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Phosphatidylinositol 3-Kinase in Cumulus Cells is Responsible for Meiotic Progression from M I to M II Stage in Porcine Follicular Oocytes

Masayuki Shimada[1], Mohamed-Kheir Idris Anas[2] and Takato Terada[1]*

[1]Faculty of Applied Biological Science and [2]Graduate School for International Development and Cooperation, Hiroshima University, Higashi-Hiroshima 739-8528, Japan

Abstract: *Porcine cumulus oocyte complexes (COCs) were cultured in an inhibitor free-medium for 24 h and then some of them were denuded. The COCs and denuded oocytes (DOs) were further cultured for 24 h in the presence of Phosphatidylinositol 3-kinase (PI 3-kinase) specific inhibitors, wortmannin and LY294002. In COCs and DOs, wortmannin (10^{-8}, 10^{-7} M) and LY294002 (5.0×10^{-5} M) significantly increased the proportion of oocytes arrested at M I and decreased that of oocytes reaching M II. Nevertheless, in DOs the differences between in the presence of the inhibitors and the control in the proportion of oocytes reaching M II substantially diminished as compared to those in the case of COCs. Cumulus cell expansion was significantly suppressed by wortmannin and LY294002. It is concluded that PI 3-kinase in cumulus cells plays a regulatory role in meiotic progression beyond M I and cumulus cell expansion in porcine COCs.*

Key words: *PI 3-kinase, Porcine oocytes, Nuclear maturation, Wortmannin, LY294002.*

Mammalian oocytes released from their follicles are induced to meiotic resumption, progression to M I and arrested at M II *in vitro* [1]. Oocyte maturation depends on activation of maturation promoting factor (MPF) which is composed of $p34^{cdc2}$ kinase and cyclin B [2–4] and possesses histone H1 kinase activity in the activated state [5]. In fact, the necessity of MPF for meiotic resumption and progressing to M II has been demonstrated in mice [6], cattle [7, 8] and pigs [9]. Moreover, it is now clear that mitogen-activated protein kinase (MAP kinase) is associated with nuclear maturation in mammalian oocytes, including those of mice [10], cattle [11] and pigs [12]. In mouse and porcine oocytes, an increase in MAP kinase activity was observed at M I and the activity was maintained at a high level until M II [12, 13]. With mouse oocytes as mammalian oocytes, Araki *et al.* [14] further reported that MAP kinase is activated by Mos kinase, but in bovine and porcine oocytes it has not yet been demonstrated what factor activates MAP kinase.

Recently, Motlik *et al.* [15] showed that co-culture with porcine follicular cells decreased $p34^{cdc2}$ kinase and MAP kinase activities in maturing bovine oocytes. In porcine follicular oocytes, cumulus cells surrounding oocytes have been shown to suppress both the GVBD process and progression from GVBD to M II [16]. On the basis of these facts, follicular somatic cells may control oocyte maturation.

In our previously experiment (unpublished data), we investigated the resumption of meiosis in porcine follicular oocytes cultured in the presence of two PI 3-kinase specific inhibitors (wortmannin and LY294002). The results of this experiment demonstrated that cumulus cell expansion and meiotic resumption of porcine follicle oocytes were blocked by the inhibition of PI 3-kinase activity within the cumulus cell itself, so it was concluded that PI 3-kinase in cumulus cells may be one of the factors regulating meiotic resumption in mammalian oocytes. In Xenopus oocytes, either insulin or IGF-I-stimulated GVBD requires PI 3-kinase activity [17] and progesterone-induced GVBD also is correlated with this kinase activity [18].

Meanwhile, it has been reported that porcine follicular fluid contains EGF [19] and IGF-I [20]. Receptors

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*To whom correspondence should be addressed.

for IGF-I have also been shown in rat granulosa cells [21] and porcine cumulus cells [22], and the presence of specific bindings of EGF to bovine and porcine cumulus cells has been observed [23]. In addition, it is generally accepted that EGF and IGF-I can activate PI 3-kinase in somatic cells [24, 25].

Very recently Lorenzo *et al.* [26] reported that the addition of EGF, IGF-I or both factors resulted in an increased proportion of cumulus oocyte complexes (COCs) reaching M II in immature rabbit oocytes, which may be regulated via the cumulus cells. Moreover, in porcine COCs EGF stimulates nuclear maturation and IGF-I enables cumulus cells to expand [22, 27]. It is therefore supposed that EGF or IGF- I facilitates progression from M I to M II and cumulus expansion through PI 3-kinase activity in the cumulus cell itself.

In order to elucidate whether PI 3-kinase cascade in cumulus cells plays a critical role in progressing from M I to M II and in enhancing cumulus cell expansion, porcine COCs were cultured in an inhibitor free-medium for 24 h or 34 h and further cultured for 24 h or 12 h in the presence of two PI 3-kinase specific inhibitors (wortmannin and LY294002). After that, the nuclear stage and cumulus cell expansion of the COCs were analyzed.

Materials and Methods

General procedures

Porcine ovaries were collected from prepubertal gilts at a local slaughterhouse and transported to our laboratory in 0.85% NaCl with 0.1 mg/ml kanamycin (Meiji Seika, Tokyo, Japan) at about 30°C within 1.5 h. The surfaces of follicles measuring from 3 to 8 mm in diameter were cut with a razor and oocytes were collected by scraping the inner surface of the follicle walls with a surgical blade. The collected oocytes were placed in prewarmed phosphate-buffered saline (pH7.4) supplemented with 0.1% polyvinyl-pyrrolidone (Sigma Chemical Co., St. Louis, USA). Oocytes with an intact, compact cumulus oophorus and evenly granulated cytoplasm were selected under a stereomicroscope and washed 3 times with maturation medium. Cultures of cumulus oocyte complexes (COCs) were carried out for 24 h in 100 μl drops of basic culture medium (about 20 oocytes/drop) covered with mineral oil (Sigma) at 39°C in a humidified atmosphere of 5% CO_2 in air. After 24 h of cultivation, half of the COCs were mechanically denuded by pipetting with flame-draw pipette tips with an inner diameter slightly larger than the oocyte diameter. COCs and denuded oocytes were continuously cultured for 24 more hour in the presence of various concentrations of the PI 3-kinase inhibitors, wortmannin (Sigma) and LY294002 (BioMol, PA, USA), as described below, in basic medium. The basic medium was NCSU37 [28] containing 7mM Turine (Sigma), 10% essential and 5% non-essential amino acids (Gibco BRL, Grand Island, NY, USA), 10% FCS (Gibco), 10 iu pregnant mares' serum gonadotrophin/ml (Teikokuzoki, Tokyo, Japan), 10 iu hCG/ml (Teikokuzoki), 1 μg 17 β-estradiol/ml (Sigma), 5 μg insulin/ml (Sigma) and 50 μg gentamicin/ml (Sigma).

Experiment 1: Effects of wortmannin on meiotic progression from M I to M II in COCs or denuded oocytes

In order to determine the role of PI 3-kinase in progression from M I to M II in porcine COCs, COCs were cultured for 24 h in the basic medium and then cultured in a medium supplemented with 10^{-8} M and 10^{-7} M wortmannin for another 24 h. Wortmannin, which had been dissolved in dimethyl sulfoxide (DMSO, Sigma) at 5×10^{-3} M and stored at –20°C in the dark, was first diluted with DMSO at 10^{-5} and 10^{-4} M, and then with the basic medium which was pre-incubated for at least 2 h to provide the final concentration (10^{-8} and 10^{-7} M) just before use. These media (10 μl) supplemented with each concentration of wortmannin were added to the 90 μl drops of the basic medium. The final concentration of DMSO was adjusted to 0.098% (v/v) in all maturation media, since this concentration had no significant effects on nuclear maturation in the preliminary experiments. Half the COCs cultured for 24 h were mechanically denuded and simultaneously cultured for 24 h in a maturation medium supplemented with 10^{-8} M and 10^{-7} M wortmannin to investigate whether PI 3-kinase acts principally on progression from M I to M II in oocytes or cumulus cells. After a total of 48 h of cultivation, the COCs were vortexed to remove cumulus cells, then the oocytes were mounted on slides, fixed with acetic acid/ethanol (1:3) for 48 h, and stained with aceto-lacmoid before being examined under a phase-contrast microscope (400 ×) for determination of their nuclear stage [29].

Since wortmannin became less effective after 6 h in a serum supplemented medium [30], we changed the wortmannin supplemented maturation medium every 5 h.

Experiment 2: Effects of LY294002 on progressing from M I to M II in COCs or denuded oocytes

Similarly to Experiment 1, with LY294002 as PI 3-kinase inhibitor, the COCs which had already been cultured in the basic medium for 24 h were cultured for

another 24 h in maturation medium with each concentration of the drug. LY294002, which had been dissolved in DMSO at 7.5×10^{-1} M and stored at –20°C in the dark, was first diluted with DMSO to 0.5 and 5.0×10^{-2} M, and then with the basic medium to provide the final concentration (0.5 and 5.0×10^{-5} M) just before use. These media (10 μl) supplemented with each concentration of LY294002 were added to the 90 μl drops of the basic medium. The final concentration of DMSO was adjusted to 0.098% (v/v) in all maturation media, since this concentration had no significant effect on nuclear maturation in the preliminary experiments. With the same objectives as Experiment 1, after the cultivation for 24 h in the basic medium and the removal of cumulus cells, denuded oocytes were simultaneously cultured in maturation medium supplemented with 0.5 and 5.0×10^{-5} M LY294002 for 24 h. After 48 h of all cultivation, the oocytes were examined to determine their nuclear stage.

Experiment 3: Effects of wortmannin and LY294002 on cumulus cell expansion in COCs

The COCs selected were cultured for 24 h in the basic medium and then cultured for another 24 h in maturation medium supplemented with 0, 10^{-8} and 10^{-7} M wortmannin or 0, 0.5 and 5.0×10^{-5} M LY294002. At the end of the culture, the degree of cumulus oophorus expansion was determined under a phase-contrast microscope. Categories of cumulus oophorus expansion were I, II, III and IV: category I (Fig. 1a) indicated only a detectable degree of expansion, category II (Fig. 1b) indicated the expansion of outer layers of cumulus cells, category III (Fig.1c) indicated the expansion of middle layers of cumulus cells, and category IV (Fig.1d) indicated the expansion of the inner layers of cumulus cells.

Experiment 4: Effects of LY294002 on progression from A/T-I to M II in COCs or denuded oocytes

In order to explore the role of PI 3-kinase in oocytes arrested at A/T-I, the COCs which had already been cultured in the basic medium for 36 h when the propor-

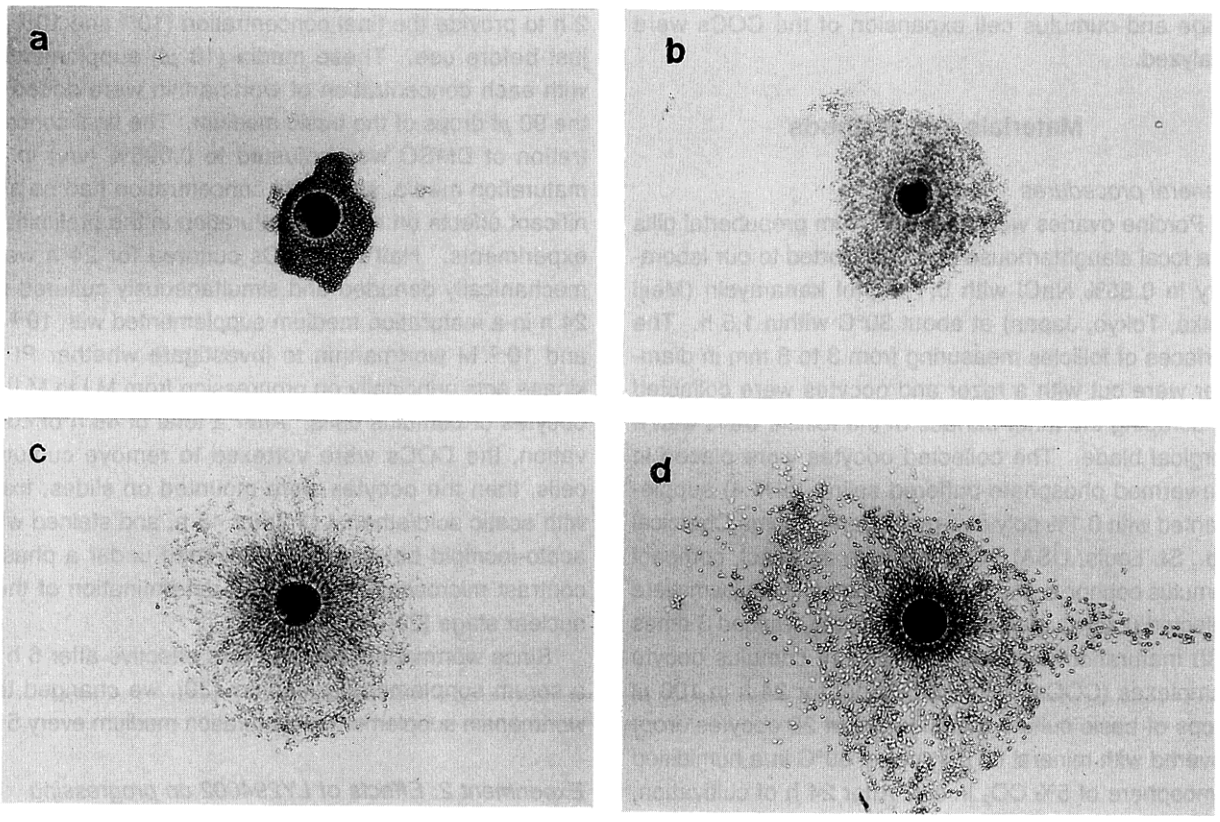

Fig. 1. Porcine COC after maturation for 48 h *in vitro*. (a) Category I: only a detectable expansion, (b) Category II: the expansion of outer layers of cumulus cells, (c) Category III: the expansion of middle and outer layers of cumulus cells, (d) Category IV: expansion of all layers of cumulus cells.

tion of oocytes arrested at A/T-I were highest [9], was further cultured for 12 h in maturation medium supplemented with 5.0×10^{-5} M LY294002. With the same objectives as Experiment 1, after the 36 h-cultivation in the basic medium and the removal of cumulus cells, denuded oocytes were simultaneously cultured in maturation medium supplemented with 5.0×10^{-5} M LY294002 for 12 h. At the end of cultivation, all oocytes were examined to determine of their nuclear stage.

Statistical analysis

Statistical analyses from three replicate trials for comparisons were carried out by analysis of variance (one-way ANOVA) and Fisher's protected least significant difference test. All percentage data were subjected to arc sine transformation before statistical analysis. A probability of $P<0.05$ was considered to be statistically significant.

Results

Experiment 1

In order to check the effects of inhibition of PI 3-kinase activity in COCs on meiotic progression from M I to M II stage, COCs (n=293) were cultured for 24 h in medium supplemented with 10^{-8} and 10^{-7} M wortmannin following the cultivation for an initial 24 h in the basic medium. As shown in Table 1, wortmannin significantly increased the proportion of oocytes arrested at M I compared to the control I. The proportion of M I oocytes in the presence of 10^{-7} M wortmannin was higher than that in the presence of 10^{-8} M wortmannin, although the difference was not significant. Regarding the proportion of oocytes reaching M II, a significant decrease was noted in the presence of 10^{-8} and 10^{-7} M wortmannin compared to the control I but there is no significant difference between 10^{-8} M and 10^{-7} M wortmannin in this proportion.

Oocytes that had been cultured for 24 h in the basic medium were denuded mechanically and cultured for another 24 h in a medium supplemented with 10^{-8} and 10^{-7} M wortmannin. 10^{-8} M wortmannin significantly increased the proportion of oocytes arrested at M I compared to the control II. The maximal proportion of the oocytes arrested at M I was noted in the presence of 10^{-7} M wortmannin. In contrast, wortmannin, at both 10^{-8} and 10^{-7} M, significantly reduced the proportion of oocytes reaching M II compared to the control II. Although not significantly different, the addition of 10^{-7} M wortmannin to a second culture medium resulted in a slightly lower proportion of oocytes reaching M II than with 10^{-8} M wortmannin.

Moreover, there was no significant difference between the controls I and II in the proportions of oocytes arrested at M I or reaching M II. These results showed that denuding of cumulus cells from COCs after 24 h cultivation in the basic medium had no effect on the proportion of oocytes arrested at M I and those reaching M II at the end of 48 h cultivation. Statistical evaluation of differences between the effects of wortmannin on the meiotic progression of either COC or denuded oocytes was done. Differences between control II and 10^{-7} M wortmannin in the proportion of denuded oocytes arrested at M I or reaching M II were significantly diminished compared to that in the case of COCs.

Table 1. Meiotic progression in porcine COCs or denuded oocytes which were cultured for 24 h in maturation medium supplemented with 0 (control), 10^{-8} and 10^{-7}M wortmannin following 24 h cultivation in basic culture medium

	Concentration of wortmannin (M)	Number of oocytes examined	Number of oocytes (%)†					
			GV	Pro-M I	M I	A,T-I	M II	Deg.
COC	Control I	102	6 (6)	2 (2)	12 (11)[a]	3 (3)	71 (70)[a]	8 (8)
	10^{-8}	95	8 (8)	5 (5)	30 (32)[b]	7 (8)	40 (42)[b]	5 (5)
	10^{-7}	96	8 (9)	4 (4)	38 (40)[c]	5 (5)	34 (35)[b]	7 (7)
DO	Control II	98	0 (0)	1 (1)	11 (12)[a]	7 (7)	70 (69)[a]	10 (10)
	10^{-8}	106	2 (2)	2 (2)	21 (19)[b]	7 (6)	63 (61)[b]	11 (10)
	10^{-7}	93	3 (3)	2 (2)	19 (21)[b]	7 (7)	54 (58)[b]	8 (9)

[a-c] Values in the same column with no common superscripts are significantly different for each treatment ($P<0.05$). COC: Cumulus oocyte complex, DO: Denuded oocyte, GV: Germinal vesicle, pro-M I: pro-Metaphase I, M I: Metaphase I, A/T- I: Anaphase and Telophase- I, M II: Metaphase II, Deg.: Degenerated. †As a percentage of the total number of oocytes examined.

Experiment 2

When COCs (n=377) were cultured for a further 24 h in the presence of 0.5 and 5.0 × 10^{-5} M LY294002 following the initial 24 h incubation in the basic medium, at all treatment levels of LY294002, the proportions of oocytes arrested at M I was significantly greater than that of control I. Conversely, the second cultivation with LY294002 significantly reduced the proportion of oocytes reaching M II compared to the control I. The proportion of oocytes reaching M II with 0.5 × 10^{-5} M LY294002 was slightly but not significantly higher than that with 5.0 × 10^{-5} M.

In the case of denuded oocytes (n=369), the presence of 0.5 × 10^{-5} M LY294002 in the second culture medium slightly increased the proportion of oocytes arrested at M I, and marginally reduced the proportion of oocytes reaching M II as compared to the control II, but those differences were not significant. Moreover, the presence of 5.0 × 10^{-5} M LY294002 in the second culture medium produced a significant increase in the proportion of oocytes arrested at M I, and significantly reduced the proportion of oocytes reaching M II as compared to that of 0.5 × 10^{-5} M LY294002 and control II.

Similarly, as in Experiment 1 with wortmannin, there was no significant difference between controls I and II in the proportions of oocytes reaching M II, indicating that denuding of cumulus cells from oocytes has no effect on meiotic progression. As a result, the differences between the control and both LY treatments in the proportions of oocytes arrested at M I or reaching M II were much larger in COCs than in denuded oocytes.

Experiment 3

The aim of this experiment was to verify whether PI 3-kinase is participates directly in the regulation of cumulus expansion. After cultivation for 24 h in the basic medium, COCs (n= 532) were further cultured for 24 h in the presence of 10^{-8} and 10^{-7} M wortmannin or 0.5 and 5.0 × 10^{-5} M LY294002. The effects of both PI 3-kinase inhibitors on the expansion are summarized in Table 3. The number of oocytes in category I was small in all treatments. 10^{-8} M wortmannin and 0.5 × 10^{-5} M LY294002 significantly increased the proportion of COCs in category II and significantly reduced COCs in category IV compared to the respective controls. Moreover, 10^{-7} M wortmannin and 5.0 × 10^{-5} M LY294002 also resulted in a higher proportion of COCs in category II and a lower proportion of COCs in category IV compared to 10^{-8} M wortmannin and 0.5 × 10^{-5} M LY294002, respectively, but the proportion of oocytes in category III, with all tested levels of wortmannin and LY294002, was statistically similar to that in the respective controls.

Experiment 4

In COCs (n=401) cultured for 36 h in the basic medium, the proportions of oocytes developed to M I, A/T-I, and M II were 37%, 24%, and 18% (Table 4), respectively. An additional 12 h-cultivation with LY294002, after the 36 h-cultivation in the basic medium, resulted in significantly lower proportions of oocytes arrested at M I and A/T-I, and a significantly higher proportion of oocytes reaching M II compared to oocytes cultured for only 36 h in the basic medium. In COCs as well as denuded oocytes, significant differences were observed

Table 2. Meiotic progression in porcine COCs or denuded oocytes which were cultured for 24 h in maturation medium supplemented with 0 (control), 0.5 and 5.0 × 10^{-5}M LY294002 following 24 h cultivation in basic culture medium

	Concentration of LY294002 (×10^{-5}M)	Number of oocytes examined	Number of oocytes (%)†					
			GV	Pro-M I	M I	A,T-I	M II	Deg.
COC	Control I	126	13 (10)[a]	5 (4)[a]	11 (9)[a]	0 (0)	86 (68)[a]	11 (9)
	0.5	121	24 (20)[b]	16 (13)[ab]	36 (30)[b]	4 (3)	32 (26)[b]	11 (9)
	5.0	130	34 (26)[b]	22 (17)[b]	39 (30)[b]	0 (0)	25 (19)[b]	9 (7)
DO	Control II	116	9 (8)	0 (0)	20 (17)[a]	4 (3)	77 (66)[a]	6 (5)
	0.5	129	10 (8)	0 (0)	34 (26)[a]	2 (2)	79 (61)[a]	5 (4)
	5.0	124	10 (8)	0 (0)	40 (32)[b]	2 (2)	63 (51)[b]	9 (7)

[a-c] Values in the same column with no common superscripts are significantly different for each treatment ($P<0.05$). COC: Cumulus oocyte complex, DO: Denuded oocyte, GV: Germinal vesicle, pro-M I: pro-Metaphase I, M I: Metaphase I, A/T-I: Anaphase and Telophase- I, M II: Metaphase II, Deg.: Degenerated. † As a percentage of the total number of oocytes examined.

between the oocytes cultured in the basic medium and those cultured in medium supplemented with LY294002 in the proportion of M II oocytes at a similar rate. Furthermore, the differences between the control group and the group of COCs cultured with LY294002 for 12 h after 36 h-cultivation in the basic medium (in this experiment) in the proportions of oocytes arrested at M I or reaching M II, were smaller than those of COCs cultured with LY294002 for 24 h after 24 h-cultivation in the basic medium (Experiment 2).

Discussion

To elucidate whether PI 3-kinase plays a critical role in meiotic progression beyond M I and in cumulus cell expansion, COCs were cultured for 24 h in a medium with wortmannin and LY294002 after an initial cultivation for 24 h in the basic medium. Results of Experiments 1 and 2 showed that wortmannin and LY294002, at all tested levels reduced the proportion of

Table 3. Effects of wortmannin (0, 10^{-8} and 10^{-7}M) or LY294002 (0, 0.5 and 5.0×10^{-5}M) on cumulus cell expansion in porcine COCs

	Concentration (M)	Number of oocytes examined	Category of cumulus expansion (%)†			
			I	II	III	IV
Wortmanin	Control	86	0 (0)	7 (8)[a]	36 (42)	43 (50)[a]
	10^{-8}	87	2 (2)	35 (40)[b]	36 (42)	14 (16)[b]
	10^{-7}	81	3 (4)	34 (40)[b]	3 7(46)	7 (9)[b]
LY294002	Control II	122	0 (0)	20 (16)[a]	41 (34)	61 (50)[a]
	0.5×10^{-5}	118	4 (3)	43 (36)[b]	43 (36)	28 (24)[b]
	5.0×10^{-5}	125	0 (0)	66 (53)[b]	41 (33)	18 (14)[b]

[a-c] Values in the same column with no common superscripts are significantly different for each drug ($P<0.05$). Category I: only a detectable expansion, Category II: expansion of outer layers of cumulus cells, Category III: expansion of middle and outer layers of cumulus cells, Category IV: expansion of all layers of cumulus cells. †As a percentage of the total number of oocytes examined.

Table 4. Meiotic progression in porcine COCs or denuded oocytes which were cultured for 12 h in maturation medium supplemented with 0 (control) and 5.0×10^{-5}M LY294002 following 36 h cultivation in basic culture medium

	Treatment	Number of oocytes examined	Number of oocytes (%)†					
			GV	Pro-M I	M I	A, T-I	M II	Deg.
	36 h[1]	82	8 (10)	4 (5)	30 (37)[a]	20 (24)[a]	15 (18)[a]	5 (6)
COC	Control I[2]	80	8 (10)	6 (8)	10 (12)[b]	2 (2)[b]	52 (65)[b]	2 (2)
	LY294002[3]	80	9 (11)	2 (2)	20 (25)[c]	2 (2)[b]	44 (55)[c]	3 (4)
DO	Control II[4]	79	4 (5)	0 (0)	14 (18)[bc]	3 (4)[b]	54 (68)[b]	4 (5)
	LY294002[5]	80	6 (8)	0 (0)	21 (26)[c]	4 (5)[b]	45 (56)[c]	4 (5)

[a-c] Values in the same column with no common superscripts are significantly different ($P<0.05$). COC: Cumulus oocyte complex, DO: Denuded oocyte, GV: Germinal vesicle, pro-M I: pro-Metaphase I, M I: Metaphase I, A/T- I: Anaphase and Telophase- I, M II: Metaphase II, Deg.: Degenerated. [1] COCs were cultured for 36 h in the basic culture medium. [2] COCs were cultured for 36 h in the basic culture medium and further cultured for 12 h in the basic culture medium. [3] COCs were cultured for 36 h in the basic culture medium and further cultured for 12 h in the medium supplemented with 5.0×10^{-5}M LY294002. [4] COCs cultured for 36 h in the basic culture medium were denuded and these denuded oocytes were further cultured for 12 h in the basic culture medium. [5] COCs cultured for 36 h in the basic culture medium were denuded and these denuded oocytes were further cultured for 12 h in the medium supplemented with 5.0×10^{-5}M LY294002. †As a percentage of the total number of oocytes examined.

oocytes reaching M II in both COCs and denuded oocytes (Tables 1 and 2). The concentrations of both drugs employed in this study are thought to not inhibit other protein kinases and lipid kinases, since they can selectively abolish only PI 3-kinase in human or mouse neutrophils [31, 32]. Therefore it can be assumed that PI 3-kinase activity in either cumulus cells or oocytes is indispensable for regulation of meiotic progression from M I to M II in porcine COCs.

Nevertheless, in denuded oocytes the differences between the two inhibitors (wortmannin at all tested levels and LY294002 at 5.0×10^{-5} M) and the control II in the proportion of oocytes reaching M II were substantially diminished compared to those in the case of COCs. This indicates that in porcine COCs, meiotic progression beyond M I is controlled more by PI 3-kinase in cumulus cells than within the oocyte itself.

Thus, our tentative theory, as described above, that PI 3-kinase in cumulus cells is a main regulator of meiotic progression beyond M I is indirectly supported by the evidence that in porcine COCs the signal determined by reverse transcriptase polymerase chain reaction of IGF- I receptor mRNA in cumulus cells appears to be much stronger than in the oocytes [22], that receptor of EGF has been observed in cumulus cells [23], and that EGF and IGF-1 stimulate nuclear maturation in COCs [27, 33].

On the other hand, Lonergan *et al.* [34] found that EGF stimulated polar body formation in bovine denuded oocytes. Moreover, in cattle, 10^{-7} M wortmannin and 5.0×10^{-5} M LY294002 significantly reduced the proportion of denuded oocytes reaching M II to a similar extent to that in COCs compared to the inhibitor-free control (unpublished data), indicating that the activation of PI 3-kinase in the bovine oocyte itself is linked to meiotic progression from M I to M II. It is therefore assumed that the localization of PI 3-kinase cascade responsible for meiotic progression from M I to M II is different in pigs and cattle.

In porcine COCs at the entry into anaphase I, MPF activity has been shown to decrease to about one-third of that in oocytes at M I and then increase again to the same level as M I at M II [9]. A transient fall in MAP kinase activity was also observed at entry into anaphase I [12]. On the basis of these observation, it is estimated that a transient fall in MPF and MAP kinase activity is necessary for transition of nuclear phase from M I to anaphase I in porcine COCs. Moreover, the results of Experiment 4 showed that in both COCs and denuded oocytes which were cultured in the medium with LY294002 for 12 h after cultured for 36 h, when a transient fall in MPF activity had been recognized [9], many more oocytes progressed beyond M I and reached M II compared to oocytes cultured for only 36 h. And because there is no significant difference between COCs and denuded oocytes in the proportion of oocytes reaching M II, it is thought that the meiotic arrest at M I due to wortmannin and LY294002 in this study might be reflected in the absence of a reduction in MPF and MAP kinase activity in the oocyte. That is PI 3-kinase in cumulus cells may regulate a fall in MPF and MAP kinase activity at an entry into anaphase I.

Meanwhile, it has been shown that oocytes arrested at M I are connected with cumulus cells by oocyte-cumulus gap junctions and these junctions are closed after releasing the first polar body in mice [35] and pig (unpublished data). Experiment 3 showed that wortmannin and LY294002, at all tested levels, significantly decreased the proportion of oocytes in category IV, which means that the oocytes have an expansion of the inner layers of cumulus cells, compared to the respective controls (Table 3). These reports and our results might indicate that the gap-junctional connection between the cumulus cells and oocyte remains unclosed by these drugs. Regulation of gap junctional communication is known to be phosphorylated on connexin-43, a major structural protein of gap junction, by stimulation of EGF and Lysophophatidic acid via either MAP kinase or $pp60^{src}$ tyrosine kinase [36–38]. There is also evidence that both MAP kinase and $pp60^{src}$ tyrosine kinase cascade consist of PI 3-kinase in CHO cells and human T cells [39, 40]. It is therefore proposed that the inhibition of PI 3-kinase activity with wortmannin and LY294002 may lead to restraint of the repression of gap-junctional communication via MAP kinase or $pp60^{src}$ tyrosine kinase.

Very recently Isobe *et al.* [16] provided evidence that further cultivation of porcine denuded oocytes for 24 h after removal of the cumulus cells from porcine COCs pre-incubated for 24 h produced a significant increase in the proportion of oocytes reaching M II compared with COCs cultured continuously for 48 h. They concluded from this result that cumulus cells surrounding oocytes suppressed meiosis of progression from GVBD to M II, and that the suppressive factor for meiotic progression produced in the cumulus cells might be transferred to the oocytes through gap junctions.

Based on their conclusion and our tentative theory derived from this study, it is possible that PI 3-kinase activated by some factors, such as IGF-1 or EGF, in cumulus cells stimulates the inhibition of gap-junctional communication with cumulus cells and oocytes, so that

suppressive factors in meiotic progression produced in cumulus cells can't be transferred to the oocytes, resulting in a transient fall in MPF and MAP kinase activity preceding the transition of nuclear phase from M I to anaphase I.

In summary, two PI 3-kinase specific inhibitors (wortmannin and LY294002) produced a significantly higher proportion of oocytes arrested at M I and a lower proportion of oocytes reaching M II in porcine COCs and denuded oocytes, but in denuded oocytes the differences between the control II and the two inhibitors in the proportion of oocytes reaching M II were substantially diminished compared to those in COCs. Moreover, in both cases in which COCs and denuded oocytes were cultured in the medium with LY294002 for 12 h after being cultured for 36 h when the proportion of oocytes arrested at A/T-I was highest, many more oocytes reached M II, indicating that PI 3-kinase in cumulus cells is a main regulator of meiotic progression beyond M I, and these drugs also inhibited cumulus cell expansion. The evidence from this study suggests that PI 3-kinase in cumulus cells may be associated with the regulation of gap-junctional communication and, indirectly, a fall in MPF and MAP kinase activity in the oocyte at the entry into anaphase I.

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