.6 | Measurement of lipid content in oocytes

After in vitro growth culture, oocytes were denuded from GCs using a narrow-pulled glass pipette, fixed in 4% paraformaldehyde, and stained with Nile Red (Wako, Osaka, Japan) following the manufacturer's protocol. After staining, fluorescence of the oocytes was examined under a fluorescence microscope (Keyence). To determine oocyte lipid content, the fluorescence intensity was measured using the ImageJ software (National Institute of Health, Bethesda, MD, USA).

## 2.7 | IVM, activation, and in vitro culture (IVC)

After in vitro growth (14 days), OGCs with an antrum cavity were used for further experiment or subsequent in vitro maturation (48 hours). Then, oocyte activation was conducted, whereby oocytes were denuded from GCs in the in vitro culture (IVC) medium droplets and treated with 10  $\mu\text{mol/L}$  ionomycin for 5 minutes followed by culture in in vitro culture (IVC) medium containing 10  $\mu\text{mol/L}$  Cytochalasin B and 10  $\mu\text{mol/L}$  cycloheximide for 5 hours. After activation, the embryos were cultured for 8 days in IVC medium, and the rate of blastulation and the total blastocyst cell numbers were determined after staining blastocysts with DAPI and by fluorescence microscopy analysis.

## 2.8 | Pimonidazole staining

The OGCs (Days 2 of culture period) were immunostained against pimonidazole. For pimonidazole staining, the Hypoxyprobe-1 kit (HPI, Burlington, MA, USA) was used according to a protocol provided by the manufacturer. OGCs were fixed with 4% paraformaldehyde in PBS for overnight by permeabilization in 0.25% Triton X-100 in PBS for 30 minutes. OGCs were blocked by incubating in PBS containing 5% BSA for 1 hour and then stained with the primary antibody for overnight following incubation with secondary antibody: anti-mouse

IgG (H + L), F(ab')<sub>2</sub> Fragment (Alexa Fluor® 555 Conjugate; Cell signaling, Danvers, MA, USA) for 1 hour. OGCs were mounted with Mounting medium (Vector Laboratories, Burlingame, CA, USA). Signals were captured under LAS AF with Leica DMI 6000B (Leica, Wetzlar, Germany).

## 2.9 | Data analysis

Among the three culture conditions, comparisons of the rate of antrum formation, granulosa cell number, survival rate of the cells, diameter of oocytes, and lipid content in cells were performed using analysis of variance followed by Tukey's post hoc test. The data related to developmental rate were compared using Chi-square test. The rates were arcsine-transformed prior to analysis. A *P* value <0.05 was considered to denote statistical significance.

## 3 | RESULTS

### 3.1 | OGCs cocultured with granulosa cells from EAFs

The volume of granulosa cells mass to be added to the OGCs was examined by measuring the diameter of the oocyte grown in vitro, because the diameter of the oocyte is a fundamental marker of oocyte growth.

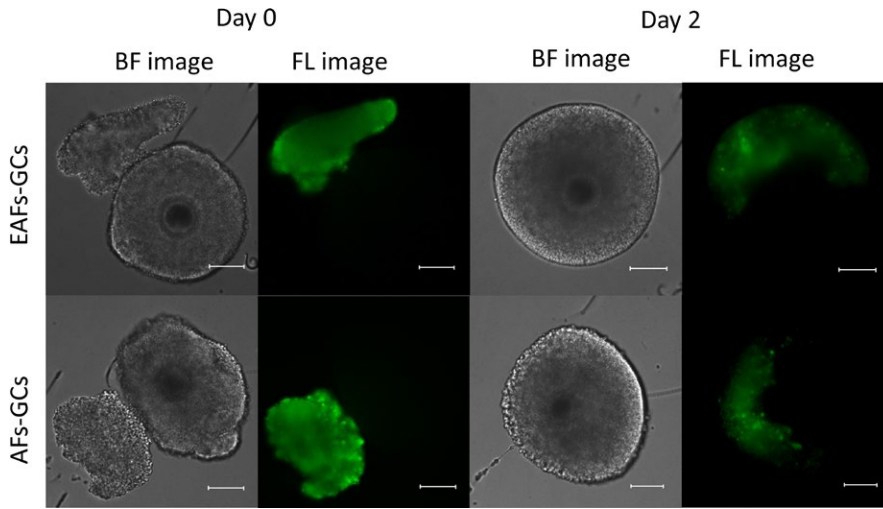
Granulosa cells were collected from EAFs and the volume of the GCs was processed into one-third (1/3GC), one-half (1/2GC), or all GCs (1/1GC), were added to the OGCs. After 16 days, oocyte diameters of 24 oocytes from each group were examined. We found that the addition of 1/2 and 1/1GC to OGCs reduced the diameter of oocytes grown in vitro in a volume-dependent manner, and there were no differences between natural and 1/3GCs (Figure 1). Therefore, we used 1/3GC in subsequent experiments.

### 3.2 | GC derived from AFs contributes to oocyte growth

When 1/3GC derived from either EAFs or AFs were added to the OGCs, the GCs and OGCs reaggregated to form “reconstructed OGCs” till day 2 of culture. To visualize the contribution of the additional GCs, GCs derived from EAFs and AFs were pre-stained with cell trackers (AAT Bioquest, Sunnyvale, CA, USA) and added to the OGCs. Two days after in vitro culture, OGCs were observed under a fluorescence microscope (Figure 2). These OGCs were morphologically similar to natural OGCs, and GCs derived from EAFs and AFs similarly contributed to the structure of new OGCs.

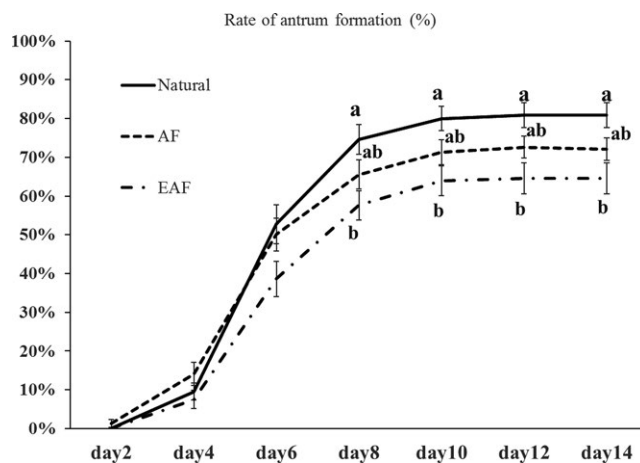
### 3.3 | Addition of GCs derived from AFs improves oocyte growth in vitro

The effect of GC addition on oocyte growth was evaluated. In the present experiment, 10 OGCs were cultured in vitro and the culture



**FIGURE 2** Representative pictures of reconstructed oocyte-granulosa cell complexes (OGCs). GCs derived from either antral follicles (AFs-GCs) or early antral follicles (EAFs-GCs) were pre-stained with cell tracker, and then co-incubated with OGCs. At 2 d of culture, GCs and OGCs were reconstructed to form new OGCs. The OGCs were observed under a fluorescence microscope. Bars represent 100  $\mu$ m

was repeated 23 times. During in vitro culture, the rate of antrum formation was comparable between “AF-GCs add OGCs” and “natural OGCs”, but these rates were significantly higher than that of “EAF-GCs add OGCs” (Figure 3). For the first four of 23 trials, the number and survival rate of the GCs in the OGCs were examined. The number of GCs was comparable among the three OGC groups, whereas survival rate of the GCs was the highest for “AF-GCs add OGCs” (Table 1). In addition, the diameter of the oocytes grown in “AF-GCs add OGCs” was significantly greater than that of oocytes developed in “EAFs-GCs add OGCs” and “natural OGCs” (Table 1). As lipid content in oocytes is a biomarker of oocyte ability,<sup>13,14</sup> we examined the lipid content in the oocytes, and we found significantly higher lipid content in oocytes grown in both “EAF-GCs add OGCs” and “AF-GCs add OGCs” compared with the natural counterparts (Figure 4).



**FIGURE 3** Antrum formation of oocyte-granulosa cell complexes (OGCs) during 14 d of culture. GCs derived from either early antral follicles (EAFs) or antral follicles (AFs) were co-incubated with an OGCs. Ten OGCs were culture for 14 d, and the experiment was repeated 17 times. Antrum formation rate was determined at every 2 d. a-b;  $P < 0.05$

In the next six trials, the oocytes developed in the three OGCs conditions were subjected to assay for in vitro maturation. Maturation rate were comparative ( $P > 0.71$ ) among the experimental groups (AF-GCs add OGCs,  $51.9 \pm 3.2$ , EAF-GCs add OGCs,  $53.2 \pm 5.8$  and natural OGCs,  $58.0 \pm 11.2$ ). In the next 13 trials, the oocytes developed in the three OGCs conditions were subjected to parthenogenetic activation to determine their developmental ability to the blastocyst stage. The rate of blastulation was 7.0%, 5.6%, and 3.5% for “AF-GCs add OGCs,” “EAF-GCs add OGCs,” and “natural OGCs,” respectively, while there was no significant difference among them (Table 2).

### 3.4 | Addition of GC induces hypoxia in the OGCs

Hypoxia-induced increase in *VEGFA* and *HIF1* occurs upstream of glycolysis activation in GCs,<sup>15</sup> and we previously found a metabolic shift in the GCs to glycolysis and activation of *HIF1a* and *VEGFA* in the GCs along with follicle development.<sup>10</sup> We therefore examined whether the addition of GCs induced hypoxic conditions in the OGCs, using pimonidazole staining, which reflects the hypoxic conditions of cell. We found that the addition of AF-GCs to OGCs significantly induced hypoxia in OGCs (Figure 5).

## 4 | DISCUSSION

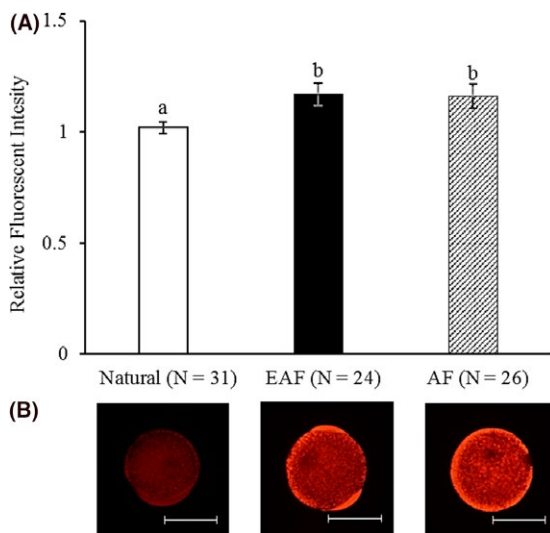
The present study shows that the addition of GCs collected from more advanced follicle stages to OGCs culture conditions supports oocyte growth. We performed in vitro oocyte growth in 200  $\mu$ L of culture medium in 96-well plates, and as it is generally accepted that an optimal concentration of cells should be present for each culture conditions. Based on the diameter of oocytes, we concluded that GC volumes greater than a certain threshold (1/3) adversely affected oocyte growth. Considering these results, we added one-third volume of GCs to OGCs. When coculturing the GCs and OGCs, GCs were incorporated into the OGCs within 2 days and new OGCs were

**TABLE 1** Effect of granulosa cells (GCs) addition to the oocyte-granulosa cell complexes (OGCs) on number and survival rate of the GCs and diameter of the oocytes grown in vitro

GCs origins	No. of OGCs	No. of replicates	No. of oocytes	Oocyte diameter ( $\mu\text{m}$ )	Granulosa cells	
					Number	Survival rate (%)
Natural	40	4	35	$116.6 \pm 1.2^\dagger$	$140\,571 \pm 6618.8$	$92.1 \pm 0.9^\dagger$
Early antral follicles	40	4	24	$116.2 \pm 1.6^\dagger$	$124\,963 \pm 7400.1$	$95.4 \pm 0.5^\ddagger$
Antral follicles	40	4	30	$121.7 \pm 1.0^\ddagger$	$116\,867 \pm 9179.5$	$97.2 \pm 0.4^\S$

Data are presented as mean  $\pm$  SEM.

P value for all " $\dagger$ ,  $\ddagger$ ,  $\S$ " is  $<0.05$ .

**FIGURE 4** Lipid content in oocytes grown in vitro. Oocyte-granulosa cell complexes (OGCs) were cultured with GCs derived from either early antral follicles (EAFs) or antral follicles (AFs) for 14 d. Oocytes grown in vitro were stained by Nile red, and fluorescent intensity was observed under a fluorescent microscope. A, Relative lipid content in oocytes (The level of "Natural" was defined as 1.0, a-b,  $P < 0.05$ ). B, representative pictures of oocytes grown in natural, EAF-GCs, and AF-GCs-added OGCs (Bar = 100  $\mu\text{m}$ )

reconstructed. We also found that the contribution of the added-GCs was morphologically similar between GCs derived from EAFs and AFs (Figure 1).

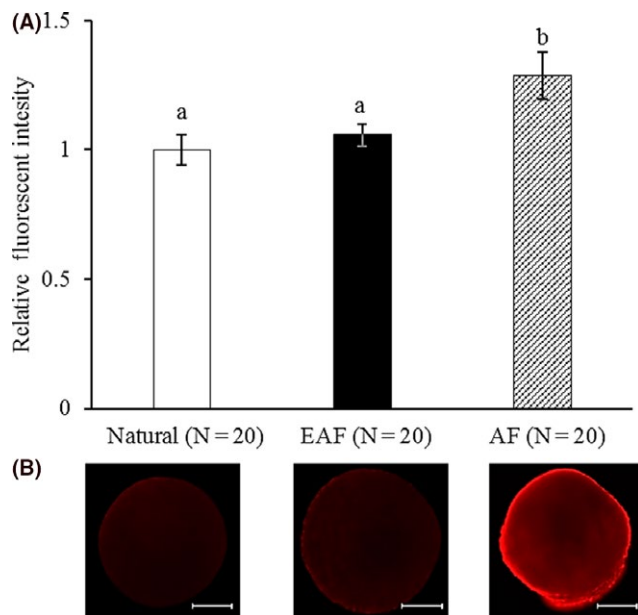
Granulosa cells surrounding the oocytes change their properties along with follicle growth, and GCs of advanced follicle

have differential gene expression profiles.<sup>10</sup> It is conceivable that proper changes in the characteristics of GCs are needed for both in vivo and in vitro oocyte growth. Interestingly, GCs collected from advanced stage follicles had higher support for oocyte growth with significantly greater diameter of oocytes grown in vitro, and oocytes tended to have high developmental ability toward the blastocyst stage compared with those developed in "EAF-GCs add OGCs" and "natural OGCs." In addition, GCs numbers did not differ among the three OGCs conditions. These results indicate that properties of the GCs may differ between EAFs and AFs. Our comprehensive gene expression analysis of GCs collected from porcine EAFs and AFs using next-generation sequencing technology (registered as DDJB: DRA004449) revealed upregulation of *HIF1A* and genes associated with glycolysis,<sup>10</sup> which is a prominent difference between the two follicular stages. Glycolytic activity and *HIF1A* expression in GCs were reported to be enhanced by hypoxic conditions.<sup>15</sup> Consistent with this notion, in this study, we found that the addition of AF-GCs to the OGCs induced a more hypoxic condition for the OGCs (Figure 5). In line with this, Hirao et al showed that low oxygen tension at the first 4 days of culture period enhanced in vitro bovine oocyte growth.<sup>16</sup> From these results, we suggested that the addition of GCs to OGCs at the start of in vitro culture provided a more hypoxic environment to the OGCs compared with those natural OGCs, and the priming effect of GC addition was greater for more advanced stage follicles. Another major impact of GCs addition to oocyte was the high lipid content in the oocytes. Lipid content is a simple but important oocyte marker associated with high developmental abilities.<sup>13,14</sup> Many factors have been reported to affect lipid content in oocytes; for example, higher lipid content in culture medium increased lipid content in oocyte and GCs.<sup>17</sup> In addition,

**TABLE 2** Effect of granulosa cells (GCs) addition to the oocyte-granulosa cell complexes (OGCs) on developmental ability of the oocytes grown in vitro

GCs origins	No. of OGCs	No. of replicates	No. of oocytes	No. of blastocysts (%)	Total cell number
Natural	110	11	85	3/85 (3.5)	$36.0 \pm 7.1$
Early antral follicles	110	11	72	4/72 (5.6)	$46.0 \pm 9.8$
Antral follicles	110	11	71	5/71 (7.0)	$43.0 \pm 9.0$

Total cell numbers are presented as mean  $\pm$  SEM.



**FIGURE 5** Levels of hypoxia in Natural, early antral follicles-granulosa cells (EAFs-GCs) and antral follicles (AFs)-GCs add Oocyte-GC (OGCs). Hypoxic condition was evaluated using pimonidazole staining. A, Levels of fluorescence intensity of pimonidazole staining (average level of “Natural” OGCs was defined as 1.0, a-b,  $P < 0.05$ ). B, Representative images of OGCs stained with pimonidazole

a greater number of GCs increased lipid content of oocytes grown in vitro,<sup>4,18</sup> suggesting that high energy conditions are associated with lipid accumulation in oocytes. In the present study, we did not find any difference in GC numbers between GC-added OGCs and natural OGCs. We, therefore, speculated that GCs derived from more advanced stage follicles induce more metabolic changes in oocytes and/or GCs resulting in oocyte growth and lipid accumulation; however, this point should be further studied in the future.

In conclusion, GCs derived from AFs contribute to the development of OGCs derived from EAFs and improve oocyte growth in vitro.

## ACKNOWLEDGEMENTS

This study was supported by a Grant-in-Aid for Scientific Research C (KAKENHI, grant number: 16K07996) and a Grant-in-Aid for JSPS Fellows (KAKENHI, grant No. JP25450400) from the Japan Society for the Promotion of Science and the Science Research Promotion Fund from the Promotion and Mutual Aid Corporation for Private Schools of Japan.

## DISCLOSURES

**Conflict of interest:** All authors declare no conflict of interest. **Human rights statements and informed consent:** This article does not contain any studies with human subjects. **Animal study:** In this study, porcine

ovaries were collected from a slaughterhouse. The ovaries were discarded without any use for edible meat, and thus, this study was approved by the Ethical Committee for Animal Experiment of Tokyo University of Agriculture.

## ORCID

Hisataka Iwata  <http://orcid.org/0000-0001-7238-8997>

## REFERENCES

- Hennet ML, Combelles CM. The antral follicle: a microenvironment for oocyte differentiation. *Int J Dev Biol.* 2012;56:819-831.
- Orisaka M, Tajima K, Tsang BK, Kotsuji F. Oocyte-granulosa-theca cell interactions during preantral follicular development. *J Ovarian Res.* 2009;2:9.
- Binelli M, Murphy BD. Coordinated regulation of follicle development by germ and somatic cells. *Reprod Fertil Dev.* 2010;22:1-12.
- Munakata Y, Ichinose T, Ogawa K, et al. Relationship between the number of cells surrounding oocytes and energy states of oocytes. *Theriogenology.* 2016;86:1789-1798.
- Landry DA, Fortin C, Bellefleur AM, et al. Comparative analysis of granulosa cell gene expression in association with oocyte competence in FSH-stimulated Holstein cows. *Reprod Fertil Dev.* 2017;29:324-2335.
- Munakata Y, Ueda M, Kawahara-Miki R, et al. Follicular factors determining granulosa cell number and developmental competence of porcine oocytes. *J Assist Reprod Genet.* 2018;12:1-11.
- Iwata H. Age-associated changes in granulosa cells and follicular fluid in cows. *J Reprod Dev.* 2017;63:339-345.
- Oi A, Tasaki H, Munakata Y, Shirasuna K, Kuwayama T, Iwata H. Effects of reaggregated granulosa cells and oocytes derived from early antral follicles on the properties of oocytes grown in vitro. *J Reprod Dev.* 2015;61:191-197.
- Sugiyama M, Sumiya M, Shirasuna K, Kuwayama T, Iwata H. Addition of granulosa cell mass to the culture medium of oocytes derived from early antral follicles increases oocyte growth, ATP content, and acetylation of H4K12. *Zygote.* 2016;24:848-856.
- Munakata Y, Kawahara-Miki R, Shiratsuki S, et al. Gene expression patterns in granulosa cells and oocytes at various stages of follicle development as well as in vitro grown oocyte-and-granulosa cell complexes. *J Reprod Dev.* 2016;62:359-366.
- Yoshida M, Ishizaki Y, Kawagishi H. Blastocyst formation by pig embryos resulting from in-vitro fertilization of oocytes matured in vitro. *J Reprod Fertil.* 1990;88:1-8.
- Yoshioka K, Suzuki C, Tanaka A, Anas IM, Iwamura S. Birth of piglets derived from porcine zygotes cultured in a chemically defined medium. *Biol Reprod.* 2002;66:112-119.
- Mohammadi-Sangcheshmeh A, Veshkini A, Hajarizadeh A et al. Association of glucose-6-phosphate dehydrogenase activity with oocyte cytoplasmic lipid content, developmental competence, and expression of candidate genes in a sheep model. *J Assist Reprod Genet.* 2014;31:1089-1098.
- Castaneda CA, Kaye P, Pantaleon M, et al. Lipid content, active mitochondria and brilliant cresyl blue staining in bovine oocytes. *Theriogenology.* 2013;79:417-422.
- Shiratsuki S, Hara T, Munakata Y, Shirasuna K, Kuwayama T, Iwata H. Low oxygen level increases proliferation and metabolic changes in bovine granulosa cells. *Mol Cell Endocrinol.* 2016;437:75-85.
- Hirao Y, Shimizu M, Iga K, Takenouchi N. Optimization of oxygen concentration for growing bovine oocytes in vitro: constant low

- and high oxygen concentrations compromise the yield of fully grown oocytes. *J Reprod Dev.* 2012;58:204-211.
17. Ogawa K, Itami N, Ueda M, et al. Non-esterified fatty acid-associated ability of follicular fluid to support porcine oocyte maturation and development. *Reprod Med Biol.* 2018;17:155-163.
  18. Munakata Y, Kawahara-Miki R, Shirasuna K, Kuwayama T, Iwata H. Polyacrylamide gel as a culture substrate improves in vitro oocyte growth from porcine early antral follicles. *Mol Reprod Dev.* 2017;84:44-54.

**How to cite this article:** Ishiguro A, Munakata Y, Shirasuna K, Kuwayama T, Iwata H. Addition of granulosa cells collected from differential follicle stages supports development of oocytes derived from porcine early antral follicles. *Reprod Med Biol.* 2019;18:65-71. <https://doi.org/10.1002/rmb2.12248>