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# Promotion of glucose utilization by insulin enhances granulosa cell proliferation and developmental competence of porcine oocyte grown *in vitro*

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## Summary

*In vitro* culture of the oocyte granulosa cell complexes (OGCs) from early antral follicles (EAFs) shows granulosa cell (GC) proliferation, but to a lesser extent than that observed *in vivo* during follicle development. As the number of GCs closely relates to energy sufficiency of the oocytes, enhancement of GC proliferation influences oocyte development. GC proliferation depends on glycolysis and insulin-mediated AKT/mTOR signaling pathway; therefore, addition of culture medium containing insulin and glucose may potentially promote GC proliferation and hence improve oocyte development. In the present study, we assessed the effect of exogenous insulin and glucose concentration on GC proliferation and oocyte energy status as well as developmental abilities of porcine oocytes grown *in vitro*. In the presence of 5.5 mM of glucose (Low), a comparison of 10 versus 20 μg/ml insulin showed that high insulin enhanced GC proliferation but exhausted glucose from the medium, which resulted in low energy status including lipid and adenosine triphosphate of the oocyte. Whereas, in the presence of 20 μg/ml insulin, medium with 11 mM glucose (High) enhanced GC proliferation and oocyte energy status as well as developmental ability up to the blastocyst stage. Considering that there was no difference in OGCs development observed with medium (10 μg/ml insulin) containing 5.5 versus 11 mM glucose, we concluded that the combination of high insulin and glucose enhanced GC proliferation and energy status of oocytes as well as the developmental ability of the oocytes grown *in vitro*.

Key words: Insulin, Glucose, Granulosa cells, Proliferation, Oocytes

## Introduction

Mammalian oocytes develop within follicles that continue to increase in size. Once the follicle reaches the antral follicle (AF) stage with a certain size, the oocytes acquire developmental competence. However, the number of follicles that successfully reach this size is limited, with almost small follicles regressing during development in the ovaries. Given appropriate culture conditions, oocytes in these small follicles can grow to full size and produce live offspring in mice and bovine (Eppig & O'Brien, 1996; Hirao *et al.*, 2004). Therefore, oocytes in small immature follicles are a useful resource for embryo production. However, currently available culture systems are inadequate and provide an extremely low success rate of growing oocyte *in vitro* (Kubo *et al.*, 2015).

The oocyte is surrounded by numerous granulosa cells (GCs) that proliferate to a great extent (Salfi *et al.*, 1979) during follicle development. The GCs support oocyte growth via complex interactions involving autocrine and paracrine factors as well as transportation of energy substrates across the gap junction (Gilula *et al.*, 1978; Brower & Schultz, 1982; Gougeon, 1996; Sutton-McDowall *et al.*, 2010). Owing to their low phosphofructokinase and hexokinase activity, oocytes are believed to directly use only a small quantity of glucose (Tsutsumi *et al.*, 1990; Cetica *et al.*, 2002) and provision of energy substrates from GCs that actively uptake glucose is important for energy status of oocytes (Biggers *et al.*, 1967;

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Sugiura & Eppig, 2005; Sutton-McDowall *et al.*, 2010). However, *in vitro* culture conditions differ from those *in vivo.* When oocyte–granulosa cell complexes (OGCs) are cultured *in vitro*, the number of GCs increases only up to one-tenth of that seen *in vivo* (Oi *et al.*, 2015). Therefore, we hypothesized that modifying the culture system to enhance GC proliferation may improve oocyte grown *in vitro.* Insulin and insulin-like growth factor-1 are known to promote GC proliferation in the follicle (Peluso *et al.*, 1991; Kadakia *et al.*, 2001). Additionally, insulin supplementation in culture media has been reported to promote GC proliferation (Savion *et al.*, 1981; Hein *et al.*, 2015). Furthermore, it has been reported that GC proliferation is regulated by PI3K/PTEN/AKT pathway, which is a downstream pathway of insulin (Goto *et al.*, 2009; Makker *et al.*, 2014). Moreover, recent RNA-sequencing of GCs showed that upregulation of HIF1α, up-stream factor of glucose metabolism and/or insulin signaling (Shiratsuki *et al.*, 2016), and glycolysis are major events in porcine follicle development (Munakata *et al.*, 2016a) and the results suggest that exogenous insulin and glucose may enhance glycolysis and induce GC proliferation and hence help in *in vitro* OGCs development.

Oocytes of cows and pigs have a large number of lipid droplets in their cytoplasm, which increases during oocyte growth (Fair *et al.*, 1997; Prates *et al.*, 2014). As lipid is an energy substrate for the oocyte during maturation and embryo development (Ferguson & Leese, 2006; Dunning *et al.*, 2010; Dunning *et al.*, 2011), the lipid content in oocytes relates to high developmental ability to form blastocysts in cows (Jeong *et al.*, 2009). In this context, we previously reported that the number of GCs surrounding the oocyte positively relates to the lipid and adenosine triphosphate (ATP) content in oocytes grown *in vitro* and *in vivo* (Munakata *et al.*, 2016b). Thus, we hypothesized that an optimal combination of exogenous insulin and glucose can improve the developmental ability of oocytes grown *in vitro* as a result of enhanced GC proliferation and oocyte energy status in terms of lipid and ATP content.

In the present study, we examined the effect of supplementing the culture medium with a combination of insulin and glucose on proliferation of GCs and oocyte development.

## Materials and methods

### Reagents and media

Unless otherwise stated, all drugs were purchased from Nacalai Tesque (Kyoto, Japan). The medium used for *in vitro* growth (IVG) of OGCs (IVG medium) was described in previous report (Itami *et al.*, 2015a). In brief, medium used for IVG was α-MEM (Sigma Aldrich, St. Louis, MO, USA), supplemented with insulin transferrin and selenium (ITS). We cultured OGCs individually using 96 well culture plate and the amount of IVG medium per 1 well was 200 µl. The combination of insulin and glucose content in the IVG medium used is presented in Table 1. As the number of OGCs collected from early antral follicles (EAFs) was insufficient to allocate all possible combinations, we used two paired combinations. The *in vitro* maturation medium (IVM medium) consisted of Medium 199 (Gibco) supplemented with 10% v/v porcine follicle fluid, 0.5 mM L-cysteine, 0.9 mM sodium pyruvate, 1 mM 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). Porcine follicle fluid was collected from antrum follicles (3–6 mm in diameter), centrifuged (10 000 *g* for 5 min), and stored at −20°C until use. Finally, porcine zygote medium 3 (PZM3, Yoshioka *et al.*, 2002) was used for *in vitro* embryo culture (IVC medium).

**Table 1** Grouping the conditions of culture medium

| | Insulin (µg/ml) | Glucose (mM) |
|---|---|---|
| Group 1 | 10 | 5.5 |
| Group 2 | 20 | 5.5 |
| Group 3 | 20 | 11 |
| Group 4 | 10 | 11 |

### Collection of OGCs, IVG, IVM, activation, and IVC

Porcine ovaries were collected from prepubertal gilts at a local slaughterhouse and were transported to the laboratory in phosphate-buffered saline (PBS) containing antibiotics at 37°C within 1 h. OGCs were collected from EAFs (500–700 µm) under a stereomicroscope using an 18-G needle connected to a syringe. The OGCs were cultured in IVG medium for 14 days. Every 4 days, half of the medium was replaced with fresh medium, at which point the OGCs were examined for antrum formation to determine antrum formation rate. At the end of the IVG period (14 days), OGCs with an antrum cavity were used for experiment or subsequent IVM culture for 44 h. Oocyte activation was conducted as previously described (Itami *et al.*, 2015b). In brief, oocytes were denuded from granulosa cells and treated with 10 µM ionomycin for 5 min followed by 6 h of incubation in IVC medium containing 10 µM cytochalasin B and 10 µM cycloheximide. After activation, parthenogenetic embryos were cultured for 7 days and the ratio

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of blastulation and total cell number of the blastocyst were determined. We stained blastocyst by DAPI and observed using fluorescence microscopy and determined total cell number. *In vitro* culture of OGCs and IVM were performed at 38.5°C in an atmosphere of 5% $CO_2$ and 95% air. *In vitro* embryo culture was performed at 38.5°C in an atmosphere of 5% $O_2$, 5% $CO_2$ and 90% $N_2$.

### Measurement of oocyte diameter and GC number of OGCs

To obtain oocyte diameter and total cell number of the OGCs, oocytes were denuded from the surrounding cells using a narrow pulled glass pipette and the GCs were dispersed in cell dispersion cocktail (Accumax, Innovation Cell Technology, Inc. San Diego, CA, USA). Total GC number was calculated using the volume of the cellular suspension and cellular concentration was determined by hematocytometer. Oocyte x and y axes diameters were measured under a digital microscope (BZ-8000; Keyence, Tokyo, Japan) and the average was defined as a diameter of the oocyte.

### Lipid staining with Nile Red

Oocytes were denuded from GCs using a narrow pulled glass pipette and fixed in 4%paraformaldehyde. Neutral lipid in oocyte cytoplasm was stained by Nile Red (Wako, Tokyo, Japan) according to the manufacturer protocol. After staining, fluorescence of the oocyte was examined under a digital fluorescence microscope (Keyence) and the fluorescence intensity was pixel transformed using ImageJ software (NIH: National Institute of Health, New York, MD, USA).

### Assessment of ATP content in oocyte

Oocytes were lysed in 50 μl distilled water and stored at −20°C until analysis. ATP content of individual oocytes was determined by measuring luminescence generated by ATP-dependent luciferin–luciferase reactions as previously described (Iwata *et al.*, 2011).

### Measurement of glucose concentration in IVG medium

The media used for glucose measurement were collected at 8 or 14 days of culture periods and stored at −20°C until analysis. Glucose concentration in IVG medium was measured using LabAssay Glucose (Mutarotase-GOD method, Wako) according to the manufacturer's protocol.

### Experimental design

*Experiment 1: Comparison of OGCs and oocyte development at high and low insulin concentrations with low glucose concentration.*

Firstly, we examined the effect of insulin in medium containing 5.5 mM glucose on development of OGCs and oocytes. Twenty OGCs were individually cultured under two conditions: medium containing 10 or 20 μg/ml insulin with 5.5 mM glucose (see Table 1, Group 1 versus Group 2). As described above, control medium was supplemented with ITS which contained 10 μg/ml insulin, thus this concentration was defined as low insulin group (Group 1). In the control medium is During IVG (day 8) and at the end of the culture period (14 days), ATP and lipid content, and oocyte diameter were measured, and the number of GCs surrounding the oocyte was determined. At day 8, all OGCs were analysed, since oocyte degeneration was not observed, whereas at the end of the culture period OGCs having antrum cavity were selected, considering that OGCs without antrum contain degenerated oocytes.

*Experiment 2: Effect of various insulin and glucose concentration on the glucose content in IVG medium*

Next, we examined glucose concentration in the medium after 8 and 14 days of OGCs incubation. OGCs were cultured in a medium containing selective combinations of insulin and glucose: Group1, 5.5 mM glucose and 10 μg/ml insulin; Group 2, 5.5 mM glucose and 20 μg/ml insulin; Group 3, 11 mM glucose and 20 μg/ml insulin. The medium was sampled on day 8 and 14, and glucose concentration of the randomly selected samples were measured.

*Experiment 3: Comparison of OGCs and oocyte development at high and low glucose concentrations with high insulin concentration*

Here, we examined the effect of glucose (5.5 or 11 mM) combined with high insulin (20 μg/ml) (See Table 1, Group 2 versus Group 3) on OGCs and oocyte development. Twenty OGCs were collected at the end of the culture period and ATP and lipid content, diameter of oocytes grown *in vitro*, and number of GCs surrounding the OGCs were determined.

*Experiment 4: Comparison of OGCs and oocyte development at high and low glucose concentrations with low insulin concentration*

Here, we examined the effect of glucose (5.5 or 11 mM) combined with 10 μg/ml insulin (See Table 1, Group 1 versus Group 4) on OGCs and oocyte development. Twenty OGCs were collected and at the end of the culture period and content of ATP and lipid, diameter

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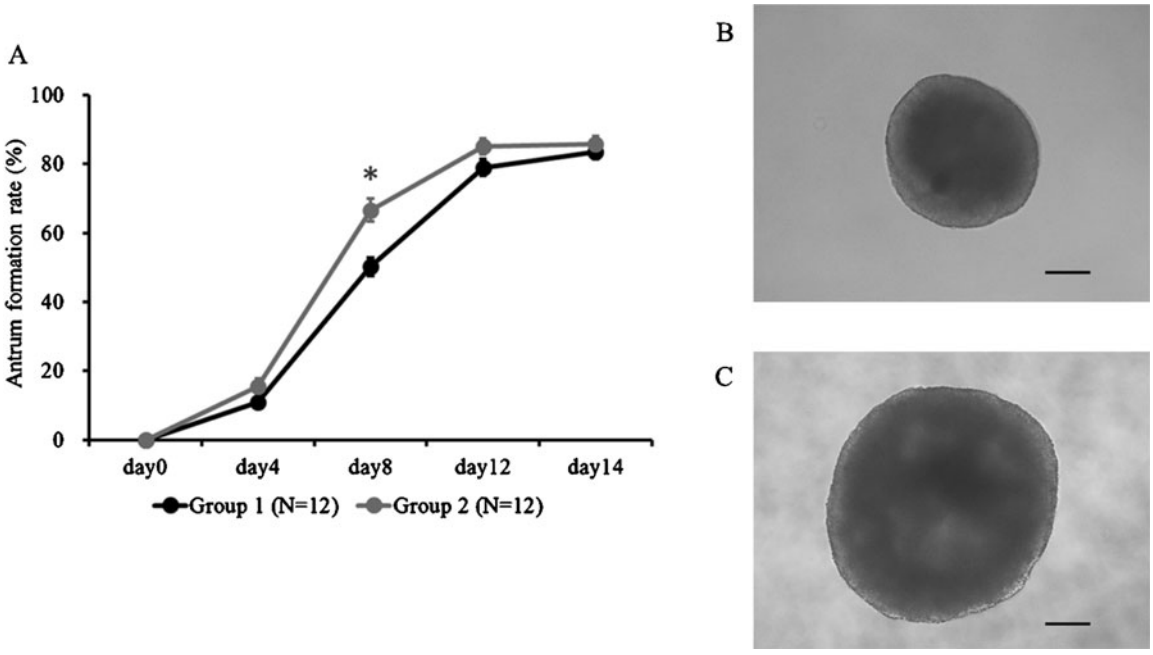


**Figure 1** Comparison of OGCs development under high and low insulin concentrations with low glucose concentration. OGCs were cultured in media containing 10 μg/ml insulin and 5.5 mM glucose (Group 1) or 20 μg/ml insulin and 5.5 mM glucose (Group 2). (*A*) Antrum formation rate of OGCs during 14 days of culture periods. (*B*, *C*) Representative images of Group 1 OGCs with antrum cavity (*B*: day 8; *C*: day 14. Black bar indicates 100 μm). Data are presented as the mean ± standard error of the mean (SEM). Statistical significance is presented as *: $P < 0.05$.

of oocytes, and number of GCs surrounding the OGCs were determined.

*Experiment 5: Comparison of lipid content in oocytes cultured in medium containing 20 μg/ml insulin and 11 mM glucose versus that containing 10 μg/ml insulin and 5.5 mM glucose*

To determine whether high insulin and glucose conditions enhanced lipid content of oocytes, we directly compared Group 1 and Group 3, based on pairwise comparison. Twenty OGCs were cultured under 2 conditions; Group1: 5.5 mM glucose and 10 μg/ml insulin, Group 3: 11 mM glucose and 20 μg/ml insulin, At the end of culture period, lipid content of oocytes was determined.

*Experiment 6: Effect of the combination of high insulin and glucose concentration on the rate of blastocyst formation after parthenogenetical activation*

Here, we cultured OGCs under three culture conditions (Groups 1, 2 and 3), and compared the developmental rate to the blastocyst stage following IVM and activation. As development of OGCs and energy status of the oocytes did not differ between Group 1 and Group 4, and considering our previous report regarding developmental rate to the blastocyst stage (Tasaki *et al.*, 2015), in this experiment, we compared Groups 1–3. Twenty OGCs were cultured under the three conditions: Group 1, 10 μg/ml insulin and 5.5 mM glucose; Group 2, 20 μg/ml insulin and 5.5 mM glucose; and Group 3, 20 μg/ml insulin and 11 mM glucose. At the end of the culture period, OGCs with an antrum cavity were subjected to IVM, while the oocytes were activated and cultured to determine their developmental ability to the blastocyst stage.

### Statistical analysis

All data obtained were analysed using Student's *t*-test. Glucose concentration in the medium and oocyte developmental rate were assessed using a one-way analysis of variance (ANOVA), followed by Tukey's post hoc test. Developmental rates were arcsine-transformed prior to the statistical analysis. Differences with *P*-values <0.05 were considered statistically significant.

## Results

### Experiment 1: In spite a temporary improvement of oocyte growth, low quality of oocytes were obtained with medium containing 5.5 mM glucose and high insulin concentration

High insulin concentration (20 μg/ml) improved antrum formation of the OGCs at day 8 of culture period (Fig. 1*A*) with greater numbers of GCs and

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**Table 2** Effect of insulin concentration on OGCs development at day 8

| Group | Oocyte diameters (μm) | Granulosa cell numbers | Lipid contents | ATP contents (pmol) |
|---|---|---|---|---|
| 1 | $129.9 \pm 0.8^{a}$ | $50222.2 \pm 2505.1^{a}$ | $1.00 \pm 0.03$ | $3.12 \pm 0.07^{a}$ |
| 2 | $133.6 \pm 1.4^{b}$ | $71547.2 \pm 3491.5^{b}$ | $0.94 \pm 0.03$ | $3.36 \pm 0.09^{b}$ |

$^{a,b}P < 0.05$. Data are shown as mean ± standard error of the mean (SEM).
OGCs numbers using each experiment between Groups 1 and 2 were described below.
Diameter: 46 versus 53. Cell number: 45 versus 53. Lipid: 46 versus 53. ATP: 58 versus 56.

**Table 3** Effect of insulin concentration on OGCs development at day 14

| Group | Oocyte diameters (μm) | Granulosa cell numbers | Lipid contents | ATP contents (pmol) |
|---|---|---|---|---|
| 1 | $140.7 \pm 0.9$ | $143518.5 \pm 6055.6^{a}$ | $1.00 \pm 0.02^{a}$ | $3.43 \pm 0.12^{a}$ |
| 2 | $140.4 \pm 1.1$ | $183000.0 \pm 5862.2^{b}$ | $0.86 \pm 0.03^{b}$ | $3.09 \pm 0.11^{b}$ |

$^{a,b}P < 0.05$. Data are shown as mean ± standard error of the mean (SEM).
OGCs numbers using each experiment between Groups 1 and 2 were described below.
Diameter: 54 versus 55. Cell number: 54 versus 60. Lipid: 38 versus 34. ATP: 61 versus 60.

higher ATP content in the oocytes compared with the results obtained with lower insulin concentration (10 μg/ml), (Table 2). At the end of the culture period (day 14), high insulin concentration caused an increase in GC number; however, lipid and ATP content in the oocytes was significantly low compared with that in oocytes cultured with low insulin concentration (Table 3).

### Experiment 2: Combination of high insulin and 5.5 mM glucose resulted in exhaustion of glucose in the medium

At day 14, the combination of 20 μg/ml insulin and 5.5 mM glucose (Group 2) reduced glucose concentration in IVG medium to 1.2 mM which was significantly different from that observed for Group 1 (10 μg/ml insulin and 5.5 mM glucose). Conversely, the combination of 20 μg/ml insulin and 11 mM glucose (Group 3) maintained glucose concentration at 5.6 mM which was significantly higher than that observed for the other groups (Group 1 and 2), (Fig. 2).

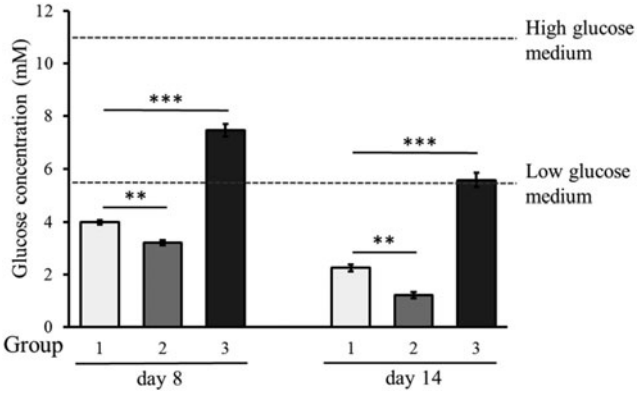


**Figure 2** Effect of various insulin and glucose concentrations on glucose content in the medium. OGCs were cultured in media containing selective combinations of insulin and glucose: Group 1, 10 μg/ml insulin and 5.5 mM glucose; Group 2, 20 μg/ml insulin and 5.5 mM glucose; Group 3, 20 μg/ml insulin and 11 mM glucose. Two dotted lines indicate the concentration of glucose: low, 5.5 mM and high, 11 mM. Data are presented as the mean ± standard error of the mean (SEM). Statistical significance is presented as **: $P < 0.01$ and ***: $P < 0.001$.

### Experiment 3: High concentration of glucose improved quality of oocytes cultured in medium containing high concentration of insulin

Adding high glucose concentration to the medium with high insulin concentration (Group 3) increased lipid and ATP content in the oocytes as well as GC number in the OGCs compared with the results observed for OGCs cultured with high insulin and low glucose concentrations (Group 2), (Table 4). High insulin condition enhance antrum formation of the OGCs at day 4 of culture period but identical at the other culture period (Fig. 3).

### Experiment 4: High concentration of glucose did not affect development of OGCs cultured in medium containing 10 μg/ml insulin

No difference was observed in either lipid and ATP content in oocytes or GC numbers of OGCs between the two culture conditions (Groups 1 and 4),

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**Table 4** Effect of glucose concentration in high insulin medium on OGCs development at day 14

| Group | Oocyte diameters (μm) | Granulosa cell numbers | Lipid contents | ATP contents (pmol) |
|---|---|---|---|---|
| 2 | 139.4 ± 0.9 | 162356.2 ± 6765.3[a] | 1.00 ± 0.03[a] | 2.92 ± 0.14[a] |
| 3 | 140.2 ± 0.8 | 191138.9 ± 5808.3[b] | 1.20 ± 0.04[b] | 3.53 ± 0.15[b] |

[a,b] $P < 0.05$. Data are shown as mean ± standard error of the mean (SEM).
OGCs numbers using each experiment between Group 2 and 3 were described below.
Diameter: 73 versus 72. Cell number: 73 versus 72. Lipid: 51 versus 50. ATP: 45 versus 50.

**Table 5** Effect of glucose concentration alone on OGCs development at day 14

| Group | Oocyte diameters (μm) | Granulosa cell numbers | Lipid contents | ATP contents (pmol) |
|---|---|---|---|---|
| 1 | 140.17 ± 0.90 | 165315.07 ± 5947.03 | 1.00 ± 0.03 | 3.33 ± 0.11 |
| 4 | 142.45 ± 0.86 | 164657.14 ± 5838.36 | 1.05 ± 0.03 | 3.52 ± 0.14 |

Data are shown as mean ± standard error of the mean (SEM).
OGCs numbers using each experiment between Groups 1 and 4 were described below.
Diameter: 71 versus 69. Cell number: 73 versus 70. Lipid: 51 versus 44. ATP: 54 versus 65.

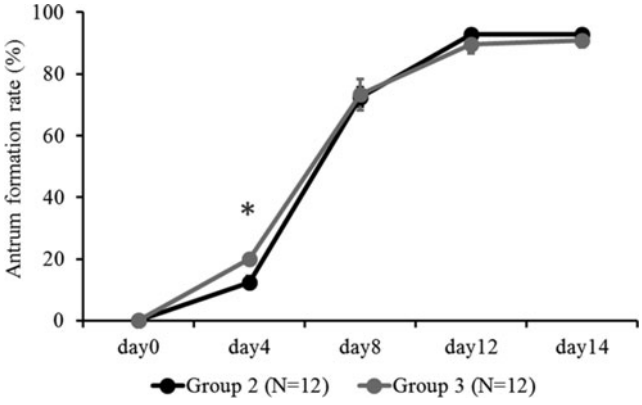


**Figure 3** Comparison of OGCs development at high or low glucose concentrations with high insulin concentration. OGCs were cultured in media containing 5.5 mM glucose and 20 μg/ml insulin (Group 2) or 11 mM glucose and 20 μg/ml insulin (Group 3). Antrum formation rate of OGCs during 14 days of culture periods. Data are presented as the mean ± standard error of the mean (SEM). Statistical significance is presented as *: $P < 0.05$.

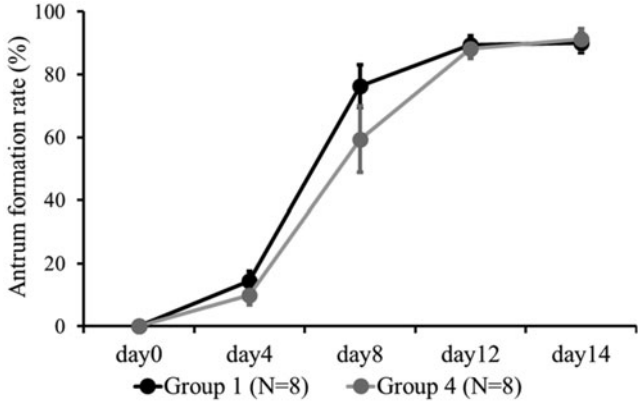


**Figure 4** Comparison of OGCs development at high or low glucose concentrations with low insulin concentration, OGCs were cultured in media containing 5.5 mM glucose and 10 μg/ml insulin (Group 1) or 11 mM glucose and 10 μg/ml insulin (Group 4). Antrum formation rate of OGCs during 14 days of culture periods. Data are presented as the mean ± standard error of the mean (SEM).

(Table 5). Additionally antrum formation was also similar between the two culture conditions (Fig. 4).

### Experiment 5: High concentration of insulin and glucose increased lipid content in oocytes

The medium containing 20 μg/ml insulin and 11 mM glucose (Group 3) showed an increase in lipid content in oocytes compared with that for Group 1 (10 μg/ml insulin and 5.5 mM glucose), (Fig. 5). The lipid content in oocytes was the highest for Group 3.

### Experiment 6: Medium containing high concentrations of insulin and glucose showed improved oocyte developmental competence after activation

Medium containing 20 μg/ml insulin and 11 mM glucose (Group 3) showed the highest developmental rate to the blastocyst stage, whereas the medium containing 20 μg/ml insulin and 5.5 mM glucose (Group 2) showed the lowest developmental rate (Table 6).

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**Table 6** Effect of combinations of insulin and glucose of developmental competence of oocyte grown *in vitro*

| Group | No. of replicates | No. of OGCs | No. of oocytes | No. of blastocysts | Rate of blastulation (/OGCs) | Rate of blastulation (/oocytes) | Total cell number |
|---|---|---|---|---|---|---|---|
| 1 | 4 | 60 | 43 | 4 | $6.7 \pm 4.7^{a,b}$ | $8.5 \pm 5.2^{a}$ | $38.6 \pm 6.7$ |
| 2 | 4 | 60 | 41 | 1 | $1.7 \pm 1.7^{a}$ | $3.1 \pm 3.1^{a}$ | 23 |
| 3 | 4 | 60 | 40 | 12 | $20.0 \pm 4.7^{b}$ | $29.2 \pm 5.9^{b}$ | $39.9 \pm 5.3$ |

$^{a,b}P < 0.05$. Data are shown as mean ± standard error of the mean (SEM).

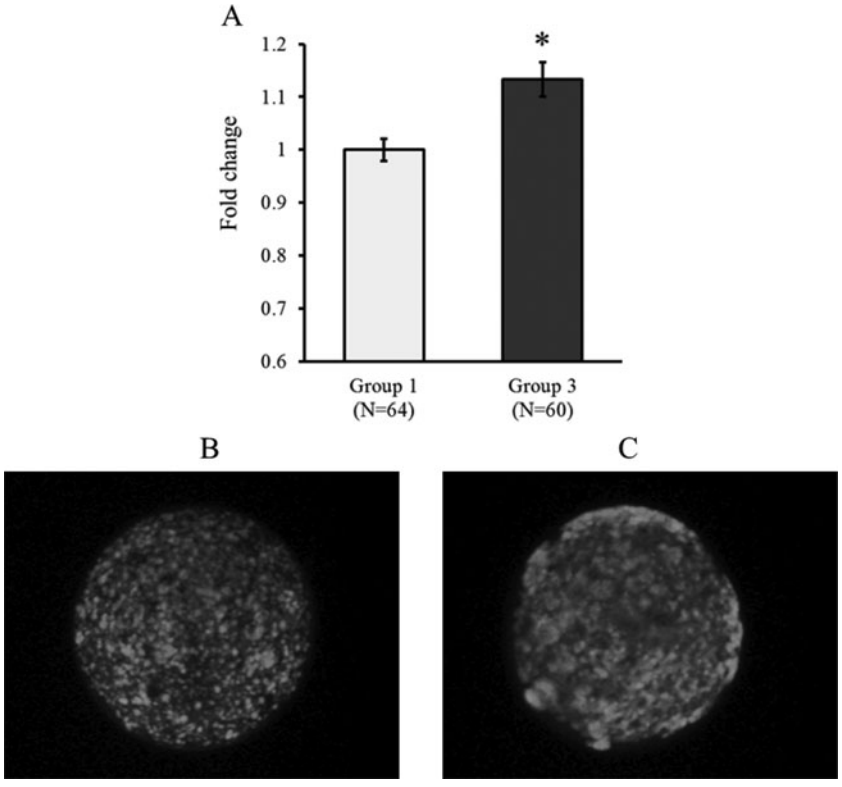


**Figure 5** Comparison of lipid content in oocytes cultured in medium containing 11 mM glucose and 20 μg/ml insulin versus that containing 5.5 mM glucose and 10 μg/ml insulin. (*A*) Comparison of lipid content in oocytes from OGCs cultured in medium containing 5.5 mM glucose and 10 μg/ml insulin (Group 1) versus that containing 11 mM glucose and 20 μg/ml insulin (Group 3). (*B*, *C*) Representative images of oocytes stained by Nile Red. [(*B*) Group 1, (*C*) Group 3)]. Data are presented as the mean ± standard error of the mean (SEM). Statistical significance is presented as $^{*}P < 0.05$.

## Discussion

The results of this study showed that insulin enhances proliferation of GCs of OGCs derived from EAFs, but does not affect developmental ability of oocytes, unless supplemented with additional glucose (11 mM). Additionally, supplementation with both insulin and glucose increases GC number as well as ATP and lipid content in the oocytes and enhances developmental ability of oocytes to the blastocyst stage.

The low quality of *in vitro*-grown oocytes is due to improper culture milieu and extremely low number of GCs surrounding the oocytes under *in vitro* culture conditions (Oi *et al.*, 2015). Recent comprehensive gene expression analysis has shown that follicle development associates with upregulation of HIF1A, vascular endothelial growth factor (VEGF), and glycolytic activity (Munakata *et al.*, 2016a). In line with these findings, it has been reported that GC proliferation depends on VEGF-mediated activation of PI3K/AKT pathway (Irusta *et al.*, 2010). Furthermore, considering the reports, activation of AKT/mTOR pathway via certain cytokines is a possible means to enhance GC proliferation of OGCs and hence improve oocyte development *in vitro*. In line with this, Serafim *et al.* (2013) reported that FSH, insulin enhanced *in vitro* follicular growth and survivability. Therefore, we examined the effect of supplementing

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the culture medium with insulin on OGC development in the present study. In experiment 1, addition of 20 μg/ml insulin increased GC proliferation and ATP content in oocytes at day 8 of culture period, however ATP and lipid content in the oocytes decreased significantly at the end of culture period even though the number of GCs was still significantly greater than that cultured in medium containing with low insulin. Insulin stimulates AKT/mTOR pathway and enhances cellular proliferation (Kido *et al.*, 2001), while activation of mTOR signaling induces follicular development and increases ovary volume *in vitro* (Cheng *et al.*, 2015). Likewise, inhibition of AKT activity increases apoptosis rate in rat GCs (Choi *et al.*, 2013). Furthermore, insulin enhances glucose uptake by human GCs (Purcell *et al.*, 2012). Therefore, we speculated that insulin may upregulate GCs proliferation and glucose utilization, which would exhaust glucose content in the culture medium, which in turn would result in low ATP and lipid content in the oocytes. As expected, we found significantly low glucose concentration in the group where the medium contained high insulin concentration (Group 2) compared with that containing low insulin concentration (Group 1). Furthermore, additional glucose supplementation of the medium containing high insulin concentration allowed for the maintenance of high glucose concentration up to 14 days of culture period (Group 3, Fig. 2). Further, we examined the effect of the combination of high insulin (20 μg/ml) and high glucose (11 mM) concentrations on OGCs and oocyte development, and found significantly higher GC number as well as ATP and lipid content in oocytes. Thus indicates that additional glucose ameliorates starvation of oocytes and increases provision of energy substrate to the oocytes. In addition, we previously reported (Tasaki *et al.*, 2015) and confirmed in this study (Experiment 4) that oocyte developmental ability as well as ATP and lipid content in oocytes did not change when OGCs were cultured in a medium supplemented with glucose alone (Group 4). To the best of our knowledge, this is the first report demonstrating that modification of culture conditions in terms of both insulin and glucose concentration increases lipid and ATP content of oocytes. In this study, oocytes grown in the medium containing both high insulin and glucose concentrations were found to have high developmental ability (Table 6). This is consistent with previous reports where oocytes with high lipid and ATP content have been shown to exhibit high developmental ability (Jeong *et al.*, 2009; Sato *et al.*, 2014). In this context, presence of sufficient glucose is required for the insulin-mediated enhanced viability of cancer cells, which depends on glycolysis (Zhang *et al.*, 2016). Thus, insulin-induced proliferation of GCs requires sufficient glucose in the culture milieu.

Lipids are known to accumulate into lipid droplets during oocyte growth; however, the factors affecting this accumulation have not yet been clarified. Recently, Munakata *et al.* (2016b) have shown that the number of GCs closely relates to the lipid and ATP content in oocyte cultured under both *in vivo* and *in vitro* conditions. Notably, insulin potentially affects both oocytes and GCs owing to the presence of receptors on both cells (Acevedo *et al.*, 2007). Although the molecular background underlying lipid accumulation in oocytes is still unclear, insulin-induced proliferation of GCs is a causal factor for greater lipid accumulation in oocytes grown *in vitro*.

In conclusion, supplementation of the culture medium with both high insulin and glucose concentrations enhances GC proliferation and oocyte energy status, which in turn, improves the developmental ability of oocytes.

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## Statement of interest

The authors have no conflicts of interest to declare.

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