—Review—

In Vitro Growth of Oocytes from Domestic Species

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Abstract: *A huge number of small oocytes are contained in the ovaries of the pig and cow. A small number of them grow from the minimal size of 30 μm in diameter to the final size of 120–125 μm, then mature, and are ovulated. A large number of the remaining oocytes do not enter the growth phase or degenerate in the ovary. Oocyte growth takes a long time and is coordinated with surrounding follicle cells. During the growth phase, oocytes are required to be arrested at prophase I (GV stage), and to acquire the meiotic competence to mature to metaphase II. IVG culture systems have been developed for domestic species, although they are still being improved. IVG culture systems for small oocytes are expected to provide a new source of oocytes for livestock production as well as a better understanding of the basic mechanisms of oogenesis/folliculogenesis in the ovary.*

Key words: *Cow, Follicle, Oocyte growth, Ovary, Pig*

Introduction

The first great advancement in *in vitro* growth (IVG) culture of small oocytes was archieved by the production of a baby mouse by Drs. Eppig and O'Brien in 1996 [1]. The baby mouse was produced from oocytes in the primordial follicles by two sequential IVG culture methods (organ culture of newborn mouse ovaries for 8 days, followed by oocyte-granulosa cell-complex culture for 14 days; see below), IVM (*in vitro* maturation), IVF (*in vitro* fertilization), and ET (embryo transfer). In the last year, two epoch-making results were published in two scientific journals, "Nature" and "Science". One was by Obata *et al.* [2], who produced baby mice derived from premeiotic female germ cells by organ-culture of gonads from 12.5-day-old fetuses followed by nuclear transfer of the nucleus from the produced oocytes to fully grown oocytes (see also Obata *et al.* in this issue). The other was by Snow *et al.* [3], who produced mature oocytes, which could subsequently be fertilized and develop into fertile adult mice, by xenotransplantation of 3-week-old mouse ovaries into immunodeficient nude rats. Although the original objectives of these two experiments were different, we now have two methods that can produce mature oocytes (eggs) from small ovarian oocytes as well as female primordial germ cells in the mouse. These methods strongly suggest the possibility of utilizing small oocytes as a potential source of mature eggs if they grow in culture or xenografted ovarian tissue from different species, including domestic animals or human beings.

Direct application of the mouse systems to large animals is quite challenging because of the relatively larger size of the oocytes and the longer growth phase. Mouse oocytes grow from 15–20 μm to 75 μm (not including the zona pellucida), whereas pig and cow oocytes grow from 30 μm to 120–125 μm to reach maturity, so that the volume of pig and cow oocytes is 4.6 times greater than that of mouse oocytes. Naturally oocyte growth accompanied by follicular development is a much lengthier process in large animals than in mice; the estimated period from the start of primordial follicle growth to the final preovulatory stage is 3 months in the pig and 6 months in the cow. We have described how pig oocytes in preantral follicles and cow oocytes in early antral follicles grow to their final size after 2 weeks of culture [4, 5]. And, a baby calf has been successfully produced from oocytes that had grown from 90–99 μm in diameter. Nevertheless, no culture system supporting the entire developmental course, as has been achieved in the case of the mouse, has yet been

Received: April 4, 2003
Accepted: May 18, 2003
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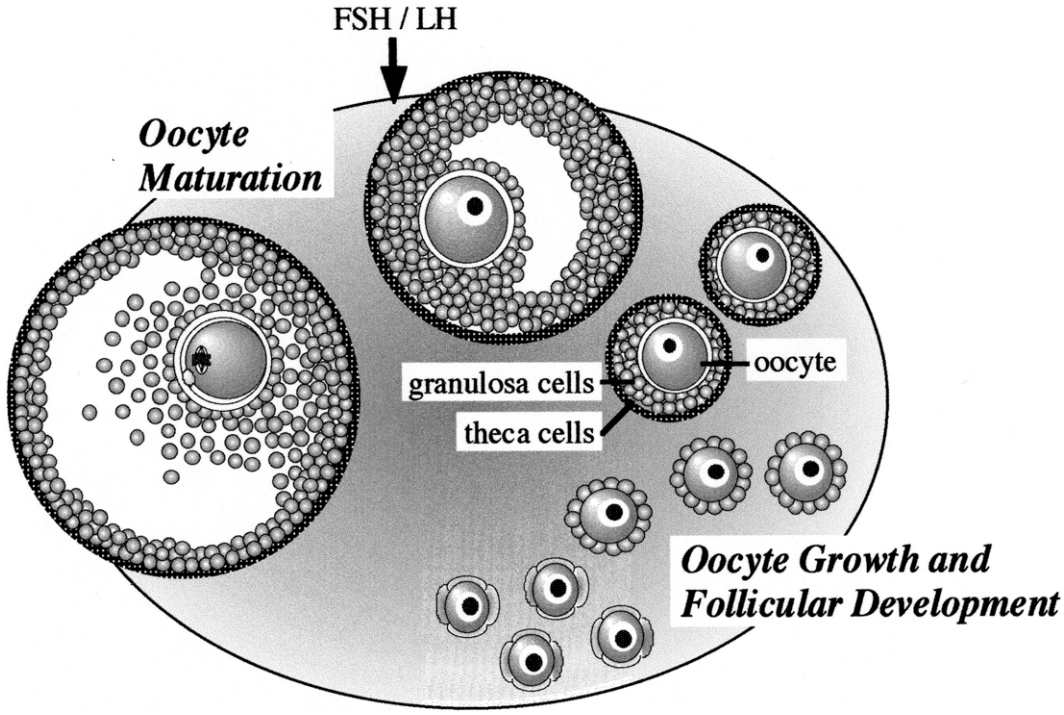

Fig. 1. Schematic description of mammalian oocyte growth and follicular development in the ovary.

developed in larger species.

In this review, a succinct account of the relevant aspects of oocyte growth in the ovaries of domestic species is first provided, and then various *in vitro* culture systems for small oocytes are introduced based on our findings. Reviews of xenotransplantation of small oocytes from large species have been provided elsewhere [6, 7].

Oocytes in the Ovary

Ovaries contain a number of small oocytes

The number of ovulated oocytes is not particularly large in mammals. For example, throughout a woman's fertile life, approximately 400 oocytes are estimated to be ovulated, and 100–200 oocytes are ovulated in a cow. These numbers are very small when compared to the lower vertebrates such as fish, but female mammals contain in their ovaries a huge number of oocytes, like fish and amphibians. In the ovaries, all oocytes exist within follicles. Follicles vary in size and morphology, as they enter the growth phase in an asynchronous manner. Over 90% of follicles are small and uniform in size. These are the "primordial" follicles, and each contains a small nongrowing oocyte (Fig. 1). The other larger follicles (primary, secondary, and antral follicles) are developing follicles that vary in size depending on the growth stage, and each contains a growing oocyte.

Approximately 4,000 primordial follicles are present in a pair of ovaries in the mouse. In the sheep and cow, as many as 100,000 primordial follicles are present in a female animal, and, surprisingly, the number is estimated at 400,000 in the pig. Because each follicle contains one oocyte, the same number of oocytes are present in mammalian ovaries. Only a limited number of oocytes in the primordial follicles are recruited for the long growth phase, with severe selection being carried out and most of these oocytes degenerate during their growth phase. As a result, a very small proportion of oocytes reach full size, mature, and are ovulated from the ovaries during the female reproductive span. Therefore, artificial growing of small oocytes taken from the ovaries will provide a new source of mature eggs for livestock production and assisted reproduction in humans and in endangered species.

Oocyte growth and follicular development

It has been thought that oocytes in the primordial follicles start growing after they are committed to growth in the ovaries. The remaining large number of oocytes

are quiescent and await recruitment. Nongrowing oocytes in primordial follicles are similar in size, depending on the species; oocytes are 15–20 μm in diameter in the rodent, and 30 μm in the pig, cow and man. The first sign of oocyte entrance into the growth phase is the morphological change in the surrounding granulosa cells. Granulosa cells are at first flat, then become cuboidal in shape when the oocytes begin to grow (Fig. 1). At this point, the follicles are called "primary" follicles. After completion of the morphological change, the granulosa cells proliferate actively, which causes the follicles to develop and increase in size. Through a series of mitotic divisions of the granulosa cells, unilaminar primary follicles are converted into multilaminar "secondary" follicles, followed by the "antral (or tertiary)" follicle stage, in which a large fluid-filled antral cavity is formed. During oocyte growth, the zona pellucida is formed around the oocyte. At the periphery of the follicles, somatic cells in the adjacent interstitial tissue differentiate into theca cells and form the external cellular envelope of the follicle. The size and stage of the follicles in which oocyte growth is completed differ among species. Mouse oocytes in early antral follicles are nearly full grown, whereas pig and cow oocytes have not completed their growth in the early antral follicles. The growth phase of these oocytes continues until they are in the late antral follicles with a diameter of approximately 3 mm.

Oocyte growth in mammals depends on communication with the surrounding granulosa cells. Heterologous gap junctions are present between oocytes and granulosa cells until oocytes complete their growth and begin maturation. The junctions are present before deposition of the zona pellucida between oocyte and granulosa cells, and after the formation of zona pellucida, granulosa cells maintain contact with the oocyte through cytoplasmic processes penetrating the zona pellucida. Granulosa cells efficiently pick up low-molecular weight precursors and metabolize them. These molecules are then passed directly into the oocyte via the junctions. Besides this nutritional role, granulosa cells regulate the meiotic arrest of oocytes as described below.

There has also been considerable speculation regarding the influence of oocytes on granulosa cell function. For example, it has been postulated that the oocyte plays a role in initiating follicular development, and in organizing the antral cavity in the latter growing period. Some of these factors originating in oocytes have been identified. Early follicular development appears to depend on oocyte-specific members of the TGFβ family, growth differentiation factor-9 (GDF-9), and bone morphogenic protein-15 (BMP-15/GDF-9B) [8].

Oocytes acquire meiotic competence during growth

Mammalian oocytes are arrested at the prophase of meiosis I during the growth phase. After reaching full size, they resume meiosis in response to gonadotrophic stimulation. Resumption of the process leading to ovulation is manifested by condensation of the nuclear chromosomes, disassembly of the oocyte nuclear membrane (germinal vesicle breakdown: GVBD), assembly of the metaphase I spindle, the first meiotic division, and progression to metaphase II. These sequential processes can also be induced *in vitro* by culturing fully grown oocytes under appropriate conditions. In an extension of this study, *in vitro*-maturation systems have been used to determine the meiotic potential of ovarian growing oocytes. Small oocytes in primordial and primary follicles have no ability to resume meiosis. Oocytes acquire the competence to resume meiosis when their size exceeds 80% of their final diameter; they then become gradually competent to progress to metaphase II as the diameter increases to over 90% of the maximum. Therefore, it is thought that oocytes acquire meiotic competence in a stepwise manner during the final growth phase.

As mentioned above, mouse oocytes in early antral follicles are already fully grown, whereas pig and cow oocytes undergo significant growth after the follicle has begun to develop an antrum. Mouse oocytes in early antral follicles are usually nearly fully competent, whereas pig and cow oocytes are not. Therefore, there is no common follicular stage at which oocytes of all species acquire meiotic competence. Simply the timing of acquisition depends on how close the oocyte growth is to its completion. Mouse oocytes acquire the competence to complete meiotic division at a diameter of 60–65 μm. Pig and cow oocytes achieve complete nuclear maturation to metaphase II at a diameter of 110–115 μm. During the growth phase, oocytes synthesize the proteins and mRNA required for resumption of meiosis and mature to metaphase II.

In ovaries, the oocytes that have reached full size do not resume meiosis until there is a surge of gonadotropins. During this waiting period, the actual resumption of meiosis is restrained by the inhibitory influence of the follicular environment, probably by inhibitory substances produced by the granulosa and theca cells. Hypoxanthine in the follicular fluid has been identified as one potential inhibitor.

In Vitro Culture of Small Oocytes from Large Species

IVG culture systems for small oocytes were developed first in the rodent and then were applied to other species, including cats, pigs, sheep, goats, cows and human beings. In our current method, IVG culture for small oocytes refers to follicle cultures or oocyte-somatic cell-complex cultures, though these methods are all "novel", and initially whole ovaries were used. Organ culture of rodent ovaries has a long history. The first successful culture was carried out by Martinovitch in 1937 [9]. With the watch-glass method, he cultured ovaries of late-fetal or neonatal rats and mice on the surface of clots composed of equal parts of chicken plasma and chicken embryo extract. Over time, complex body fluid media were replaced by tissue culture media, and rodent ovaries and oocytes at different stages have been cultured under various improved conditions, but ovarian organ culture has not been successfully applied to large animals, as the intact ovaries of domestic species are too large for this technique.

It takes a relatively long time for small oocytes of large animals to reach the fully grown stage. Based on the results of various IVG experiments, it is essential for the survival and growth of oocytes that metabolic coupling with oocytes and surrounding granulosa or cumulus granulosa cells be maintained. Oocytes are required to be arrested at prophase I (GV stage) during the culture period, and to acquire the meiotic competence to mature to metaphase II. The following methods and culture systems for large species have been designed specifically to maintain the junctions between oocytes and granulosa cells during the culture period, although these methods still require improvement.

Materials

Pig and cow ovaries can be collected at slaughterhouses. Secondary, preantral, and early-antral follicles that contain growing oocytes are used as the materials for IVG culture. Occasionally, primordial and primary follicles have been used, but significant growth of oocytes has not been observed in culture.

Secondary and preantral follicles are composed of an oocyte at the center, then layers of granulosa cells, a layer of basement membrane, and theca cell-layers on the periphery of the follicle. In the antral follicles, granulosa cells have differentiated into parietal (or mural) granulosa cells and cumulus granulosa cells. Because oocytes cannot grow without associating with granulosa cells, the following three types of complexes have been obtained from secondary and preantral follicles and used for culture: 1) intact follicles with or without ovarian connective tissue, 2) oocyte-granulosa cell-basement membrane complexes, or 3) oocyte-granulosa cell-complexes (OG complexes). Oocyte-cumulus-parietal granulosa cell-complexes (OCG complexes) collected from antral follicles are also sometimes used for culture (Fig. 2d).

Follicle isolation

The ovary is a complex organ composed of follicles and different types of somatic cells. Ovaries of large species contain well-developed connective tissues, especially in their central region. Therefore, small follicles are collected from slices (approximately 1-mm thick) of the cortical region of the ovaries (Fig. 2a). Two methods are used for follicle isolation. One is enzymatic digestion of connective tissues and the other is a micro-dissection method.

A mixture of collagenase and deoxyribonuclease (DNAse) is used for the enzymatic method. Connective tissues are rich in fibrous materials that are digestible by collagenase. DNAse is supplemented for the digestion of sticky DNA derived from damaged cells during enzymatic treatment. By this treatment, oocyte-granulosa cell-basement membrane complexes without thecal layers are obtained. Importantly, the treatment must be as short as possible to avoid excessive damage to the follicles. Therefore, the pieces must not be too large. And washing tissues thoroughly with medium after the digestion is important, as collagenase retained in the follicles causes continued degradation during IVG culture.

We prefer the micro-dissection method. When slices of the ovarian cortex are observed under a dissecting microscope, some small follicles are soon found (Fig. 2b). Preantral and early antral follicles are approximately 0.2–0.7 mm in diameter and can be dissected out manually under the microscope with 1) a pair of fine forceps and a needle (25G) (Fig. 2c) , 2) a pair of fine forceps and a blade (#21), or 3) crossing two tapered blades (#11).

Oocyte-cumulus complexes containing parietal granulosa cells (OCGs) were also used in a series of our previous experiments. They were collected from early antral follicles approximately 0.5 mm in diameter. Under the dissecting microscope, follicles with ovarian connective tissues were held with forceps at the point closest to the cumulus oophorus. The opposite side of the follicle wall was punctured and cut open with a fine

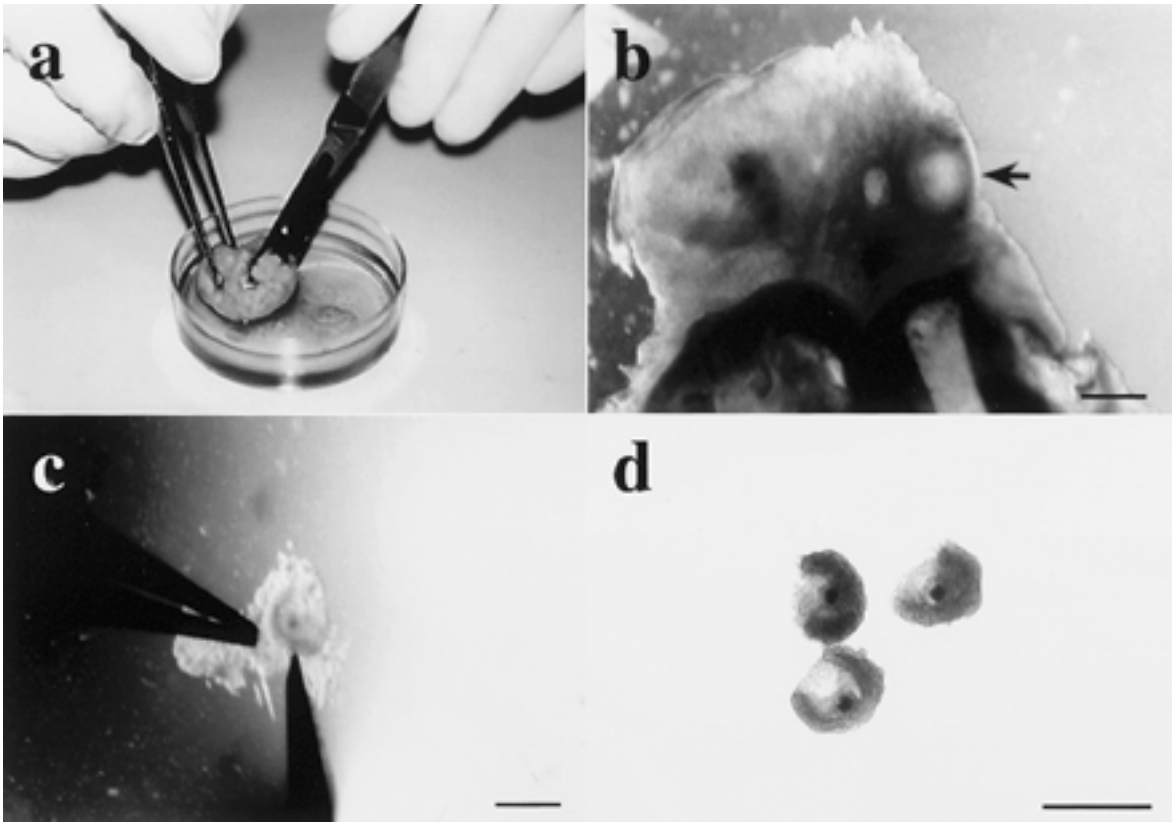

Fig. 2. Micro dissection of pig early antral follicles and oocyte-cumulus-granulosa cell complexes. Each bar represents 500 μm.

needle (25G). The follicle was then pushed gently all around its periphery by the needle until an OCG was pushed out from the opening of the follicle (Fig. 2d).

Dish and matrix

The culture systems need some modifications to prevent the oocytes from becoming denuded as a results of migration of the surrounding theca and granulosa cells to the substrate. These follicular cells prefer the bottom of the plastic culture dish. When follicles or OCGs are cultured in a nontreated flat dish, follicular cells spread onto the bottom, the follicles disintegrate, and subsequently oocytes become denuded and degenerated.

For the culture of mouse oocyte-granulosa cell (OG) complexes, membrane inserts treated with a mixture of type I and type III collagens are used to prevent migration of the granulosa cells (Fig. 3a, Table 1). Collagen-treated plastic dishes are also used for large species (Fig. 3b), and V- or U-shaped 96-multi-well plates are used for individual culture of follicles (Fig. 3c).

We have also used an alternative approach, culturing complexes "in the collagens". In this method, pig and cow follicles or OCGs from early antral follicles are embedded into collagen gels (Fig. 3d). The number of follicles or complexes in each gel is less than 10 (Fig. 3f). The mixture is prepared by mixing a 0.3% acid-soluble collagen solution (Cell matrix type I-A, Nitta Gelatine Co. Ltd., Osaka, Japan), a ten-times concentrated culture medium without bicarbonate, and 0.05 M NaOH containing 22 mg/ml $NaHCO_3$ and 47.7 mg/ml HEPES at an 8/1/1 (v/v/v) ratio in culture dishes (# 1008, 35 $\times$ 10 mm, Falcon, Becton Dickinson Labware, NJ, USA). A half volume of the prepared mixture solution is put on the bottom, then follicles/OCGs are placed on the gel solution and covered with another half volume of the collagen mixture to make a sandwich of the follicles/OCGs (Fig. 3e). After the collagen has set to gel for 15 min at 38.5°C, 3–4 ml of culture medium is added to each culture dish. In the gels, the three-dimensional integrity of the follicles/OCGs is maintained during the culture, and granulosa

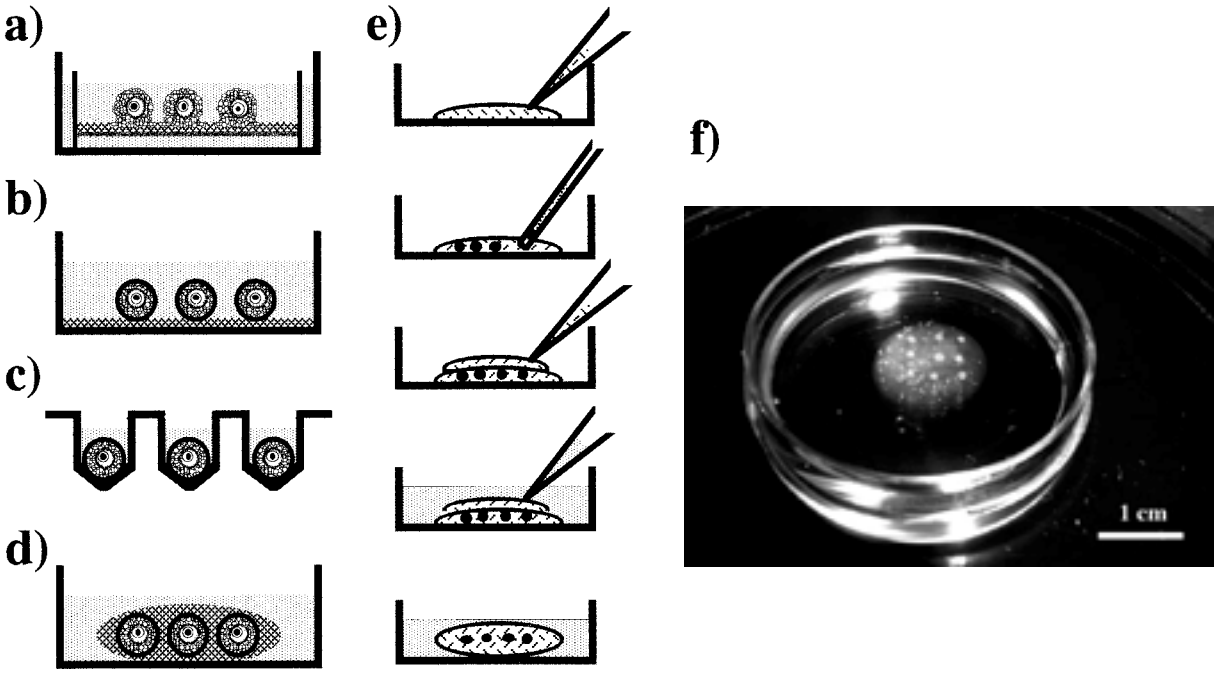

Fig. 3. Culture systems for mammalian oocytes. Follicles and oocyte-granulosa cell complexes are cultured on a collagen-treated transwell (a), a collagen-treated Petri dish (b), a 96-well multi-well plate (c), or in a collagen gel (d). Follicles and oocyte-granulosa cell complexes are sandwiched by the collagen gel (e), and cultured in a Petri dish (f).

cells proliferate in the complexes. Some of the pig preantral follicles form antra [4], and cow OCGs from early antral follicles form antrum-like structures in the gels [5]. Furthermore, some oocytes become fully grown after 2–3 weeks (Table 1).

Culture medium

As the growth of mammalian oocytes is dependent on the surrounding somatic cells, the culture medium must support the viability of the somatic cells in addition to the oocyte for a long culture period. Complex tissue-culture media such as TCM199, McCoy's medium, and Waymouth's medium have been used for the culture of small follicles and OCGs (Table 1). These media contain an energy source, amino acids, nucleosides, and vitamins as well as being balanced salts.

In general, fetal calf/bovine serum (FCS or FBS) is added to the medium at a concentration of 5 or 10%(v/v). One of the benefits of serum addition may be a supply of various growth factors, but FCS is rich in platelet-derived growth factor (PDGF), and its concentrations could be excessive. As the follicles contain three types of cells, an oocyte, granulosa cells, and theca cells, FCS and perhaps PDGF appear to work in different ways according to the cell type. A growth factor suitable for a particular cell type is not always appropriate for other types.

We have recently reassessed the effects of our previous serum-supplemented culture medium on the cow follicle culture, and compared these effects with those of a serum-free but bovine serum albumin (BSA)-containing medium [10]. We collected 0.5–0.7 mm early antral follicles with surrounding theca cells (oocyte diameter was approximately 95 μm), embedded them in collagen gels, and cultured them for 16 days. In the serum-supplemented medium, follicular cells grew into the collagen gels and lost their follicular structure, whereas in serum-free medium, the three-dimensional structures of the follicles were preserved for 16 days. In those follicles, 91% of the oocytes were morphologically normal and had grown to 117.6 $\pm$ 5.7 μm in diameter. These results suggest that the overall health of a follicle in culture is definitely independent of the serum components. It should be noted that in this case none of the purified growth factors was added as a serum substitute, as that seems to create a highly artificial condition.

IVG cultures in various mammals have revealed that oocyte growth and the development of follicular somatic cells are highly coordinated through bi-directional

Table 1. *In vitro* growth (IVG) culture systems for mammalian oocytes

Species	Follicle stage	Oocyte size	Materials (duration)	Dish and matrix	Medium	Additives	Final size	Maturation
Pig [4]	preantral	70–90 μm	Follicle (16 days)	Petri dish collagen gel	Waymouth's (5% FCS)	pFSH, estradiol	90–120 μm	Yes
Pig [11]	early antral	100 μm	OCG (7 days)	Petri dish collagen gel	Waymouth's (5% FCS)	pFSH, hypoxanthine	120 μm	Yes
Cow [12]	preantral	56 μm	Follicle (14 days)	96-well	McCoy's (1 mg/ml BSA)	androstenedione, ITS, bFSH, EGF, IGF-I	66 μm	n.d.
Cow [13]	preantral	59 μm	Follicle (13 days)	24-well collagen gel	TCM199 (1 mg/ml BSA)	hypoxanthine, aprotinin, bFSH, bLH, insulin	72 μm	n.d.
Cow [5]	early antral	95 μm	OCG (14 days)	Petri dish collagen gel	TCM199 (10% FCS)	hypoxanthine	110 μm	Yes
Cow [10]	early antral	95 μm	OCG (14 days)	Petri dish collagen gel	TCM199 (3 mg/ml BSA)	hypoxanthine	118 μm	Yes
Mouse [1]	primordial	15–20 μm	Ovary (8 days) + OG (14 days)	Ttanswell collagen	Waymouth's (3 mg/ml BSA)	EGF, FSH, ITS, fetuin	70 μm	Yes

OCG: oocyte-cumulus-mural granulosa cell complex, OG: oocyte-granulosa cell complex, ITS: mixture of insulin, transferrin and selenium, n.d.: not determined.

communication between oocytes and somatic cells [8]. The growth of oocytes and their extrinsic regulation of meiotic progression/arrest depend on the surrounding granulosa and theca cells. In turn, oocytes promote granulosa cell proliferation and differentiation throughout follicular development. Such bi-directional communication is maintained through the gap junctions constructed between the oocyte and granulosa cells, and is carried out in a paracrine fashion. Perhaps in our serum-free IVG system, the follicle functions as a compartment in which local interactions between the oocyte and somatic cells are sufficient to support oocyte growth and maintain follicular integrity without serum-derived growth factors.

Additives

It has been reported that the proliferation of granulosa cells from preantral follicles can be stimulated by basic FGF, FSH, and EGF in the cow [13, 14]. FSH is essential for the growth of pig oocytes, as FSH maintains the viability of granulosa cells, probably by its anti-apoptotic action, although it does not play a critical role in the survival of follicles in the cow (Table 1). EGF was found to stimulate proliferation of granulosa cells of pig and cow OCGs under our experimental conditions, although it does not exhibit any beneficial effect on oocyte growth. There have been some reports demonstrating that insulin or insulin-like growth factor-I (IGF-I) promotes follicular development in culture, although the effects are still controversial. As discussed earlier, follicles consist of three different cell types, and it is difficult to determine the effects of these factors, and the three-dimensional complexity of the follicles makes the experiments more complicated than those conducted with cell monolayers. It should also not be overlooked that follicles and their constituent cells at different stages may behave in different manners in response to the same growth factors. Therefore, there seems to be no master factor that improves the overall health of all oocytes in any follicle at all growth stages.

Hypoxanthine, a component of the follicular fluid, seems to be essential to the growth of IVG culture under our experimental conditions. In the cow, hypoxanthine promotes a continued association between oocytes and granulosa cells, and increases the percentages of surviving oocytes, regardless of serum supplementation. This continued association is also important because meiotic arrest at the GV stage is maintained in granulosa cell-enclosed oocytes grown to their final size, whereas denuded oocytes sometimes have already resumed meiosis. Meiotic arrest must be maintained in cultured oocytes as mentioned above, otherwise they degenerate during the culture period after the resumption of meiosis. The levels of cAMP in granulosa cells are thought to affect both their differentiation and function. Hypoxanthine has been identified as one of the meiotic-arresting substances in the oocyte, and in culture medium it is thought to maintain cAMP levels by its inhibitory action on cAMP-phosphodiesterase in granulosa cells as well as in oocytes. Interestingly, hypoxanthine may also participate in the acquisition of meiotic competence by pig oocytes during the final growth stage *in vitro* [11].

Perspectives

Although there have been several reports of the production of living young from small oocytes in primordial or developing follicles of mice, there has been only one report regarding a domestic species, which produced only one calf derived from IVG-cultured 135 growing oocytes (90–99 μm in diameter) in ovarian early antral follicles [5]. Even though IVG methods are still at the experimental stage, this result demonstrate that *in vitro*-grown oocytes acquire full developmental competence in the cow. Another method, xenotransplantation of small oocytes to nude mice or SCID (severe combined immunodeficiency) mice may be a substitute for long-term culture systems [6, 7] or may be used in combination with culture methods. We are currently establishing a xenotransplantation system to SCID mice with bovine secondary follicles [14]. After transplantation under the kidney capsules of female SCID mice, the follicles develop to the antral stage, and some oocytes grow to 120 μm in diameter, although we have not yet assessed the ability of the oocytes to be fertilized and develop to term.

Establishment of IVG culture systems for oocytes would provide a large population of mature eggs for livestock production. It will also contribute to assisted reproduction in humans and in endangered species. A combination of cryopreservation and IVG systems, if successful, would provide the desired number of mature eggs from a preserved small amount of ovarian tissue by using existing assisted-reproduction techniques. Primordial oocytes are quite useful for these techniques because they are present in huge numbers and are cryo-resistant. Improved understanding of the growth-initiation factors of the oocytes could lead to successful establishment of such a system [7].

Finally it should be emphasized that IVG culture is able to reveal to us the dynamic changes that take place in follicles and oocytes in the ovaries. As such, IVG technology is providing us with a revolutionary change in analyzing oocyte growth and follicular development, as these events have long been described in terms of "histology". As progress in IVF technology during the last half century has given us greater knowledge regarding oocyte maturation, fertilization and early embryonic development, we believe that IVG culture will provide us with a better understanding of the basic mechanisms of oocyte growth and follicular development in the ovaries.

Acknowledgements

This work was supported by a Grant-in-Aid for Creative Scientific Research (13GS0008) and by a Grant-in-Aid for Scientific Research (14360170) to T. M. from the Ministry of Education, Culture, Sports, Science and Technology in Japan.

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