Involvement of meiotic resumption in the disruption of gap junctions between cumulus cells attached to pig oocytes

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The present study was undertaken to examine the mechanisms by which cumulus cells regulate meiotic resumption in pig oocytes using microinjections of lucifer yellow into cumulus–oocyte complexes combined with a fluorescent assay. Some cumulus–oocyte complexes cultured for 0, 8, 16, 24, 32, 40 and 48 h were denuded to assess the nuclear status of oocytes; the remaining complexes were injected with lucifer yellow and monitored for the transfer from the oocyte to the surrounding cumulus cells using confocal laser scanning microscopy. The proportion of oocytes undergoing germinal vesicle breakdown at 16, 24 and 32 h of cultivation was much higher than that of cumulus–oocyte complexes in which all gap junctions within cumulus cells and between cumulus cells and oocyte were disrupted (16 h: 22.7% versus 8.8%, 24 h: 66.7% versus 40.3%, 32 h: 84.0% versus 69.4%), showing that the disruption does not trigger meiotic resumption in the pig oocyte. A significant positive correlation ($r = 0.99$, $P < 0.01$) was established between the proportion of germinal vesicle breakdown oocytes and that of cumulus–oocyte complexes exhibiting loss of gap junctions within all cumulus cell layers excluding the innermost layer. From these results, it is concluded that meiotic resumption in pig oocytes is induced by the disruption of gap junctions within cumulus cells, rather than that between the oocyte and cumulus cells, which blocks the conduction of meiosis inhibitory signals from the outer cumulus cells to the oocyte.

Introduction

Using electron microscopy, electrophysiological measurements and dye transfer experiments, Gilula *et al.* (1978) showed that, in the preovulatory and postovulatory cumulus–oocyte complexes (COCs) of rats, numerous small gap junctional contacts and bidirectional ionic coupling were present between cumulus cells and oocytes, although those gap junction contacts and the coupling were reduced as the time of ovulation approached. They postulated that cumulus–oocyte communication regulates the maturation of oocytes during follicular development before ovulation. Dekel and Beers (1980) also observed that germinal vesicle breakdown (GVBD) in rat denuded oocytes is accelerated in the presence or absence of LH, and its time course is very similar to that of the cumulus-enclosed oocytes cultured in medium with gonadotrophin. They concluded that removal of cumulus cells could mimic the effect of LH on spontaneous maturation by interrupting the communication between cumulus cells and oocyte. In addition, Dekel *et al.* (1981) found that meiotic arrest and cumulus–oocyte communication were maintained for at least 20 h when intact rat follicles were cultured in hormone-free medium. However, the

inclusion of LH in the culture medium resulted not only in the resumption of meiosis, but also in termination of the communication, suggesting that an interruption in the transmission of a substance from the cumulus to the oocytes, produced by exposing the COCs to gonadotrophins, allows meiosis to proceed. From these studies, it has been hypothesized that the interruption of cumulus–oocyte gap junctions leads to the relief of meiotic arrest (Tsafirri and Dekel, 1994).

According to Eppig (1982), who evaluated cumulus–oocyte communication by incorporating [³H]uridine and [³H]choline into COCs, maturation of rat oocytes is already initiated by 3 h after hCG injection, whereas gap junctions between oocyte and cumulus cells were not disrupted at 3 and 6 h after hCG injection. A similar observation was reported by Salustri and Siracusa (1983) who demonstrated that at 3 h of culture of mouse COCs in the presence of FSH, the metabolic coupling remained high but meiotic maturation had already resumed. Larsen *et al.* (1986, 1987) observed a close temporal correlation between the loss of cumulus cell–cumulus cell gap junctions, rather than cumulus–oocyte gap junctions, and the resumption of meiotic maturation in superovulated rats. These results have emphasized that gap junction loss within the cumulus cells *per se* induces the resumption of oocyte meiosis in rodents. However, the precise mechanism of this gap junction loss-dependent meiotic resumption is unclear, although a gap

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junction loss independent pathway was reported by Byskov *et al.* (1997).

In pigs, Motlik *et al.* (1986) provided evidence that the oocyte and corona radiata (the innermost cumulus cells) remain tightly coupled, even when the oocyte has already resumed meiosis. However, to date, the precise role that cumulus cells play in the resumption of meiosis in pig COCs has not been fully elucidated. The aim of the present study was to investigate the regulatory mechanisms involved in meiotic resumption in pig oocytes by determining the timing of the disruption of cell–cell gap junctions using a fluorescent dye (lucifer yellow).

Materials and Methods

General procedures

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. Oocytes were collected from follicles 3–9 mm in diameter with a 21 gauge needle attached to a disposable 10 ml syringe. Oocytes possessing a complete and compact cumulus mass were selected and washed twice in Dulbecco's PBS supplemented with 5% bovine serum (Nacalai Tesque, Kyoto) and 0.1 mg kanamycin ml^{-1} . They were further washed several times in the culture medium. Ten to fifteen COCs were transferred to a droplet of 100 μl of culture medium covered with warm mineral oil (E. R. Squibb and Sons, Inc. Princeton, NJ) in a 35 mm polystyrene culture dish and cultured for 0, 8, 16, 24, 32, 40 and 48 h at 39°C in a humidified atmosphere of 5% CO_2 in air. The culture medium was TCM-199 with Earle's salts (Gibco BRL, Grand Island, NY) supplemented with 10 iu hCG ml^{-1} (Teikokuzouki, Tokyo), 10 iu PMSG ml^{-1} (Teikokuzouki), 1 μg oestradiol ml^{-1} (Sigma Chemical Co., St Louis, MO), 50 μg gentamicin sulfate ml^{-1} (Sigma) and 10% fetal calf serum (Gibco). The medium was sterilized by filtration through 0.45 μm Millipore filters (Millipore Ltd, Bedford, MA).

After a given period of cultivation, some of the COCs were denuded mechanically for determination of meiotic status using a finely drawn Pasteur pipette and the remainder of the COCs was prepared for the dye transfer test. The denuded oocytes were fixed for 48 h in acetic acid: ethanol (1:3, v/v) at 10°C, and stained with 1% (v/w) 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 (GV) or having undergone germinal vesicle breakdown (GVBD).

Dye transfer experiments

Oocytes surrounded by cumulus cells were injected with 10 mmol Hepes l^{-1} containing 10% (w/v) lucifer yellow CH (Sigma) after incubations of 0, 8, 16, 24, 32, 40 and 48 h on a heated stage (37°C) of an inverted microscope at $\times 100$ magnification using a Hoffmann Modulation contrast system (Modulation Optics Inc., Greenvale, NY). The holding and injection pipettes were made by drawing glass capillary tubes

with a pipette puller (Model PC-84, Sutter Instrument Company, Novato, CA) and were further processed on a micropipette beveler (Model BV-10, Sutter Instrument Company) and a microforge (WILDM3D, Leica, Heerbrugg). The outer and inner diameters of the holding and injection pipettes were 150 and 120 μm , and 10 and 7 μm , respectively. The injection pipette had a bevel angle of 35° and a sharp spike to assist penetration through the oolemma. The holding and injection pipettes were fixed to a tool holder and were connected to a microinjector (CIJ-1; Shimadzu, Kyoto). The injection press and duration were 20–30 kPa and 0.05 s, respectively and 4 μl of lucifer yellow solution was injected into each oocyte. The volume of the injected solution was calculated on the basis of the diameter of a droplet of the solution expelled from the injection pipette into mineral oil. During injection and subsequent fluorescent observation, COCs were kept in PBS containing 5% (v/v) bovine serum. The spread of the dye into surrounding cumulus cells was monitored using confocal laser scanning microscopy (ACAS 570, Meridian Instruments, Inc., Okemos, MI) with a $\times 100$ oil objective. An argon ion laser adjusted at 488 nm wavelength was used. The emitted light was separated by a dichroic mirror (575SP); a 530/30 band pass filter was placed in front of the detector. The scanning was carried out in the section where the largest diameters of oocytes were observed at a field depth of about 14.5 μm . No oocytes were damaged by the injection of lucifer yellow solution, although degenerated oocytes at the time of injection could not be used because the injected lucifer yellow spilled out of these oocytes easily due to disintegration of their ooplasm. However, observation of the lucifer yellow fluorescence more than 15 min after injection showed a spontaneous spilling of lucifer yellow out of the oocytes, so the fluorescent monitoring was carried out within 15 min after injection.

Cumulus–oocyte complexes 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 oocytes (Fig. 1a); category 2, the dye was transferred to more than two inner layers of cumulus cells but not all layers of the cumulus cells (Fig. 1b); category 3, the dye was transferred only to the innermost layer of cumulus cells (Fig. 1c); and category 4, the dye was not transferred to cumulus cells (Fig. 1d).

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. A probability of $P < 0.05$ was considered to be statistically significant.

Results

Time course of germinal vesicle breakdown in pig cumulus–oocyte complexes cultured in vitro

Cumulus–oocyte complexes cultured for 0–48 h were denuded and their nuclear configuration was observed to

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

Fig. 2. Time course of germinal vesicle breakdown (GVBD) in pig cumulus-oocyte complexes cultured *in vitro*. Values with different superscripts are significantly different ($P < 0.05$).

explore the relationship between the loss of intercellular communication and induction of meiotic resumption. The proportion of oocytes undergoing GVBD at the various culture times are shown (Fig. 2). Up to 8 h of culture, most oocytes (63 of 65 at 0 h and 120 of 121 at 8 h) were at GV stage. After 16 h of cultivation, GVBD had occurred in 22.7% of the oocytes (29 of 128). The incidence of GVBD significantly increased after 24 h cultivation. With further culture, the proportion of oocytes with GVBD increased progressively, and reached 93.5% (116 of 124) after 48 h of culture.

Transfer of the lucifer yellow from oocytes to cumulus cells

After 0–48 h of culture, COCs were injected with lucifer yellow and the degree of dye transferred from oocytes to cumulus cells was observed (Table 1). When oocytes at the onset and after 8 h of cultivation were injected with lucifer yellow, the dye was transferred into all cumulus cells surrounding oocytes in more than 90% of COCs (category 1). After 16 h of cultivation, the proportion of COCs in category 1 had significantly decreased. In oocytes microinjected with lucifer yellow after 16 h of cultivation the dye was transferred into more than two inner cumulus cell layers but not to outermost cumulus cells (category 2) in 61.8% of COCs. The COCs in category 3 (dye transferred only to the innermost layer of cumulus cells) and category 4 (no dye transferred to cumulus cells) were first observed after 16 h of cultivation. After 24 h of cultivation, 40% of COCs showed no transport of lucifer yellow from oocytes to cumulus cells. When COCs were cultured for more than 32 h, none was recorded in category 1. The proportions of COCs in category 4 increased from 8.8% of COCs at 16 h of cultivation to 92.1% of COCs at 40 h of cultivation. After 48 h of cultivation, all COCs were found to be in category 4.

Relationship between germinal vesicle breakdown and the degree of the disruption of cumulus-cumulus cell or oocyte-cumulus cell gap junctions

The time course of the proportion of GVBD oocytes, the total number of category 2, 3 and 4 COCs, the total numbers

of category 3 and 4 COCs and category 4 COCs were calculated at each time of cultivation to examine the relationship between GVBD and the degree of the disruption of cumulus-cumulus cell or oocyte-cumulus cell gap junctions (Fig. 3). 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 layer to at least two inner layers of cumulus cells were disrupted. Category 4 COCs represent the COCs in which the gap junctions between oocytes and cumulus cells were disrupted. A significant positive correlation was established between the proportion of GVBD oocytes and those COCs that fell into categories 3 and 4 ($r = 0.99$, $P < 0.01$). The temporal pattern of COCs in category 4 was delayed compared with that of GVBD oocytes. However, after 40 and 48 h of cultivation, the proportions of category 4 COCs were higher than the proportion of GVBD oocytes.

Discussion

At the onset and after 8 h of cultivation, disruption of gap junctions between the outermost cumulus cells was found in 9.0 and 8.5% of COCs, respectively. After 16 h of cultivation, the proportion of COCs in which the injected lucifer yellow was transferred through the gap junction from oocytes to all surrounding cumulus cells (category 1 COCs) was significantly decreased compared with the proportion after 0 and 8 h of culture. However, cultivation of COCs for 16 h resulted in a significantly higher proportion of COCs in which gap junctions between cumulus cells was disrupted (category 2 and 3 COCs). In agreement with the present findings, Motlik *et al.* (1986) showed that the uptake of [3 H]uridine as well as the coupling index decreased markedly in pig COCs cultured *in vitro* for 16 h. These results indicate that the disruption of gap junctions within the cumulus cells in pig COCs is initiated between 8 and 16 h of cultivation and the progression of this uncoupling advanced very rapidly toward 16 h.

The observation that GVBD in denuded rat oocytes cultured in the presence or absence of LH was accelerated and that its timing was very similar to that of COCs cultured in the presence of LH, led Dekel and Beers (1980) to suggest that LH fostered maturation of the oocyte by interrupting its communication with the cumulus oophorus. Similarly, Dekel *et al.* (1981) found that both cumulus-oocyte communication and meiotic arrest were maintained for at least 20 h of culture without gonadotrophin. However, exposure of rat oocytes to LH caused the interruption of ionic coupling between cumulus cells and the oocyte and induced the resumption of meiosis. Therefore, they concluded that the maintenance of meiotic arrest may be dependent upon the integrity of cell-to-cell communication between the cumulus cells and the oocyte. These studies strongly suggest that the disruption of communication between cumulus cells and the oocyte leads to the GVBD of the oocyte.

However, in the present study, the proportion of oocytes undergoing GVBD at 16, 24 and 32 h of cultivation was much higher than the proportion of COCs in category 4 (16 h: 22.7% versus 8.8%, 24 h: 66.7% versus 40.3%, 32 h: 84.0% versus

Table 1. Time course of lucifer yellow transfer in pig cumulus-oocyte complexes (COCs)

Duration of culture (h)	Number of COCs	Category (%)			
		1	2	3	4
0	100	91 (91.0) ^a	9 (9.0) ^a	0 (0.0) ^a	0 (0.0) ^a
8	71	65 (91.5) ^a	6 (8.5) ^a	0 (0.0) ^a	0 (0.0) ^a
16	68	12 (17.6) ^b	42 (61.8) ^b	8 (11.8) ^{bc}	6 (8.8) ^b
24	77	6 (7.8) ^b	28 (36.4) ^c	12 (15.6) ^{bd}	31 (40.3) ^c
32	72	0 (0.0) ^c	4 (5.6) ^{ad}	18 (25.0) ^d	50 (69.4) ^d
40	63	0 (0.0) ^c	2 (3.2) ^{ad}	3 (4.8) ^{ac}	58 (92.1) ^e
48	66	0 (0.0) ^c	0 (0.0) ^d	0 (0.0) ^a	66 (100.0) ^f

Category 1, dye transferred to all layers of cumulus cells surrounding oocytes; 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 only to the innermost layer of cumulus cells; category 4, dye not transferred to cumulus cells.

^{a,b,c,d,e,f}Values with different superscripts within a column are significantly different ($P < 0.05$).

Fig. 3. Relationship between germinal vesicle breakdown (GVBD) of pig oocytes and the degree of the lucifer yellow dye transfer in pig cumulus-oocyte complexes (COCs). ■, Percentage of GVBD oocytes; □, percentage of COCs exhibiting 2, 3 and 4 degree of dye transfer; ●, percentage of COCs exhibiting 3 and 4 degree of dye transfer; ▲, percentage of COCs exhibiting 4 degree of dye transfer. Category 1, dye transferred to all layers of cumulus cells surrounding oocytes; 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 only to the innermost layer of cumulus cells; category 4, dye not transferred to cumulus cells.

69.4%). This finding indicates that the disruption of the gap junctions between cumulus cells and the oocyte is preceded by the meiotic resumption of the oocyte. Therefore, the disruption of the cumulus-oocyte gap junctions does not lead to the resumption of meiosis in the pig COCs.

A significant positive correlation ($r = 0.99$, $P < 0.01$) was established between the proportion of GVBD oocytes and the proportion of COCs in either category 3 or 4. Since all gap junctions within cumulus cells except for those of the inner layer had disappeared in COCs in categories 3 and 4, the correlation reveals that the meiotic resumption in pig oocytes involves the disruption of gap junctions within cumulus cells

rather than those between the oocyte and cumulus cells. Similar conclusions that the losses of cumulus-to-cumulus cell gap junction preceded the induction of meiotic resumption have been derived from studies with rat COCs *in vivo* (Larsen *et al.*, 1986, 1987) and *in vitro* (Wert and Larsen, 1989), and mouse COCs *in vivo* (Eppig, 1982).

Larsen *et al.* (1986, 1987), who compared the degree of gap junctional cell-to-cell interaction in several distinct regions of the rat follicle at various times during the preovulatory period by using a more direct quantitative freeze-fracture analysis, have provided evidence that, although a small decrease in the mean area of membrana gap junctions was noted, the area of cumulus cell gap junctions was markedly reduced 2–3 h after an ovulatory injection of hCG (the time of meiotic resumption in the oocytes). Considering other experiments in which inhibition of meiotic maturation by membrana granulosa cells in isolated COCs has been demonstrated (Tsafirri and Channing, 1975; Sato and Ishibashi, 1977; Sirard and Bilodeau, 1990a, b; Larsen *et al.*, 1986, 1987) suggested that the cumulus oophorus regulates the conduction of meiosis inhibitory signals from the membrana granulosa to oocyte *in vivo*. In addition, quantitative freeze-fracture analysis in rat COCs by Wert and Larsen (1989) showed that downregulation of cumulus cell gap junctions was initiated before meiotic resumption under various *in vitro* conditions. These results supported the earlier suggestion (Larsen *et al.*, 1986, 1987) that gap junction loss within the cumulus oophorus is instrumental in releasing the oocyte from the inhibitory influence of membrana granulosa cells so that meiotic maturation can be initiated in intact preovulatory follicles.

However, since COCs without membrana granulosa cells were examined in the present study, it is clear that oocyte meiotic resumption is not induced by terminating the transmission of an inhibitory factor from granulosa cells. Moreover, Isobe *et al.* (1996) reported that the culture of denuded oocytes for a further 24 h, after the removal of their cumulus cells after 24 h of cultivation, significantly increased the proportion of oocytes undergoing GVBD (90%) compared with oocytes cultured continuously for 48 h without removing the cumulus cells (80%), and that the amount of GVBD of denuded oocytes co-cultured with COCs for a second 24 h of cultivation was

similar to that of denuded oocytes cultured without COCs (93% and 88%, respectively). From these results, Isobe *et al.* (1996) suggested that the cumulus cells surrounding pig oocytes cultured *in vitro* suppress GVBD, and that the suppressive factor produced by the cumulus cells is transferred to the oocytes through gap junctions. Therefore, the cumulus cells cultured *in vitro* in the present study might also have produced the GVBD suppressive factor, since the present culture system was similar to that used by Isobe *et al.* (1996). Furthermore, on the basis of the conclusion that the disruption of gap junctions within cumulus cells evokes meiotic resumption, which is derived from the evidence of a significant positive correlation between the proportion of GVBD oocytes and the proportion of category 3 and 4 COCs, the meiotic inhibitory signal must be produced by all cumulus cell layers except the innermost layer. This suggestion is supported by the evidence that, when COCs were cultured in a medium supplemented with various concentrations of conditioned medium (prepared previously by culturing 20 COCs in 10 µl droplet), increasing the concentrations of the medium reduced both the proportion of GVBD oocytes and the proportion of COCs in which some of the outermost layers of cumulus cells were expanded (N. Isobe, T. Maeda and T. Terada, unpublished).

Even at the onset of cultivation, a low frequency of oocytes undergoing GVBD was observed (3.1%) despite the application of strict oocyte selection criteria. This result was consistent with the study by Motlik and Fulka (1976), who found that some oocytes had already undergone GVBD immediately after isolation from ovaries. These oocytes undergoing GVBD before cultivation seem to be isolated from atretic follicles since the progression of atresia is accompanied by nuclear maturation of the oocyte in those follicles (Ingram, 1962). In the present study at the onset of cultivation, the lucifer yellow injected into the oocyte was not transferred to all layers of cumulus cells in part of COCs (9.0%). On the basis of the conclusion that the disruption of gap junctions within cumulus cells induced the meiotic resumption, this GVBD before cultivation may be due to the interruption of transfer through gap junctions that is independent of the cumulus cell expansion, since only oocytes with unexpanded cumulus cells were used in the present study. This hypothesis is supported by the fact that a decrease in the gap junction permeability is the result of the phosphorylation of connexin 43, one of gap junction proteins (Granot and Dekel, 1994).

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