In vitro growth and maturation of pig oocytes

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Preantral follicles containing oocytes of 70–89.5 µm in diameter were isolated from pig ovaries and cultured in collagen gel for up to 16 days, in the presence of serum, FSH and oestradiol. Formation of follicular antra occurred as the culture proceeded. The oocytes had been enclosed by granulosa cells and contacts between the oocytes and processes of the enclosing cumulus cells were maintained over the culture period. After 16 days of culture, 30–40% of the oocytes were of normal appearance, and the diameters of about half of these oocytes were larger than 100 µm. When the oocytes grown *in vitro* were liberated from the follicles and cultured for a further 48 h in modified Krebs–Ringer bicarbonate solution, 6, 30 and 60% of the oocytes larger than 90, 100 and 110 µm underwent germinal vesicle breakdown, respectively. Progression to metaphase II was observed in 40% of oocytes that were over 110 µm in diameter, whereas no oocyte less than 90 µm in diameter resumed meiosis. The relationship between the size and meiotic competence of oocytes was similar for oocytes grown *in vitro* or *in vivo*. Oocytes grown and matured *in vitro* were penetrated by spermatozoa and formed a female pronucleus, but decondensation of the sperm head was incomplete. The results demonstrate for the first time that pig oocytes from preantral follicles can grow up to their final size, acquire meiotic competence, and be penetrated by spermatozoa *in vitro*.

Introduction

In mammalian ovaries, oocytes begin to grow and increase in diameter while the nucleus remains in the first meiotic prophase. After the preovulatory surge of gonadotrophin, only fully grown oocytes can resume meiosis, complete the first meiotic division, and are ovulated. Although a large number of oocytes are present in ovaries (Gosden and Telfer, 1987), only a very small population of oocytes becomes available for ovulation, and most of the oocytes are lost at various stages of growth in the ovary.

In vitro culture of oocytes isolated from ovaries has been thought to be potentially useful for oocytes that are lost owing to atresia of follicles (Eppig *et al.*, 1990). The fully grown mammalian oocytes isolated from antral follicles can mature, be fertilized and develop into live young in mice (Schroeder and Eppig, 1984), rats (Vanderhyden and Armstrong, 1989), sheep (Staigmiller and Moor, 1984), cattle (Goto *et al.*, 1988) and pigs (Mattioli *et al.*, 1989).

Recently, *in vitro* culture techniques for growing oocytes of rodents have been improved to assist investigation of the mechanisms of oocyte and follicular development. Under appropriate conditions, meiotically incompetent oocytes from

preantral follicles can grow to final size and complete nuclear maturation *in vitro* (Eppig, 1977; Eppig and Downs, 1987; Daniel *et al.*, 1989; Hirao *et al.*, 1990; Carroll *et al.*, 1991). Furthermore, successful production of live young from oocytes that have been grown and matured *in vitro* demonstrates that the oocytes can acquire full developmental capacity *in vitro* (Eppig and Schroeder, 1989; Eppig *et al.*, 1992).

For livestock production, culture of growing oocytes could provide a large population of female germ cells. In the study reported here, a culture system is described that will support the growth of pig oocytes from mid-growth stage to final size. Such oocytes acquire the capacity to resume and complete the first meiotic division and be penetrated by spermatozoa.

Materials and Methods

Collection of preantral follicles

Ovaries were collected from prepubertal gilts at a local abattoir and transported to the laboratory within 1 h, while preventing any abrupt temperature changes. The ovaries were washed with PBS and Eagle's minimum essential medium (MEM; Nissui Pharmaceutical Co., Ltd, Tokyo), Hepes (Nacalai Tesque, Inc., Kyoto) buffered with, and cut into small pieces

(2–3 mm) with a blade. The pieces were incubated in Hepes-buffered MEM containing 0.1% collagenase (Wako Pure Chemical Industries, Ltd, Osaka) for 1 h at 39°C. After the treatment, preantral follicles were removed from the connective tissues and freed from adherent thecal cells using fine forceps and a needle. Follicles with diameter of 0.2–0.3 mm were collected with a mouth-operated fine pipette and washed three times with Hepes-buffered MEM containing 2% fetal calf serum (FCS; Filtron Ltd, Victoria). Each follicle was transferred into a microdrop of the medium under paraffin oil, and the diameters of oocytes (excluding zona pellucida) were measured with an ocular micrometer attached to an inverted microscope. Only follicles containing oocytes with diameters of 70–89.5 µm were used for the culture.

Culture of follicles and recovery of oocytes

Freshly isolated follicles were separated into two classes according to oocyte diameter, 70–79.5 and 80–89.5 µm, and were cultured for 16 days and 12 days, respectively. Collagen gel solution was prepared by mixing 0.3% acid-soluble collagen solution (Cellmatrix-I; Nitta Gelatine Co. Ltd., Osaka), ten times concentrated Waymouth MB752/1 medium (Sigma Chemical Co., St Louis, MO) without bicarbonate, and 0.05 mol NaOH l⁻¹ containing 22 mg NaHCO₃ ml⁻¹ and 47.7 mg Hepes ml⁻¹, at concentration 8:1:1 (v:v:v). Isolated follicles were washed three times in the culture medium (described below), and five follicles in a small volume of medium were placed in the centre of a plastic dish (35 mm × 10 mm; Falcon, Becton Dickinson Labware, Lincoln Park, NJ) and overlaid with 0.4 ml of the collagen gel solution. When the collagen gels had set after incubation for 20 min at 39°C, 3 ml of the culture medium was poured into the dishes. The follicles were cultured at 39°C under an atmosphere of 5% CO₂ and 95% air. The culture medium used was Waymouth MB752/1 supplemented with 50 µg sodium pyruvate ml⁻¹ (Nacalai Tesque, Inc.), 100 µg penicillin ml⁻¹ (Penicillin G potassium; Meiji Conf. Co., Tokyo), 50 µg streptomycin ml⁻¹ (Streptomycin sulfate; Meiji Conf. Co.), 5% FCS, 2 miu FSH ml⁻¹ (from porcine pituitary; Sigma Chemical Co.) and 1 µg oestradiol ml⁻¹ (Sigma Chemical Co.). Supplementation of both hormones supported follicular growth *in vitro* in the preliminary experiments. One-half of the medium was changed every 4 days. At the end of the culture, the collagen gel matrices were treated with 0.1% collagenase solution and the follicles were recovered. Oocytes collected from the follicles were denuded by pipetting and their growth and morphology were examined under an inverted microscope. Oocytes in which the ooplasm was deformed and disintegrated were considered as degenerated. The diameters of oocytes with normal appearance were measured with an ocular micrometer.

Some follicles were fixed in 4% formaldehyde in phosphate buffer after culture for histological examination. For light microscopy, the fixed follicles were dehydrated and embedded in JB-4 (Polysciences Inc., Warrington, PA). They were serially sectioned by 2 µm, stained with periodic acid–Schiff and haematoxylin, and examined. For electron microscopy, follicles were post-fixed in osmium tetroxide. They were then dehydrated, embedded in epoxy resin (Luveak 812; Nacalai Tesque,

Inc.), and serially sectioned into ultrathin sections. The follicles were stained with uranyl acetate and lead citrate, and examined under an electron microscope (1200EX; Jeol, Tokyo).

In vitro maturation and fertilization of oocytes

In the second experiment, the maturational competence of oocytes grown *in vitro* was assessed. The follicles containing oocytes with diameters of 70–89.5 µm were cultured for 16 days as described previously. After culture, the oocytes enclosed by one or two layers of cumulus cells were collected from the follicles, washed three times with modified Krebs–Ringer bicarbonate (mKRB) solution (Toyoda and Chang, 1974) and transferred into microdrops (10 µl) of the solution covered with paraffin oil in a plastic dish (60 mm × 15 mm; Falcon). They were cultured for 48 h at 39°C in 5% CO₂ and 95% air; oocytes that had acquired meiotic competence would undergo spontaneous maturation. As a control, oocyte–cumulus cell complexes isolated from ovarian follicles with diameters less than 3 mm were used. After measurement of diameters of the oocytes, they were cultured in the same manner. After 48 h, some of the oocytes were denuded mechanically and mounted on slides. They were then fixed in acetic alcohol, stained with 1% aceto–orcein and examined for the stage of meiotic maturation under a phase-contrast microscope.

The remaining oocytes were transferred into microdrops (100 µl) of the fertilization medium, BO solution (Brackett and Oliphant, 1975) containing 10 mg BSA ml⁻¹ (Sigma Chemical Co.) and 2 mmol caffeine l⁻¹ (Sigma Chemical Co.), and covered with paraffin oil. The oocytes were inseminated by epididymal boar spermatozoa using the method described by Nagai *et al.* (1988) with some modifications. Frozen spermatozoa were thawed in TCM 199 (Gibco, Grand Island, NY) containing 3 mg BSA ml⁻¹ at 37°C. The suspension was centrifuged for 3 min at 700 g, and the supernatant was discarded. The spermatozoa were resuspended in TCM 199 (pH 7.8) containing 12% FCS. After another washing with centrifugation, the spermatozoa were diluted with the same medium to a concentration of 2–4 × 10⁸ cells ml⁻¹, and the suspension was incubated for 30 min at 37°C under an atmosphere of 5% CO₂ and 95% air. A portion of the preincubated sperm suspension was introduced into the fertilization medium containing oocytes grown *in vitro*, so that the final concentration of spermatozoa was 2 × 10⁶ cells ml⁻¹, and cultured at 39°C in 5% CO₂ and 95% air. Ten hours after insemination, the oocytes were mounted on slides, fixed in acetic alcohol and stained with 1% aceto–orcein to ascertain sperm penetration and formation of pronuclei.

Results

Oocyte growth in vitro

Oocytes at mid-growth phase were clearly visible in all preantral follicles isolated from the ovaries (Fig. 1). In the follicles embedded in the collagen gels, oocytes were enclosed in granulosa cells throughout the culture period. The follicles grew in the gels, in particular those that developed an antral

Fig. 1. Preantral follicles isolated from pig ovaries. Oocytes are visible in follicles. Bar represents 200 μm .

Fig. 2. Pig oocyte-cumulus cell complexes obtained after 16 days of culture. Bar represents 100 μm .

cavity (19%) became two to three times larger than before culture. When these antral follicles were opened with fine forceps, oocytes enclosed by one or two layers of cumulus cells were liberated (Fig. 2).

Oocyte growth was obvious after the culture *in vitro*. Sixty follicles containing oocytes with diameter of 70–79.5 μm were cultured for 16 days, and 26 oocytes (43%) were of normal morphology at the end of the culture. The mean diameter of the oocytes increased from $74.0 \pm 3.2 \mu\text{m}$ to $95.3 \pm 7.3 \mu\text{m}$. However, when 84 follicles containing oocytes with diameters of 80–89.5 μm were cultured for 12 days, 27 oocytes (32%) were normal. The mean diameter increased from $83.9 \pm 3.3 \mu\text{m}$ to $100.5 \pm 6.9 \mu\text{m}$. Moreover, in both groups there were a few oocytes that were near to final size, with diameter greater than 115 μm .

Histological examination of sections of antral follicles fixed after the growth culture showed that the morphology of follicles developing in ovaries was similar, except that the cultured follicles had no thecal cells (Fig. 3). The pycnotic nuclei

of granulosa cells were rarely observed, and the oocytes had normal cytoplasm and an intact germinal vesicle with nucleolus. Another noticeable characteristic of the cultured oocytes was the considerably thinner zona pellucida compared with that of oocytes of similar size that were grown *in vivo*. A number of oocyte microvilli and processes of the enclosing cumulus cells projected into the zona pellucida (Fig. 4). Contacts between the oocytes and processes of the cumulus cells were observed. An intact nuclear membrane was also visible (not shown).

Maturation of oocytes grown *in vitro* and *in vivo*

The meiotic competence of oocytes grown *in vitro* was assessed and compared with that of oocytes grown *in vivo*. A total of 486 follicles containing oocytes with diameters of 70–89.5 μm were cultured for 16 days, and the 151 (31%) oocytes recovered were cultured for a further 48 h. A portion of oocytes was fixed and examined before maturation culture, and oocytes were confirmed to be in the germinal vesicle stage. After maturation culture, emission of a first polar body was obvious in four oocytes under the inverted microscope. Cumulus expansion was not observed.

The meiotic competence of the oocytes grown both *in vitro* and *in vivo* was correlated with oocyte size (Table 1). After culture for 48 h, all of the oocytes with diameters of 70–89.5 μm isolated from ovaries remained at the germinal vesicle stage, indicating that they were meiotically incompetent. In contrast, 6, 53 and 77% of the oocytes grown *in vivo* that had diameters greater than 90, 100 and 110 μm , respectively, underwent germinal vesicle breakdown. Four and 41% of the oocytes greater than 100 and 110 μm , respectively, progressed to metaphase II. However, 6, 30 and 60% of oocytes grown *in vitro* that were larger than 90, 100 and 110 μm , respectively, underwent germinal vesicle breakdown. None of the oocytes smaller than 90 μm resumed meiosis. Progression to metaphase II was observed in 40% of oocytes with diameters greater than 110 μm (Fig. 5).

Fig. 3. Histological section of a pig follicle that developed an antral cavity during 12 days of culture in the presence of FSH and oestradiol. Note the absence of thecal cells. The section was stained with periodic acid–Schiff and haematoxylin. Bar represents 100 μ m.

In vitro fertilization

When oocytes grown *in vitro* ($n = 36$) were inseminated after 48 h of maturation culture, sperm penetration was observed in three oocytes. In all of these oocytes, the female pronucleus was observed with sperm head(s) detached from their corresponding sperm tails, whereas enlargement of the sperm head was incomplete (Fig. 6), and emission of the second polar body was never observed.

Discussion

Growth of oocytes *in vitro* has been studied in mice (Eppig, 1977; Eppig and Downs, 1987; Eppig and Schroeder, 1989; Hirao *et al.*, 1990; Carroll *et al.*, 1991) and rats (Daniel *et al.*, 1989). Live young were produced from mouse oocytes grown and matured *in vitro* after transfer to foster mothers (Eppig and Schroeder, 1989; Eppig *et al.*, 1992). However, *in vitro* culture techniques for growing oocytes of domestic animals have not been established yet.

Fig. 4. Electron micrograph of a pig oocyte cultured for 16 days *in vitro*. Note the presence of a contact (inset) between the oocyte and cumulus cell process that traverses the zona pellucida (ZP). Bar represents 2 μ m.

Table 1. Relationship between oocyte diameter and meiotic maturation in pig oocytes grown *in vivo* and *in vitro*

Oocyte growth	Oocyte diameter (µm)	Examined	Number of oocytes (%)				
			Resuming meiosis				Degenerating
			Total	LD	Met I	Met II	
<i>In vivo</i>	70–89.5	34	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)
	90–99.5	32	2 (6) ^a	2 (6)	0 (0)	0 (0)	2 (6)
	100–109.5	47	25 (53) ^b	8 (17)	15 (32)	2 (4) ^a	0 (0)
	110–120	34	26 (77) ^b	7 (21)	5 (15)	14 (41) ^b	1 (3)
<i>In vitro</i>	70–89.5	35	0 (0)	0 (0)	0 (0)	0 (0)	1 (3)
	90–99.5	51	3 (6) ^a	3 (6)	0 (0)	0 (0)	3 (6)
	100–109.5	50	15 (30) ^c	7 (14)	8 (16)	0 (0)	3 (6)
	110–120	15	9 (60) ^{bc}	1 (7)	2 (13)	6 (40) ^b	1 (7)

LD: late diakinesis; Met I: metaphase I; Met II: metaphase II.

^{abc} Values with different superscripts in the same column are significantly different. ($P < 0.05$, chi-square test). **Fig. 5.** Photomicrograph of a pig oocyte after maturation culture for 48 h following 16 days of growth culture. The whole-mounted oocyte was fixed in acetic alcohol and stained with 1% aceto-orcein. Note the metaphase II spindle and an emitted polar body. Bar represents 20 µm. **Fig. 6.** Photomicrograph of a pig oocyte grown and matured *in vitro* that was fixed 10 h after insemination with frozen-thawed epididymal spermatozoa. The whole-mounted oocyte was fixed in acetic alcohol and stained with 1% aceto-orcein. Within the vitellus, a female pronucleus (FP) and a neighbouring sperm head (arrowhead) are seen. Bar represents 20 µm.

Pig oocytes from antral follicles were used for *in vitro* maturation experiments (see Moor *et al.*, 1990), in which duration of culture was less than 48 h. In the study reported here, a long-term culture system was developed for pig oocytes at mid-growth phase; and this culture system supported the growth and acquisition of meiotic competence of the oocytes. From studies in rodents, intimate association of granulosa cells with the oocyte was demonstrated as essential for oocyte growth. Results on the growth of pig oocytes reported here are in agreement with the finding that the enclosing granulosa cells are required. In a preliminary experiment, in which preantral pig follicles were settled on the surface of the dish, the surrounding granulosa cells migrated away from the oocyte as reported in mouse oocyte-granulosa cell complexes (Eppig, 1977). Eventually, most of the pig oocytes

became denuded, and failed to grow. A culture system using collagen gel which supports mouse follicular development up to multilaminar stages was reported by Torrance *et al.* (1989). When the preantral pig follicles were embedded in collagen gels, the oocytes were held within the layers of granulosa cells and grew to nearly final size during the culture. Moreover, ultrastructural observation indicated that contacts between the oocytes and processes of cumulus cells were maintained after the culture. Throughout the oocyte growth in the ovary, granulosa cells are coupled with oocytes via heterologous gap junctions (Anderson and Albertini, 1976) through which small molecules such as energy substrates, nucleotides and amino acids are transferred into the oocytes (Heller *et al.*, 1981;

Brower and Schultz, 1982). It is therefore possible that active metabolic cooperativity is maintained between the pig oocytes and granulosa cells through gap junctions under the culture conditions used in the present study.

In a previous study, we cultured pig preantral follicles for 4 days in the presence and absence of FSH, and found that FSH enhances the viability and growth of oocytes (Hirao *et al.*, 1992). In the study reported here, preantral follicles were cultured for much longer. Not only oocyte growth to nearly final size but also formation of follicular antra were observed in the presence of FSH, whereas antrum formation never occurred in the absence of FSH (data not shown). In hamsters (Roy and Greenwald, 1989) and mice (Nayudu and Osborn, 1992), antrum formation does not occur in preantral follicles unless FSH is present in the culture medium. In rats, FSH stimulates an antrum-like reorganization of the granulosa cells in preantral follicles (Gore-Langton and Daniel, 1990). Antrum formation of preantral follicles *in vitro* therefore seems to be profoundly affected by FSH. However, it is considered that antrum formation is not necessary for the acquisition of maturational competence of mouse oocytes, and that FSH has little effect on oocyte growth, since oocytes can grow to full size *in vitro* without formation of an antrum in the absence of hormones (Eppig, 1977). In pigs, the antrum begins to differentiate in follicles with diameters of 0.4–0.8 mm (Crozet *et al.*, 1981), in which oocytes are still growing and have a limited ability to resume meiosis (Motlik *et al.*, 1984). Our finding of a beneficial effect from FSH on the growth of pig oocytes suggests that follicular differentiation by FSH involves functional changes, which affect the final growth of the oocytes. Alternatively, FSH could stimulate formation of gap junctions between oocytes and granulosa cells, as reported in the homologous gap junctions between porcine granulosa cells cultured with FSH (Amsterdam *et al.*, 1988).

In the study reported here, pig preantral follicles were used, which contained oocytes with diameters of 70–89.5 μm . When these oocytes were liberated from the follicles, they did not resume meiosis after maturation culture. Although this finding suggests that the oocytes are meiotically incompetent, they resumed meiosis after growth culture and, moreover, some of them completed the first meiotic division. The meiotic competence of pig oocytes seems to be acquired in two steps as they approach full size in the ovary: (1) resuming meiosis and (2) completing the first meiotic division (Motlik *et al.*, 1984). This assumption was elucidated from evidence that oocytes with a mean diameter of 100 μm did not progress beyond metaphase I, whereas those with a mean diameter of 115 μm appeared to complete meiotic maturation. In the study reported here, similar results were obtained for oocytes grown both *in vitro* and *in vivo*. Meiotic resumption was observed in oocytes with diameter greater than 90 μm , and the rate of germinal vesicle breakdown increased with increase in oocyte diameter. Completion of the first meiotic division was observed only in those oocytes near full size. Hence, it is clear that oocytes grown under the culture conditions acquired maturational competence sequentially at similar growth stages to those observed *in vivo*.

In pig oocytes, protein synthesis is required for both germinal vesicle breakdown and the subsequent transition from

metaphase I to metaphase II (Fulka *et al.*, 1986; Motlik *et al.*, 1991). Oocytes grown *in vitro* are therefore equipped to synthesize the protein(s) required for maturation-promoting factor activation (Hashimoto and Kishimoto, 1988).

There have been reports concerning the fertilization of pig oocytes matured *in vitro*. Since oocytes have a limited ability to form male pronuclei under insufficient maturational conditions (Motlik and Fulka, 1974; Moor *et al.*, 1990), it has been assumed that, even though oocytes complete the first meiotic division, many do not complete cytoplasmic maturation. This was also observed in the study reported here in which in all oocytes the development of male pronuclei was arrested. This failure was probably due to the inadequacy of the so-called cytoplasmic maturation of oocytes. Considerable improvements in the culture systems for growth of pig oocytes, suggested from mouse studies (Eppig *et al.*, 1990), or for oocyte maturation (Yoshida *et al.*, 1992), could help to ensure normal post-penetration changes in pig oocytes grown and matured *in vitro*.

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