Hypoxanthine Promotes the Acquisition of Meiotic Competence in Pig Oocytes from Early Antral Follicles during Growth Culture

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Abstract: *Pig early antral follicles (0.5–1.0 mm in diameter) contain oocytes approximately 100 μm in diameter with limited competence for meiotic resumption. Oocyte-cumulus complexes containing parietal granulosa cells (OCGs) were dissected from the ovarian follicles, embedded in collagen gels and cultured in Waymouth's medium containing 5% fetal calf serum. In the first experiment, OCGs were cultured in medium containing 0, 1, 10 or 100 ng/ml of FSH for 8 days, and the viability and growth of oocytes were examined. The mean diameter of the surviving oocytes in each experimental group significantly increased, although the percentage of surviving oocytes was highest in the 10 ng/ml FSH-supplemented group (41% vs. 16–22%). In the second experiment, OCGs were cultured in medium containing 10 ng/ml FSH with or without 2 mM hypoxanthine for 7 days. Regardless of hypoxanthine supplementation, about 45% of the oocytes in each group had normal morphology after the culture and grew to almost the full size of 120 μm. All of these were at the germinal vesicle stage. Recovered oocytes were further cultured for 48 hours so that their meiotic competence could be compared. Of the oocytes cultured in hypoxanthine-free medium, 13% underwent germinal vesicle breakdown, although no oocytes progressed beyond metaphase I. On the other hand, 47% of the oocytes cultured in hypoxanthine-supplemented medium underwent germinal vesicle breakdown, and 9% progressed to metaphase II. These results suggest that hypoxanthine promotes the acquisition of meiotic competence in growing pig oocytes in the medium containing FSH, which supports oocyte viability.*

Key words: *Growing oocyte, Hypoxanthine, In vitro culture, Meiotic competence, Pig*

Mouse oocytes in primordial follicles can be grown in culture to their final size, undergo fertilization *in vitro*, and then develop into live born pups [1]. The birth suggests the possibility that small oocytes in the primordial follicles are a potential source of oocytes for IVF (*in vitro* fertilization) in the mouse as well as other mammalian species [2]. In large species, no culture systems supporting the entire developmental course from the primordial to the fully-grown stage have yet been established, although some culture methods have been developed to support growing oocytes from the mid-growth phase to final size [3–6]. In the pig, Hirao *et al.* [3] reported that oocytes at the mid-growth phase (70–90 μm) in preantral follicles grew to their final size and matured to metaphase II (MII), but the efficiency of the procedure was low.

Pig oocytes in early antral follicles less than 1 mm in diameter are still in their growing phase, and cannot resume meiosis even under the appropriate culture conditions in which fully-grown oocytes mature to MII [7, 8]. As early antral follicles develop into large antral follicles 4–6 mm in diameter in the ovary, pig oocytes reach their fully-grown size, 120 μm, and acquire meiotic competence. *In vitro* growth culture systems must support both the viability of oocytes and the

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acquisition of meiotic competence during the culture period. It has been suggested that tissue culture medium supplemented essentially solely with fetal calf serum can support neither growth nor acquisition of meiotic competence of growing pig oocytes [9]. FSH was reported to promote follicular development in cultured bovine preantral follicles [6, 10–12], and to support oocyte viability in cultured pig preantral follicles [13]. Hypoxanthine, a naturally occurring cAMP-phosphodiesterase inhibitor, is a component of pig and mouse follicular fluid [14, 15], and maintains the association between a mouse oocyte and the surrounding granulosa cells *in vitro* [16]. In addition, hypoxanthine increased the number of morphologically normal oocytes in cultured bovine preantral follicles [17].

In this study, we cultured 100 μm-diameter oocytes from early antral follicles and examined their growth and the acquisition of meiotic competence during the culture. The presumably oocyte-growth stimulating factors described above were added to the medium. Of those, however, the optimal concentration of FSH for oocyte culture may depend on the preparation and purity of the hormone, and the culture system. Therefore, in the first experiment, growing oocytes were cultured in a medium supplemented with FSH at various concentrations to determine its optimal concentration in our culture system. It was clearly suggested that FSH at a concentration of 10 ng/ml significantly supported oocyte viability and growth, but it was also indicated that only a small number of the oocytes resumed meiosis. In the second experiment, we therefore cultured oocytes from early antral follicles in FSH- and hypoxanthine-supplemented medium, and examined the growth of the oocytes and their meiotic competence to mature.

Materials and Methods

Collection of growing oocytes from early antral follicles

Ovaries were obtained from cross-bred gilts slaughtered at a local abattoir. After three washes with Dulbecco's phosphate-buffered saline containing 0.1% polyvinylalcohol, early antral follicles with diameters of 0.5–1.0 mm were dissected from the ovarian cortices in Waymouth's medium (pH 7.2; Waymouth 752/1MB; Sigma Chemical Co., St Louis, MO, USA) containing 1 mg/ml bovine serum albumin (Intergen Co., NY, USA), 0.05 mg/ml sodium pyruvate, 0.336 mg/ml $NaHCO_3$, 0.1 mg/ml kanamycin (Sigma Chemical Co.), and 5 mg/ml HEPES. Connective tissues surrounding the follicles were stripped off with forceps, and healthy early antral follicles, in which no granulosa cells were detached from the follicle wall, were selected under a dissection microscope. Follicles were then opened with fine forceps, and oocyte-cumulus complexes containing parietal granulosa cells (OCGs) were collected. Each of the OCGs was washed in the medium and transferred into a 10 μl drop of HEPES-buffered Waymouth's medium under paraffin oil, where the diameters of the oocytes (excluding zona pellucida) were measured to the nearest 1 μm under an ocular micrometer (Nikon, Tokyo, Japan).

Culture and examination of oocytes

The culture of the oocytes was based on the method for pig preantral follicles described by Hirao *et al.* [3]. Instead of culturing intact preantral follicles, OCGs were dissected out from early antral follicles, and only those containing growing oocytes 90–110 μm in diameter were embedded in collagen gels. The collagen mixture consisted of 0.3% acid collagen solution (Cellmatrix Type I; Nitta Gelatine, Tokyo, Japan), 10 times-concentrated Waymouth's medium, and 0.05 N sodium hydroxide solution containing 22 mg/ml $NaHCO_3$ and 47.7 mg/ml HEPES at a ratio of 8:1:1. The mixture was placed on the bottom of a Petri dish (#1008, Falcon, Becton Dickinson Labware, Lincoln Park, NJ, USA), and OCGs were transferred into the mixture with a small volume of the medium. The gel was put in an incubator at 39°C for 20 min. After gelatinization, 4 ml of the culture medium was poured onto the gel, and the OCGs were cultured at 39°C in a humidified atmosphere of 5% CO_2 and 95% air for 7 or 8 days. The basic culture medium was Waymouth's medium containing 5% fetal calf serum (Bio Whittaker, Walkersville, ML, USA), 22 mg/ml $NaHCO_3$, and 0.05 mg/ml sodium pyruvate. Every third day half the volume of the culture medium was exchanged for fresh medium.

Experimental design

In the first experiment, FSH from pig pituitary glands (UCB-Bioproducts SA, Belgium) was added to the basic culture medium at concentrations of 0, 1, 10 and 100 ng/ml, and the viability and growth of the oocytes were examined. After 8 days of culture, the collagen gels were torn with fine forceps, and the oocytes were recovered. The oocytes were then denuded completely by pipetting, and transferred into 10 μl drops of HEPES-buffered Waymouth's medium. Oocytes that showed evidence of cytoplasmic degeneration were excluded from further analysis. Oocytes with normal morphology were found to be surviving oocytes, and their diameters

Table 1. Growth of pig oocytes from early antral follicles in medium supplemented with FSH at various concentrations

Growth of oocytes	FSH (ng/ml)	No. of oocytes examined	Mean diameter of oocytes before culture (μm)	No. of oocytes surviving [d] (%)	Mean diameter of oocytes after culture [e] (μm)
In vivo [a]		25	103.3 ± 3.6 [h]		
In vivo [b]		25	119.5 ± 1.8 [i]		
In vitro [c]	0	67	103.7 ± 4.2 [h]	11 (16) [f]	115.4 ± 10.1 [i]
In vitro [c]	1	69	104.9 ± 3.9 [h]	15 (22) [fg]	120.0 ± 8.5 [ij]
In vitro [c]	10	68	103.5 ± 4.0 [h]	28 (41) [g]	121.4 ± 8.8 [ij]
In vitro [c]	100	67	103.8 ± 3.7 [h]	14 (21) [fg]	123.4 ± 7.4 [j]

[a] Oocytes from early antral follicles 0.5–1.0 mm in diameter. [b] Oocytes from large antral follicles 4.0 mm in diameter. [c] Oocyte-cumulus complexes with a piece of parietal granulosa tissue from early antral follicles 0.5–1.0 mm in diameter were cultured for 8 days. [d] Oocytes with normal morphology after 8 days of culture. [e] Mean diameter ± SD of surviving oocytes after 8 days of culture. [f, g] Values with different superscripts in the same column differ significantly ($P<0.05$, chi-square test). [h–j] Mean oocyte diameters with different superscripts differ significantly ($P<0.05$, Student's *t*-test).

were measured as described above.

In the second experiment, OCGs were cultured in the basic culture medium supplemented with 1 μg/ml estradiol-17β (Sigma Chemical Co.), 10 ng/ml FSH and with or without 2 mM hypoxanthine (Kohjin Co. Ltd., Tokyo, Japan) for 7 days. After the culture, the oocytes were recovered, denuded, and assessed for degeneration. The diameters of the oocytes that had normal morphology were measured. Some of these oocytes were mounted on slides, fixed in acetic ethanol (1:3), stained with 1% aceto-orcein, and examined for their nuclear morphology under a differential interference microscope. The precise nuclear stage was determined on the basis of changes in the configuration of chromosomes and the nuclear membrane [18]. Other oocytes were further cultured to evaluate their maturational competence. They were cultured in 2 ml of the basic culture medium for 48 hours at 39°C in a humidified atmosphere of 5% CO_2 and 95% air. The oocytes were then stained and examined as described above.

As the control, growing oocytes from early antral follicles of 0.5–1.0 mm in diameter and fully grown oocytes from large antral follicles 4 mm in diameter were collected from ovaries, their diameters were measured, and their nuclear morphology was examined. Some of the oocytes were cultured, and their maturational stage was examined in the same manner.

Statistical analyses

To assess the effects of FSH or hypoxanthine on the growth of oocytes and the maturational competence of *in vitro*-grown oocytes, at least three independent experiments were conducted. Results for all replicates were pooled and analyzed. Statistical differences in the mean diameters of oocytes were analyzed by Student's *t*-test. The other values were analyzed by the chi-square test. A probability of less than 0.05 was considered statistically significant.

Results

Optimal concentration of FSH for oocyte growth

From each ovary, 15–20 early antral follicles with diameters of 0.5–1.0 mm were collected. Granulosa cells of OCGs grew into collagen gels during culture, and oocytes were maintained in the granulosa cells. From the cultured OCGs, denuded oocytes were recovered. Some of the oocytes had degenerated, but 16–41% of the oocytes had normal morphology (Table 1). The rate of survival of the oocytes was significantly higher in 10 ng/ml FSH-supplemented medium than in FSH free medium (41% vs. 16%, $P<0.05$). Surviving oocytes in three FSH-supplemented groups grew to full size, although no significant differences were observed among the mean diameters of the groups.

We first cultured the *in vitro*-grown oocytes so that they would mature for 48 hours, but 15% (4/26) of the oocytes resumed meiosis and reached the diakinesis stage (D), and no oocytes progressed to MI or beyond, so that although the percentage of oocytes surviving the culture was highest in the 10 ng/ml FSH-supplemented medium, FSH alone did not support normal development of the meiotic competence in the oocytes. In the next experiment, we examined the combined effect of FSH and hypoxanthine on the acquisition of meiotic competence in oocytes during growth culture.

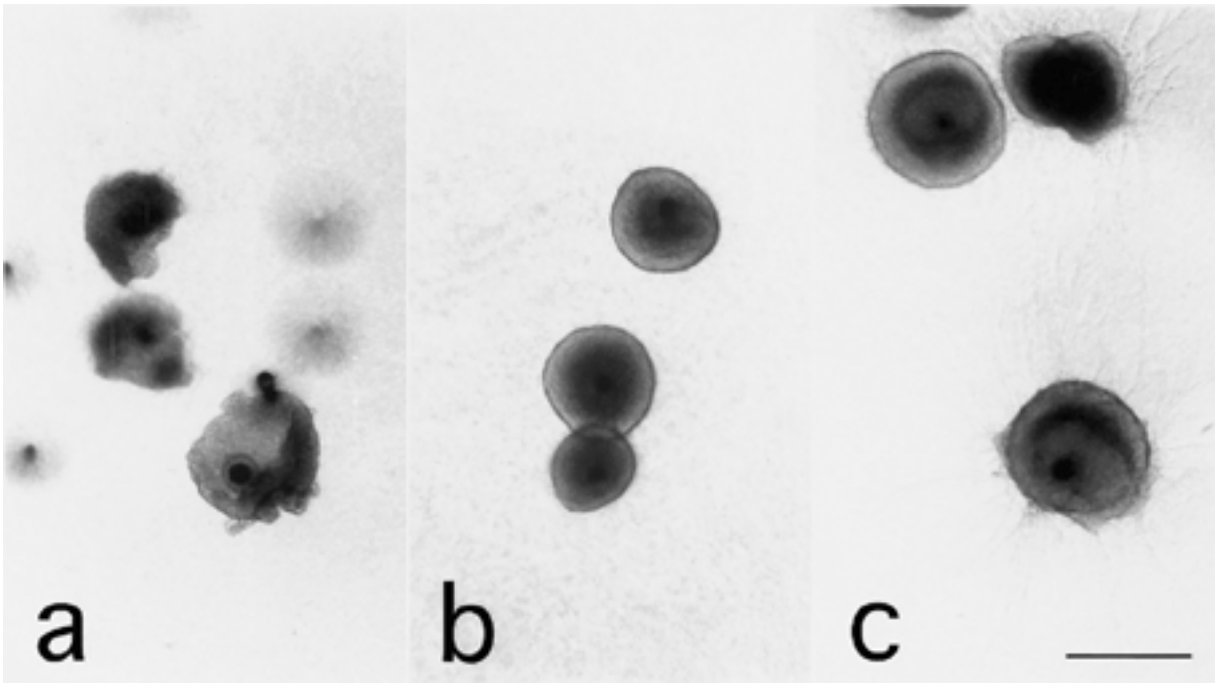

Fig. 1. Pig oocytes-cumulus complexes with parietal granulosa cells (OCGs) from early antral follicles before culture (a). The OCGs were embedded in collagen gels and cultured in 2 mM hypoxanthine- and 10 ng/ml FSH-supplemented medium for 7 days. After 1 day of culture, parietal granulosa cells surrounded the oocytes, and the OCGs formed compact spherical structures (b). The OCGs then formed an antrum-like structure (c). Scale bar: 500 μm.

Effect of hypoxanthine on the growth of oocytes

To assess the maturational competence of *in vitro*-grown pig oocytes, OCGs were cultured for 7 days in medium containing 10 ng/ml FSH and 2 mM hypoxanthine. In this experiment 1 μg/ml estradiol-17β was also added to the medium to promote granulosa cell proliferation. After 1 day of culture, parietal granulosa cells surrounded the oocytes completely, and the OCGs changed into compact spherical structures (Fig. 1b). No apparent morphological differences were observed between the hypoxanthine-free and hypoxanthine-supplemented groups. After 2 days of culture, a single cavity formed inside some of the complexes, specifically those that had an antral follicle-like structure (Fig. 1c). The antral cavities were maintained in some of the OCGs throughout the culture period. After 7 days of culture, denuded oocytes and oocytes loosely attaching to granulosa cells were recovered from the structures. Similar percentages of oocytes survived the culture in hypoxanthine-free (44%,12/27) and hypoxanthine-supplemented (45%, 13/29) groups (Table 2). The mean diameters of the oocytes were 124.6 ± 7.0 and 115.8 ± 5.6 μm in the hypoxanthine-free and hypoxanthine-supplemented groups, respectively. These values were significantly higher than those before the culture.

Effect of hypoxanthine on the meiotic competence of oocytes

The configuration of chromatin in the germinal vesicle (GV) of fully-grown oocytes from 4 mm follicles was different from that in small oocytes from early antral follicles (Table 2). Oocytes from early antral follicles had a GV that contained spreading fine filamentous chromatin (GV 0). On the other hand, fully-grown oocytes had a horseshoe-like heterochromatin rim around a nucleolus (GV I). In OCGs cultured for 7 days, chromatin had started to condense around a nucleolus, and nearly half of the oocytes were at the GV I stage in both experimental groups. All of the oocytes were at the GV stage, and no meiotic resumption was observed during the culture period.

Meiotic competence of the oocytes was found to be significantly different in these two groups cultured with and without hypoxanthine (Table 3). Only 13% (3/24) of the oocytes grown in hypoxanthine-free medium underwent germinal vesicle breakdown; one oocyte reached metaphase I (MI), and no oocytes progressed

Table 2. Growth and nuclear morphology of pig oocytes derived from early antral follicles after culture in hypoxanthine-supplemented medium

Growth of oocytes	Hypoxanthine (mM)	No. of oocytes examined	Mean diameter of oocytes before culture (μm)	No. of surviving oocytes [d]	Mean diameter of oocytes after culture (μm)	Nuclear morphology of oocytes [e] (%)					
						GV 0	GV I	GV II-IV	D	MI	MII
In vivo [a]		20	101.9 ± 1.9 [f]			20 (100)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)
In vivo [b]		20	120.3 ± 2.2 [g]			2 (10) [j]	13 (65) [j]	5 (25)	0 (0)	0 (0)	0 (0)
In vitro [c]	0	27	101.1 ± 3.8 [f]	12	124.6 ± 7.0 [h]	5 (42) [jk]	7 (58) [jk]	0 (0)	0 (0)	0 (0)	0 (0)
In vitro [c]	2	29	101.0 ± 3.1 [f]	13	115.8 ± 5.6 [i]	7 (54) [j]	6 (46) [j]	0 (0)	0 (0)	0 (0)	0 (0)

[a] Oocytes from early antral follicles 0.5–1.0 mm in diameter. [b] Oocytes from large antral follicles 4.0 mm in diameter. [c] Oocyte-cumulus complexes with a piece of parietal granulosa tissue from early antral follicles 0.5–1.0 mm in diameter were cultured in 10 ng/ml FSH-supplemented medium for 7 days. [d] Oocytes showing normal morphology after 7 days of culture were examined for nuclear morphology. [e] Nuclear morphology was determined on the basis of the criteria of Motlik and Fulka [18]. Nuclear morphology before GV I was classified as GV 0. [f–i] Mean oocyte diameters with different superscripts differ significantly ($P<0.05$, Student's *t*-test). [j, k] Values with different superscripts in the same column differ significantly ($P<0.05$, chi-square test).

Table 3. Maturational stage of pig oocytes after growth culture followed by maturation culture

Growth of oocytes	Hypoxanthine (mM)	No. of oocytes examined	Mean diameter of oocytes before culture (μm)	No. of surviving oocytes [d]	Mean diameter of oocytes after culture (μm)	Nuclear morphology of oocytes [e] (%)					
						GV 0	GV I	GV II-IV	D	MI	MII
In vivo [a]		20	100.9 ± 4.9 [f]			20 (100)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)
In vivo [b]		20	119.1 ± 3.8 [g]			0 (0)	2 (10) [h]	1 (5)	1 (5)	3 (15) [hi]	13 (65) [h]
In vitro [c]	0	52	102.5 ± 3.7 [f]	24	117.8 ± 4.8 [g]	5 (21)	14 (58) [i]	2 (8)	2 (8)	1 (5) [h]	0 (0)
In vitro [c]	2	52	101.9 ± 4.0 [f]	23	120.7 ± 5.7 [g]	5 (22)	5 (22) [hi]	2 (9)	1 (4)	8 (34) [i]	2 (9) [i]

[a–c, e] See footnotes to Table 2. [d] Oocytes showing normal morphology after 7 days of culture were further cultured to maturation, and examined for nuclear morphology. [f, g] Mean oocyte diameters with different superscripts differ significantly ($P<0.05$, Student's *t*-test). [h, i] Values with different superscripts in the same column differ significantly ($P<0.05$, chi-square test).

beyond that stage. On the other hand, about half of the oocytes grown in hypoxanthine-supplemented medium resumed meiosis, and 43% (10/23) of the oocytes reached MI or beyond. Moreover, two oocytes matured to MII with the emission of the first polar body. Since the oocytes just after isolation from early antral follicles were not competent to resume meiosis, it is clear that some oocytes had acquired the meiotic competence during the growth culture period.

Discussion

In the first experiment, pig oocytes approximately 100 μm in diameter grew to their final size after 8 days of culture in all of the experimental groups, but the number of oocytes surviving the culture was significantly higher in the 10 ng/ml FSH-supplemented group. FSH stimulates DNA synthesis and the proliferation of pig granulosa cells in small antral follicles [19], and stimulates estrogen production in granulosa cells [20, 21]. Low concentrations of FSH have been reported to support oocyte viability in cultured pig preantral follicles [13]. In addition, FSH prevents apoptosis in cultured pig granulosa cells [22]. FSH was thought to stimulate the proliferation of granulosa cells and to prevent their apoptosis in cultured OCGs in this experiment. Consequently, enclosed oocytes kept their viability during the culture period.

Oocytes maintained the GV stage during the growth culture period, but they changed the chromatin configuration in the germinal vesicles. In the typical pattern found in the small pig oocytes before culture, decondensed filamentous chromatin is distributed throughout the germinal vesicles. On the other hand, in fully-grown oocytes in ovaries, a chromatin ring forms around the nucleolus, showing the morphology of "GV I" staged-oocytes as characterized by Motlik and Fulka [18]. About a half of the surviving oocytes developed to stage GV I. In the mouse, the acquisition of meiotic competence during oocyte growth is closely correlated with a change in chromatin morphology from the diffuse to the perinucleolar condensed state [23]. Pig oocytes

with a GV I configuration of chromatin grown in the ovary can mature to MII [8]; therefore, we supposed the *in vitro*-grown oocytes to have acquired meiotic competence, but they had only limited ability to resume meiosis in our preliminary experiment. This finding suggests that the acquisition of meiotic competence by the oocytes was dissociated from their growth and from the change in chromatin configuration in the FSH-supplemented condition in this experiment. *In vitro*-grown mouse and bovine oocytes resume meiosis spontaneously when the culture media lack meiosis-inhibiting substances, such as IBMX (3-isobutyl-1-methylxanthine) or hypoxanthine [4, 24]. On the other hand, no pig oocytes resumed meiosis during the growth culture period in this study (see Table 2). This finding suggests that oocytes of this species require some other factor(s) if they are to acquire meiotic competence.

Hypoxanthine has been reported to maintain the association between a cultured oocyte and the surrounding granulosa cells in the mouse [15] and cow [4], and to promote the growth of oocytes, but most of the recovered pig oocytes had been denuded of granulosa cells after culture regardless of the presence of hypoxanthine in the culture medium, so that from this point of view, the positive effect of hypoxanthine was not evident under our culture conditions. To our surprise, however, hypoxanthine did promote the acquisition of meiotic competence. The exact mechanism(s) underlying the promotion of maturational competence found in this study is not known. Since hypoxanthine is a naturally occurring cAMP-phosphodiesterase inhibitor, hypoxanthine in our culture medium was thought to maintain the cAMP level in granulosa cells as well as in oocytes. If so, it may be a case similar to that of cultures supplemented with dibutyryl cAMP or forskoline, an adenylate cyclase activator, that are shown to promote the acquisition of meiotic competence in naked growing mouse oocytes [25]. But it has not yet been made clear how the increase in cAMP in oocytes promotes the acquisition of the competence. Rather, it seems to be more important that our data might suggest that hypoxanthine plays several different roles in the final stage of oocyte growth *in vivo*. Antrum formation by granulosa cells and the acquisition of meiotic competence of oocytes appear to be well coordinated in the ovary, which is the period in which hypoxanthine is accumulated in the follicular fluid. The follicular fluid eventually contains hypoxanthine at a high concentration of 1.4 mM [14]. It is well known that this high level of hypoxanthine is one of the meiosis-inhibiting factors contained in follicular fluid, and is crucial in preventing the spontaneous meiotic resumption of oocytes in their follicles [25]. In addition to this role of maintaining meiotic arrest in meiotic-competent oocytes, as suggested in this study, hypoxanthine may participate in the acquisition of meiotic competence by pig oocytes during their final growth stage in the ovary.

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