Effect of the factor inhibiting germinal vesicle breakdown on the disruption of gap junctions and cumulus expansion of pig cumulus–oocyte complexes cultured *in vitro*

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The present study was undertaken to explore the regulatory mechanisms for meiotic resumption of pig cumulus–oocyte complexes (COCs) by assessing the nuclear status of oocytes, the degree of gap junction cell-to-cell communication and cumulus expansion after culture of various numbers of COCs in 10 μ l droplets of medium for 24 h. Gap junction communication was examined by confocal laser scanning microscopy after injection of a fluorescent dye, lucifer yellow, into the oocytes. When one, three or six COCs were cultured in a 10 μ l droplet, germinal vesicle breakdown was observed in >70% of oocytes; increasing the number of COCs in a droplet further actually decreased the proportion of oocytes undergoing germinal vesicle breakdown (10 COCs: 49%; 20 COCs: 21%; 40 COCs: 13%). When six COCs were cultured in a

10 μ l droplet of conditioned medium (prepared previously by culturing 20 intact and oocyctomized COCs for 24 h), the proportion of oocytes undergoing germinal vesicle breakdown was significantly reduced compared with the proportion in fresh medium. An increase in the proportion of category 1 COCs (all gap junctions within cumulus cells and between cumulus cells and oocyte are functionally maintained) was achieved by increasing the number of COCs cultured in a 10 μ l droplet. The addition of conditioned medium to the fresh medium at a concentration of 50% significantly inhibited cumulus expansion. From these results, it is concluded that the factors secreted by cumulus cells regulate the disruption of gap junctions and cumulus expansion, and concurrently control the incidence of germinal vesicle breakdown in pig COCs.

Introduction

According to Eppig (1982), maturation of mouse oocytes has already started 3 h after hCG injection, whereas the gap junctions between oocytes and cumulus cells are not disrupted in the first 6 h after hCG injection. Culture of mouse cumulus–oocyte complexes (COCs) for 3 h in the presence of FSH initiates the resumption of meiotic maturation despite intact metabolic coupling between cumulus cells and oocytes (Salustri and Siracusa, 1983). Larsen *et al.* (1986, 1987) observed a close temporal correlation between the loss of cumulus cell–cumulus cell gap junctions and resumption of meiotic maturation in superovulated rats. Isobe *et al.* (1998) found that, in pigs, the proportions of oocytes undergoing germinal vesicle breakdown (GVBD) at 16, 24 and 32 h of cultivation were much higher than the proportion in COCs in which all gap junctions within cumulus cells and between cumulus cells and oocytes were disrupted. Furthermore, a significant positive correlation ($r = 0.99$, $P < 0.01$) was observed between the propor-

tion of oocytes undergoing GVBD and the proportion of COCs that had lost gap junctions, at least within all cumulus cell layers other than the innermost layer. From these results, it was concluded that meiotic resumption in pig oocytes is induced by disruption of gap junctions within cumulus cells (Isobe *et al.*, 1998).

Petr *et al.* (1989) reported that when 40 pig COCs were cultured in a 10 μ l droplet of medium for 24 h, GVBD in the oocytes was suppressed, and a significantly higher proportion of oocytes undergoing GVBD was observed in denuded oocytes compared with COCs when ten denuded oocytes were cultured with 40 COCs in a 10 μ l droplet. Increasing the number of bovine COCs to 40 per 10 μ l medium also resulted in a significant inhibition of meiotic resumption compared with controls containing 10 COCs per 50 μ l medium (Sirard *et al.*, 1992). From these results, these authors proposed that the factor inhibiting GVBD is generated in the cumulus cells and secreted into culture medium and might cause suppression of GVBD through the mediation of cumulus cells.

On the basis of the study by Isobe *et al.* (1998) and the reports showing that there is a GVBD inhibitor that is transferred directly from cumulus cells to oocytes via gap junctions (Eppig, 1982; Larsen *et al.*, 1987; Isobe *et al.*,

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1998), it is possible that intact gap junction communication allows direct transfer of the GVBD inhibitor from cumulus cells to oocytes, resulting in inhibition of GVBD. However, the mechanism by which the factor of cumulus cell origin maintains the gap junction communication between cumulus cells surrounding oocytes is still not understood completely.

The aim of the present study was to explore the regulatory mechanism for meiotic resumption of pig COCs further by investigating the relationship between the nuclear status of oocytes and the degree of gap junction cell-to-cell interaction when various numbers of COCs were cultured in 10 μ l medium for 24 h.

Materials and Methods

Oocyte collection

Ovaries were collected from prepubertal gilts at a local abattoir and were transported to the laboratory in 0.85% (w/v) NaCl with 0.1 mg kanamycin ml^{-1} (Meiji Seika, Tokyo) at about 35°C within 2 h. The surfaces of follicles ranging from 3 to 8 mm in diameter were cut with a razor and oocytes were collected by scraping the inner surface of the follicle wall using a surgical blade. The oocytes were placed in prewarmed PBS supplemented with 5% (v/v) bovine serum (Nacalai Tesque, Kyoto). Oocytes with a complete and compact cumulus mass were selected and washed several times in medium (TCM-199 with Earle's salts (Gibco BRL, Grand Island, NY) supplemented with 10 iu hCG ml^{-1} (Teikokuzouki, Tokyo), 10 iu pregnant mare serum gonadotrophin ml^{-1} (Teikokuzouki), 1 μ g oestradiol ml^{-1} (Sigma Chemical Co, St Louis, MO), 50 μ g gentamicin sulphate ml^{-1} (Sigma) and 10% (v/v) fetal calf serum (Gibco)).

Effect of numbers of COCs cultured in a 10 μ l droplet on GVBD and the degree of gap junction disruption

Groups of one, three, six, ten, 20 and 40 COCs were transferred to 10 μ l droplets of culture medium covered with warm mineral oil (E. R. Squibb and Sons Inc., Princeton, NJ) in 35 mm polystyrene dishes and cultured for 24 h at 39°C in a humidified atmosphere of 5% CO_2 in air.

At the end of cultivation, some COCs were denuded by vortexing for 1.5 min in PBS supplemented with 5% bovine serum and the remaining cumulus cells attached to oocytes were removed mechanically by repeated pipetting with a small-bore pipette. The oocytes were fixed for > 48 h in acetic acid:ethanol (1:3; v/v) at room temperature and stained with 1% (w/v) lacmoid in 45% (v/v) acetic acid for

examination by phase-contrast microscopy. The oocytes were classified according to the method of Hunter and Polge (1966) as having an intact germinal vesicle or having undergone GVBD. Nuclear status of germinal vesicle arrested oocytes was further divided into GV I, GV II, GV III or GV IV according to Motlik and Fulka (1976).

The remaining COCs were used for assessment of gap junction status by the injection of fluorescent dye. The dye transfer experiment was conducted as described by Isobe *et al.* (1998). In brief, oocytes surrounded by cumulus cells were injected with about 4 μ l of 10% (w/v) lucifer yellow CH (Sigma) on a heated stage (37°C) of an inverted microscope at $\times 100$ magnification using a Hoffman Modulation contrast system (Modulation Optics Inc, Greenvale, NY). The spread of the dye from the oocyte into surrounding cumulus cells was monitored using confocal laser scanning microscopy (ACAS 570; Meridian Instruments Inc, Okemos, MI) with a $\times 100$ oil objective within 10–15 min after injection.

The COCs were classified into four categories according to the degree of dye transferred into the cumulus cells: category 1: the dye was transferred to all layers of cumulus cells surrounding the oocyte (Fig. 1a,b); category 2: the dye was transferred to more than two inner layers of cumulus cells but not to all layers of the cumulus cells (Fig. 1c,d); category 3: the dye was transferred to the innermost layer of cumulus cells only (Fig. 1e,f); and category 4: the dye was not transferred to cumulus cells (Fig. 1g,h).

Effect of the factor secreted by cumulus cells on expansion of cumulus cells

After 20 COCs were cultured in each 10 μ l droplet of medium for 24 h, the medium was collected in an Eppendorf tube and stored frozen at -30°C as conditioned medium until used. Six COCs were cultured in 10 μ l droplets of 0% (fresh medium), 50% (5 μ l fresh medium and 5 μ l conditioned medium) or 100% conditioned medium for 24 h. At the end of cultivation, the cumulus cell expansion was scored on the subjective scales of $\pm$ (no observable expansion; Fig. 2a), + (enlargement of cumulus mass but no cumulus cells separated; Fig. 2b) and ++ (some cumulus cells separated; Fig. 2c). Cumulus cells of ++ expansion were a little sticky.

Effect of conditioned medium prepared with oocyctomized COCs on GVBD of oocytes

Oocyctomy was conducted as described by Buccione *et al.* (1990). Each COC was held with the holding pipette using negative pressure. A lancing pipette, fashioned in the manner of a standard microinjection pipette, was pushed

Fig. 1. Light micrographs (a, c, e and g) and confocal laser scanning microscopic images (b, d, f and h) of various categories of lucifer yellow transfer in pig cumulus–oocyte complexes. (a,b) Category 1: dye transferred to all layers of cumulus cells surrounding oocytes. (c,d) Category 2: dye transferred to more than two inner layers of cumulus cells but not all layers of the cumulus cells. (e,f) Category 3: dye transferred to only the innermost layer of cumulus cells. (g,h) Category 4: dye not transferred to cumulus cells. Scale bars represent 50 μ m.

through the layers of cumulus cells and completely through the oocyte and into the holding pipette. After withdrawal of the lancing pipette, negative pressure in the holding pipette aspirated most or all of the oocyte. The complexes deprived of the ooplasm will hereafter be called oocyctectomized COCs. Twenty oocyctectomized COCs were cultured in a 10 μ l droplet of medium for 24 h and the medium was defined as the oocyctectomized COC-conditioned medium. Control conditioned medium was prepared concomitantly using fresh, intact COCs as described earlier. After 24 h cultivation of six COCs in 10 μ l droplets of both control conditioned and oocyctectomized COC-conditioned medium, the oocytes were fixed, stained and their nuclear status was assessed as described above.

Reversibility of meiotic inhibition in COCs cultured in a 10 μ l droplet of medium

After primary incubation of 20 COCs for 24 h in a 10 μ l droplet of fresh medium, COCs were washed several times in medium and cultured for a further 24 h in a 100 μ l droplet of fresh medium. The COCs were denuded, fixed and stained as above. As a control, 20 COCs were cultured in a 100 μ l droplet of fresh medium for 24 and 48 h and the nuclear status of each COC was also examined.

Statistical analysis

Each experiment was replicated at least three times. The data from each experiment were pooled for presentation and a chi-squared analysis was used to test significance among groups. The percentage data were subjected to arc sine transformation before correlation analyses were carried out using Student's *t* test. The level of significance was $P < 0.05$.

Results

Petr *et al.* (1989) reported that the incidence of GVBD decreases with increasing numbers of COCs cultured in a 10 μ l droplet. In the present study, various numbers of COCs were cultured in 10 μ l droplets for 24 h to determine whether this quantitative inhibitory influence of cumulus cells on GVBD of oocytes is also observed under our culture conditions. The proportions of oocytes undergoing GVBD after cultivation are shown (Table 1). Culture of a small number ($n = 1, 3$ and 6) of COCs in each 10 μ l droplet resulted in higher GVBD (72–81%). However, when ten COCs were cultured in a 10 μ l droplet, the proportion of oocytes with GVBD decreased significantly. Moreover, cultivation of > 20 COCs in a 10 μ l droplet produced a more marked decrease in the proportion of GVBD

Fig. 2. Light micrographs of various degrees of cumulus expansion in pig cumulus-oocyte complexes. (a) $\pm$ Degree: no expansion was observed. (b) + Degree: cumulus mass was enlarged but no cumulus cells were separated. (c) ++ Degree: some cumulus cells were separated. Scale bars represent 50 μ m.

Table 1. Effect of the number of pig cumulus–oocyte complexes (COCs) cultured in a 10 µl droplet of medium on germinal vesicle breakdown of oocytes

Number of COCs in 10 µl medium	Number of oocytes examined	Number (%)* of oocytes with		Number (%)** of degenerated oocytes
		Germinal vesicles	GVBD	
1	74	16 (23.9) ^a	51 (76.1) ^a	7 (9.5) ^{ab}
3	77	14 (18.7) ^a	61 (81.3) ^a	2 (2.6) ^a
6	58	14 (27.5) ^a	37 (72.5) ^a	7 (12.1) ^b
10	113	53 (51.0) ^b	51 (49.0) ^b	9 (8.0) ^{ab}
20	71	50 (79.4) ^c	13 (20.6) ^c	8 (11.3) ^b
40	138	109 (87.2) ^c	16 (12.8) ^c	13 (9.4) ^{ab}

GVBD: germinal vesicle breakdown.

*Percentage of the number of oocytes examined except for degenerated oocytes.

**Percentage of the number of oocytes examined.

^{abc}Values within a column with different superscripts are significantly different ($P < 0.05$).**Table 2.** Effect of the number of pig cumulus–oocyte complexes (COCs) cultured in a 10 µl droplet of medium on the degree of dye transfer

Number of COCs cultured in 10 µl droplet	Number of COCs examined	Number (%) of COCs in each category			
		1	2	3	4
1	30	0 (0.0) ^a	23 (76.7) ^a	4 (13.3) ^a	3 (10.0) ^{ab}
3	49	1 (2.0) ^a	35 (71.4) ^a	7 (14.3) ^a	6 (12.2) ^{ab}
6	49	5 (10.2) ^{ab}	29 (59.2) ^a	8 (16.3) ^a	7 (14.3) ^{ac}
10	58	12 (20.7) ^b	33 (56.9) ^a	3 (5.2) ^{ab}	10 (17.2) ^a
20	43	31 (72.1) ^c	11 (25.6) ^b	0 (0.0) ^b	1 (2.3) ^b
40	65	60 (92.3) ^d	2 (3.1) ^c	0 (0.0) ^b	3 (4.6) ^{bc}

Category 1: dye transferred to all layers of cumulus cells surrounding oocyte; category 2: dye transferred to more than two inner layers of cumulus cells but not all layers of the cumulus cells; category 3: dye transferred to the innermost layer of cumulus cells only; and category 4: dye not transferred to cumulus cells.

^{abcd}Values within a column with different superscripts are significantly different ($P < 0.05$).

oocytes and all the differences in these proportions between the 20 or 40 COCs groups and the < 10 COCs groups were significant. With regard to the proportion of degenerated oocytes, there were significant differences between some groups, but a proportional increase together with an increase in the number of COCs in a 10 µl droplet was not detected.

After cultivation of various numbers of COCs in a 10 µl droplet for 24 h, COCs were injected with lucifer yellow and the degree of dye transfer from the oocyte into cumulus cells was observed (Table 2). When one, three or six COCs were cultured in a 10 µl droplet, more than half of the COCs were in category 2, whereas only a few COCs underwent a category 1 degree of dye transfer. When ten COCs were cultured in 10 µl droplets, a significantly higher proportion of COCs remained in category 1 compared with the group of one or three COCs in a 10 µl droplet. Further increasing the number of COCs cultured in a 10 µl droplet led to a marked increase in the proportion of COCs in category 1, reaching a maximum value in the group of 40 COCs cultured in a 10 µl droplet (92%).

The proportion of GVBD oocytes and each percentage of the total number of category 2, 3 and 4 COCs, the total

number of category 3 and 4 COCs, and the total number of category 4 COCs, were calculated for each group to examine the relationship between induction of GVBD and the degree of disruption of cumulus–cumulus cell or oocyte–cumulus cell gap junctions (Fig. 3). The category 2, 3 and 4 COCs represent the COCs in which gap junctions within at least the outermost cumulus cell layers were disrupted. Category 3 and 4 COCs represent the COCs in which the gap junctions from within the outer cumulus layers to at least two inner layers of cumulus cells were disrupted. Category 4 COCs represent the COCs in which the gap junctions between the oocyte and the cumulus cells were disrupted. The percentages of category 3 and 4 COCs and the percentages of category 4 COCs were much lower than the proportion of oocytes undergoing GVBD in any of the groups. In contrast, the percentage of total COCs with category 2, 3 or 4 disruption was slightly higher than the proportion of GVBD oocytes in all groups, except for the group of 40 COCs in a 10 µl droplet. A significant positive correlation was established between the percentage of total COCs with category 2, 3 or 4 disruption and the proportion of GVBD oocytes ($r = 0.98$, $P < 0.01$).

Fig. 3. Relationship between germinal vesicle breakdown (GVBD) and the degree of dye transfer in pig cumulus-oocyte complexes (COCs). ○: Percentage of GVBD oocytes. ●: Percentage of the total number of COCs with category 2, 3 and 4 degrees of dye transfer. ▲: Percentage of the total number of COCs with category 3 and 4 degrees of dye transfer. ■: Percentage of the total number of COCs with category 4 degrees of dye transfer.

The degree of cumulus cell expansion of COCs that were cultured in a 10 µl droplet of different medium containing various volumes of the medium conditioned by culturing 20 intact COCs in the same volume droplet is shown (Table 3). Culture of six COCs in a 10 µl droplet of fresh medium (with no conditioned medium) resulted in expansion of outer cumulus cells in most COCs. However, an increase in the concentration of conditioned medium significantly reduced the percentage of COCs with a ++ degree of cumulus cell expansion. Expansion of cumulus cells in nearly all COCs (99%) was inhibited completely by cultivation in 100% conditioned medium, and highly significant differences in the percentage of COCs with ++ expansion were observed between the COCs cultivated in 100% conditioned medium and other groups.

The proportion of oocytes undergoing GVBD when six COCs were cultured for 24 h in medium conditioned by 20 oocyctomized COCs is shown (Table 4). Culture of COCs in the oocyctomized COC-conditioned medium resulted in a similar level of GVBD as did culture in the medium conditioned by 20 intact COCs. The proportions of oocytes with GVBD in both the oocyctomized COC- and intact COC-conditioned medium groups were significantly lower than the proportion in the fresh medium group. When six denuded oocytes were cultured in a 10 µl droplet of COC-conditioned medium, 66.7% of oocytes underwent GVBD (data not shown).

When 20 and 40 COCs were cultured in 10 µl droplets of medium, nuclear status of germinal vesicle-arrested oocytes was divided further into GV I, GV II, GV III or GV IV (Table 5). More than 70% of oocytes in both groups were kept at the GV I stage. Progression to GV II and GV IV stages was also observed in 6 and 24% of germinal

vesicle-arrested oocytes in the 20 COC group, and 3 and 14% of germinal vesicle-arrested oocytes in the 40 COC group.

After culture of 20 COCs in a 10 µl droplet of medium for 24 h, the COCs were transferred to a 100 µl droplet and cultured for a further 24 h. Culture of 20 COCs in a 100 µl droplet for 24 h led to meiotic progression from prometaphase I to telophase I in 61% of oocytes, and 31% of oocytes remained at the germinal vesicle stage (Table 6). About 48 h was required for oocytes cultured in a 100 µl droplet to reach metaphase II (74% of COCs at metaphase II). When 20 COCs were pre-cultured in a 10 µl droplet for 24 h, transfer of COCs to a 100 µl droplet resulted in resumption of meiotic maturation in oocytes. More than half of these oocytes reached metaphase II stage within 24 h after transfer of COCs from the 10 µl droplet to the 100 µl droplet.

Discussion

Petr *et al.* (1989) observed a marked decrease in the proportion of GVBD oocytes as the number of COCs cultured in a 10 µl droplet increased, and they postulated that pig cumulus cells produce a factor (or factors) that inhibits GVBD and which is secreted into the surrounding medium. A similar finding was also detected in bovine COCs by Sirard *et al.* (1992). However, the mechanism of action of the GVBD inhibitor has not been explored at all. The present study was undertaken to elucidate the regulatory mechanism by which this factor (or factors) inhibits GVBD.

In the experiment for confirming the secretion of GVBD-inhibiting factors from COCs into medium, GVBD occurred in >70% of oocytes when one, three or six COCs were cultured in a 10 µl droplet. In contrast, a large decrease in the proportion of GVBD oocytes was detected when large numbers of COCs were cultured in the same volume of medium (ten COCs: 49.0%; 20 COCs: 20.6%; 40 COCs: 12.8%). Furthermore, culture of six COCs in a 10 µl droplet of conditioned medium also suppressed GVBD of oocytes. Hence, these results indicate strongly that COCs produce a GVBD-inhibiting factor and secrete it into the medium, but it is not possible to determine whether this factor is secreted from the cumulus cells or from the oocyte itself. Therefore, oocyctomized COCs were used to prepare a conditioned medium and six COCs were cultured in the oocyctomized COC-conditioned medium for 24 h. The conditioned medium led to inhibition of GVBD at the same level as was observed in the medium conditioned by intact COCs, indicating that the GVBD-inhibiting factor (or factors) is secreted by cumulus cells *per se*. The secretion of a granulosa cell- or theca cell-derived factor that inhibits GVBD of oocytes into medium has also been reported in cattle (Richard and Sirard, 1996a,b; Sirard and Bilodeau, 1990).

Culture of many COCs in a small amount of medium raises the possibility that nutrients for oocyte GVBD may

Table 3. Effect of conditioned medium on cumulus expansion in pig cumulus–oocyte complexes (COCs)

Concentration of conditioned medium	Number of COCs examined	Percentage of GVBD oocytes	Number (%) of COCs at each degree of cumulus expansion		
			±	+	++
0	104	72.5 ^a	3 (2.9) ^a	8 (7.7) ^a	93 (89.4) ^a
50	91	nd	1 (1.1) ^a	41 (45.1) ^b	49 (53.8) ^b
100	102	32.8 ^b	101 (99.0) ^b	0 (0.0) ^c	1 (1.0) ^c

GVBD: germinal vesicle breakdown. nd: not determined.

Cumulus cell expansion was scored on the subjective scales of ± (no observable expansion), + (enlargement of cumulus mass but no cumulus cells separated) and ++ (some cumulus cells separated).

^{abc}Values within a column with different superscripts are significantly different ($P < 0.05$).

Table 4. Effect of culture in oocyctomized cumulus–oocyte complex-conditioned medium on germinal vesicle breakdown of pig oocytes

Medium	Number of oocytes examined	Number (%) * of oocytes with		Number (%)** of degenerated oocytes
		Germinal vesicles	GVBD	
OOX conditioned	71	63 (92.6) ^a	5 (7.4) ^a	3 (4.2)
Intact COC conditioned	113	95 (88.0) ^a	13 (12.0) ^a	5 (4.4)
Fresh	105	30 (30.6) ^b	68 (69.4) ^b	7 (6.7)

COC: Cumulus–oocyte complex. OOX: oocyctomized cumulus–oocyte complex. GVBD: germinal vesicle breakdown.

*Percentage of the number of oocytes examined except for degenerated oocytes.

**Percentage of the number of oocytes examined.

^{ab}Values within a column with different superscripts are significantly different ($P < 0.05$).

Table 5. Nuclear status in germinal vesicle stage of pig cumulus–oocyte complexes (COCs) cultured in 10 µl droplets of medium for 24 h

Number of COCs per 10 µl medium	Total number of oocytes with intact germinal vesicles	Number (%) * of oocytes at each stage			
		GV I	GV II	GV III	GV IV
20	50	35 (70.0)	3 (6.0)	0	12 (24.0)
40	109	91 (83.5)	3 (2.8)	0	15 (13.8)

*Percentage of the total number of oocytes with intact germinal vesicles.

Table 6. Reversibility of germinal vesicle-arrested pig cumulus–oocyte complexes (COCs) cultured in 10 µl droplets of medium for 24 h

Volume (µl) of medium for culture of 20 COCs		Number of oocytes examined	Number (%) of oocytes at each stage			Number of degenerated oocytes
First 24 h	Second 24 h		Germinal vesicle	ProM I–T I	Metaphase II	
100*	–	100	31 (31.0) ^a	61 (61.0) ^a	4 (4.0) ^a	4 (4.0) ^a
10**	100	98	5 (5.1) ^b	27 (27.6) ^b	55 (56.1) ^b	11 (11.2) ^b
100***	100	61	5 (8.2) ^b	8 (13.1) ^c	45 (73.8) ^c	3 (4.9) ^a

ProM I–T I: Prometaphase I, metaphase I, anaphase I and telophase I.

*Twenty COCs cultured in a 100 µl droplet of medium for only 24 h.

**Twenty COCs cultured in a 10 µl droplet of medium for 24 h and then cultured in a 100 µl droplet for another 24 h.

***Twenty COCs cultured in a 100 µl droplet of fresh medium for 48 h.

^{abc}Values within a column with different superscripts are significantly different ($P < 0.05$).

be depleted from the medium. For example, in mouse COCs, decreasing concentrations of glucose in medium enhance GVBD of oocytes in which GVBD was inhibited by hypoxanthine (Downs and Mastropolo, 1994). If the conditioned medium was short of glucose in the present study, the medium could have promoted GVBD of oocytes. Downs and Mastropolo (1994) reported that when denuded oocytes were cultured in medium containing various concentrations of glucose, glucose had no promoting effect on GVBD of oocytes; however, GVBD of denuded oocytes cultured in a 10 μ l droplet of conditioned medium was not inhibited. Therefore, in the present study, glucose is probably not limiting in the medium.

When ten and 20 COCs were cultured in a 10 μ l droplet in the present study, the proportions of oocytes undergoing GVBD were much lower than those observed in the study by Petr *et al.* (1989). This difference is probably attributable to the different composition of the culture media in each study. In the present study, hormones (hCG, PMSG and oestradiol) were added to the culture media, which was not the case in the study of Petr *et al.* (1989). Alternatively, the difference in the proportions of GVBD oocytes between the present study and that of Petr *et al.* (1989) could be due to the number of cumulus cells surrounding the oocyte used. Only oocytes attached to at least three layers of cumulus cells were used in the present study, whereas Petr *et al.* (1989) did not describe how many layers of cumulus cells surrounded the oocytes.

The decrease in the proportion of oocytes undergoing GVBD that resulted from increasing the number of COCs cultured in a 10 μ l droplet indicates that a larger quantity of the GVBD-inhibiting factor(s) was accumulated in the culture medium containing higher numbers of COCs. Concomitant with this reduction in the proportion of GVBD oocytes, an increase in the proportion of category 1 COCs (dye transferred to all layers of cumulus cells surrounding the oocyte) was observed as the number of COCs cultured in a 10 μ l droplet increased. As category 1 COCs were defined as oocytes with entirely functional gap junctions between cumulus cells or cumulus cells and oocytes, it is possible that, in pigs, the GVBD-inhibiting factor(s) inhibits the disruption of gap junctions, thus preventing oocytes from undergoing GVBD by continuous transfer of the inhibitor that was produced by cumulus cells and transported directly to the oocyte (Isobe *et al.*, 1998). However, there is no evidence that the factors that inhibit GVBD and inhibit disruption of gap junctions are the same.

The degree of cumulus cell expansion after culture of six COCs in a 10 μ l droplet of conditioned medium was assessed to examine the effect of the GVBD-inhibiting factor(s) on expansion of cumulus cells. As the concentration of conditioned medium increased, there was a concomitant significant decrease in the proportion of COCs with ++ expansion and no sign of cumulus cell expansion was detected in 99% of COCs cultured in 100% conditioned medium. Furthermore, the conditioned medium produced

a significant reduction in the proportion of oocytes undergoing GVBD. These findings indicate that the GVBD-inhibiting factor(s) is present in the conditioned medium and that the expansion of cumulus cells in pig COCs is also regulated by factor(s) secreted by cumulus cells. These observations raise the possibility that the blocking of cumulus cell expansion by GVBD-inhibiting factor(s) is accompanied by functional maintenance of gap junctions between cells, thus preventing oocytes from undergoing GVBD. This interpretation regarding GVBD prevention due to the factor is also supported by the fact that an unknown factor inhibits GVBD of oocytes via cumulus cells (Petr *et al.*, 1989; Richard and Sirard, 1996b).

In the present study, the proportion of category 4 COCs (dye not transferred to cumulus cells) was much lower than the proportion of oocytes undergoing GVBD in each COC group (1–40 per 10 μ l droplet), indicating that GVBD of oocytes occurred before disruption of cumulus–oocyte gap junctions. In contrast, Dekel and Beer (1980) and Dekel *et al.* (1981) reported that the interruption of cumulus–oocyte gap junctions could lead to relief of meiotic arrest. The results of the present study also indicate that the proportions of total COCs with category 2, 3 or 4 disruption (which were considered as the disruption of gap junctions within at least the outermost cumulus cells) were considerably higher than the proportion of GVBD oocytes in all groups, except for a group of 40 COCs cultured in a 10 μ l droplet, and that the positive correlation between these proportions was highly significant. These results indicate strongly that loss of gap junction within cumulus cells triggers the resumption of meiosis in pig oocytes. Similar conclusions regarding the induction of meiotic resumption in mammalian oocytes were derived from studies of rat COCs *in vivo* (Larsen *et al.*, 1986, 1987) and *in vitro* (Wert and Larsen, 1989), mouse COCs *in vivo* (Eppig, 1982) and pig COCs *in vivo* (Motlik *et al.*, 1986) and *in vitro* (Isobe *et al.*, 1998).

When 20 and 40 COCs were cultured in a 10 μ l droplet for 24 h, GV I oocytes were dominant. This finding demonstrates that such culture conditions can block meiosis of oocytes completely. Furthermore, when 20 COCs were cultured in a 100 μ l droplet after cultivation in a 10 μ l droplet for 24 h, oocytes completed meiosis in another 24 h only. This finding indicates that, after cultivation in a 10 μ l droplet for 24 h, GV I-arrested oocytes are alive and can reach metaphase II faster than oocytes freshly isolated from follicles. It remains to be determined whether COCs cultured in a 10 μ l droplet for 24 h and followed by a 100 μ l droplet for 24 h can be fertilized normally and develop better than those cultured in a 100 μ l droplet for 48 h. This contention is supported by Funahashi *et al.* (1997), who found that exposure of COCs to dibutyl cAMP for the first 20 h of culture followed by culture in the absence of the drug for 28 h for maturation, improves the efficiency of production of pig embryos *in vitro*. Lonergan *et al.* (1997) reported that a premature treatment may be necessary to allow the oocytes from

small follicles in which the final phase of folliculogenesis has been shortened artificially by removal from the follicle, to 'catch up' with those from larger follicles or those matured totally *in vivo*, which also supports the contention.

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