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Novel Approach for the Three-Dimensional Culture of Granulosa Cell–Oocyte Complexes

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ABSTRACT

The *in vitro* culture of immature ovarian follicles is used to examine the factors that regulate follicle development and may ultimately provide options for reproductive infertility. The objective of this study was to develop a three-dimensional *in vitro* culture system for the growth and development of individual granulosa cell–oocyte complexes. An alginate hydrogel was used to encapsulate immature mouse granulosa cell–oocyte complexes (GOCs) that were subsequently maintained in a serum-free *in vitro* culture. An overall incorporation efficiency of 50% was achieved. The complexes were assessed by transmission electron microscopy for changes in ultrastructure during *in vitro* growth. The architecture of the follicular complex was maintained during the encapsulation and the subsequent culture. The granulosa cells proliferated, and the oocytes also grew in volume and obtained the structural characteristics of mature oocytes including cortical granule formation, a well-developed zona pellucida with microvilli, normal mitochondria, and lattice-like structures in the cytoplasm. Oocytes retrieved and matured were able to resume meiosis, a necessary step for proper development. Thus, this system represents a new *in vitro* methodology for growth of individual granulosa cell–oocyte complexes.

INTRODUCTION

THE *IN VITRO* CULTURE of immature ovarian follicles isolated from the ovary has become a useful tool to study follicle development and offers the potential to preserve reproductive options in cases of polycystic ovarian syndrome (PCOS), premature ovarian failure, or definitive sterility (postoncotherapy).[1] The fundamental role of the ovary is to produce oocytes capable of fertilization and subsequent development into viable offspring. This task is accomplished through the cooperative development of various cells of the ovarian follicle: both somatic cells (granulosa and theca) and germline cells (oocyte). The paracrine interaction between these cells along with endocrine input from the pituitary gland, allow development to occur.[2] The earliest stage follicles, termed primordial, are formed postnatally in mouse ovaries and consist of an oocyte surrounded by a single layer of pregranulosa cells.[3] Unknown factors stimulate primordial follicle growth to subsequent stages: preantral (primary and secondary) follicles, and antral (tertiary and Graafian) follicles. Preantral follicles are characterized by granulosa cell proliferation, oocyte growth, and acquisition of a thecal cell layer, while antral follicles display granulosa cell differentiation and steroidogenesis. Ovulation is stimulated by a midcycle surge of the pitu-

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itary gonadotropins luteinizing hormone (LH) and follicle-stimulating hormone (FSH). Stimulation by the gonadotropins results in the resumption of meiosis of the oocyte as well as luteinization of the granulosa cells.[1,4]

Strategies for the *in vitro* maturation of ovarian follicles range from the culture of individual oocytes to the culture of the whole organ.[1,5] Several mouse culture systems have been developed with varied aims (reviewed in Refs. 6 and 7), such as the production of oocytes for *in vitro* fertilization and the analysis of basic mechanisms of follicle development.[8–16] Some of these systems have been successful in producing live offspring from immature oocytes grown *in vitro*.[10,15] However, *in vitro*-grown oocytes are different from native oocytes. The differences between these *in vitro* systems and the *in vivo* environment may originate in the communication between somatic cells and the oocyte. Culture of primary GOCs on collagen transwells has enabled maturation.[17] Even if the individual components remain functional, the architecture may not be retained as the follicles may flatten on plastic substrates.[18] Alternatively, the culture of whole organs can maintain the three-dimensional architecture, maintain the structural integrity of the tissue, and allow for follicle–follicle interactions, which are essential to normal development.[5] These systems may have significant nutrient limitations as the tissue mass develops, or the native stroma may exert inhibitory control over the initiation of primordial follicle growth.[19] In addition, all stages of folliculogenesis occur simultaneously, which complicates analysis of follicle growth. For example, the ability to discriminate between follicles that will be recruited from the quiescent pool or those that will become dominant ovulatory follicles is difficult in intact ovaries or ovarian sections.

For this study, a simplified culture system for the study of individual granulosa cell–oocyte complexes (GOCs) was investigated that combines the positive attributes of the current *in vitro* methodology into a single system. These include (1) maintenance of the biological architecture of the granulosa cells with the oocyte, (2) serum-free growth conditions, (3) large-scale preparation by enzymatic digestion of the ovary, and (4) the ability to directly and easily study individual granulosa cell–oocyte complexes during *in vitro* development. For this system, individual GOCs were incorporated into a three-dimensional culture system based on an alginate hydrogel. The results of this study show that ovarian GOCs develop within the three-dimensional alginate matrix.

MATERIALS AND METHODS

Animal care

CD1 mice were purchased (Charles River Laboratories, Wilmington, MA) and maintained as a breeding colony at Northwestern University (Evanston, IL). Animals were housed in a temperature- and light-controlled environment on a 12 h:12 h light:dark photoperiod and provided with food and water *ad libitum.* All animals were treated in accordance with the National Institutes of Health (NIH, Bethesda, MD) *Guide for the Care and Use of Laboratory Animals* and the established IACUC protocol at Northwestern University.

GOC isolation and culture

Granulosa–oocyte complexes were isolated as described[10] with minor modifications. Briefly, ovaries from 12-day-old female mice were dissected and dissociated in α-modified minimum essential medium (α-MEM; Life Technologies, Grand Island, NY) containing 0.3% bovine serum albumin (BSA; ICN Biomedicals, Aurora, OH), 0.1% type I collagenase (Worthington Biochemical, Lakewood, NJ), and 0.02% deoxyribonuclease I (Worthington Biochemical) at 37°C and 5% O_2, 5% CO_2, 90% N_2 for approximately 1 h with intermittent pipetting. Granulosa–oocyte complexes were collected by micropipettes and washed three times in α-MEM, manually counted, and encapsulated into alginate. Growth medium was α-MEM containing 0.3% BSA, bovine pancreatic insulin (5 μg/mL), human transferrin (5 μg/mL), sodium selenite (ITS, 5 ng/mL; Sigma-Aldrich, St. Louis MO), penicillin (5 units/mL), and streptomycin (5 μg/mL) (Life Technologies).

Alginate encapsulation and culture of alginate beads

Sodium alginate (NutraSweet Kelco, Chicago, IL) was dissolved in Dulbecco's phosphate-buffered saline (PBS) without calcium or magnesium (Mediatech, Herndon, VA) and sterilized by filtration. Granulosa–oocyte complexes were suspended within the alginate at a concentration of 2 μL of alginate solution to 1 GOC and mixed by gently pipetting with a wide-bore plastic pipette tip. The volume of GOCs never exceeded 1/20th the volume of alginate and the final concentration of the alginate solution was never less than 1.9% (w/v). The alginate–cell mixture was loaded into a 1-mL syringe and extruded through a 23-gauge needle under a continuous stream of 5% O_2, 5% CO_2, 90% N_2 (flow rate of gas, 17 std L/min) into a warmed beaker of 50 mM $CaCl_2$–150 mM NaCl. Beads were retrieved from the $CaCl_2$ solution within 5 min and washed three times in αMEM–0.3% BSA. For group culture, beads were placed into petri dishes with growth medium (α-MEM-0.3% BSA, fetuin [1 mg/mL; JRH Biosciences, Lenexa, KS]). For individual cultures, beads containing one GOC were transferred with a large-bore pipette tip and placed (one bead per well) into a Costar 96-well tissue culture dish (Corning Life Sciences, New York, NY) and cultured in 100 μL of growth

medium. After plating, beads were placed into modular incubation chambers (Billups-Rothenberg, Del Mar, CA), equilibrated with 5% O_2, 5% CO_2, 90% N_2, and incubated at 37°C. Gas was exchanged every 12 h. Cultures were fed every other day by replacement of one-third the volume of growth medium.

Light and electron microscopy

For light microscopy, live samples were imaged on a TE300 inverted microscope (Nikon Instruments, Melville, NY) equipped with a SenSys Photometrics digital camera (Roper Scientific, Tucson, AZ). Measurements were taken by calibration to a stage micrometer, using Metamorph Software (Universal Imaging Corporation, Downingtown, PA). For electron microscopy, beads were fixed in 4% paraformaldehyde, 1% glutaradehyde in 0.2 M sodium cacodylate buffer and osmicated in 1% osmium tetroxide. Before embedding, beads were stained with hematoxylin and dehydrated through a graded ethanol series. Beads were flat embedded between Aklar sheets in epoxy resin. Semithin sections (1 μm) were stained with toluidine blue. Ultrathin section (light yellow interference color) were cut and mounted on Formvar-coated slot grids. Sections were stained with 2.5–3% uranyl acetate for 30 min and with 2.66% Reynold's lead citrate for 5 min and viewed through a 100CX-II electron microscope.

Oocyte in vitro *maturation*

In vitro maturation was performed as described.[10,17,20] For recovery of GOCs in beads, 20 mM sterile EGTA was added directly to the medium and incubated at 37°C in 5% O_2, 5% CO_2, 90% N_2, until the beads dissolved (approximately 5 min). GOCs were collected by micropipetting, washed three times in α-MEM–0.3% BSA, and matured overnight for 17.5–18 h in α-MEM, 0.3% BSA, 0.1% fetuin plus human follicle stimulating hormone (hFSH, 100 ng/mL; National Hormone and Peptide Program, Torrence, CA) and human epidermal growth factor (EGF, 10 ng/mL; Sigma-Aldrich). After incubation, oocytes were released by repeated pipetting with a 1-mL pipette tip to remove cumulus cells and oocytes collected by the micropipette. Oocytes were washed three times in minimum essential medium (MEM; Gibco-BRL, Grand Island, NY) supplemented with 0.3% BSA and scored for the presence or absence of the germinal vesicle (GV) or signs of degeneration.

RESULTS

Granulosa cell–oocyte complex incorporation into alginate beads

Enzymatic digestion of ovaries harvested from 12-day-old mice yielded GOCs from preantral follicles. The average diameter of the GOCs was 82 ± 17 μm (n = 83) (Fig. 1A). Often stroma remained attached to the outer surface of the GOC, depending on the timing of digestion and the amount of mechanical force applied during preparation. Oocytes from the complexes averaged 47 ± 6 μm in diameter (n = 77). Once the GOCs were collected, they were encapsulated into an alginate matrix (Fig. 1B) to form alginate beads. The bead sizes used in these experiments ranged from 0.5 to 1.0 mm, and can be readily controlled if necessary (data not shown). Approximately half of the initial GOCs were recovered in the incorporation procedure (Table 1). The number of GOCs within each bead depended on the volume of alginate gel used in the initial preparation. The incorpora-

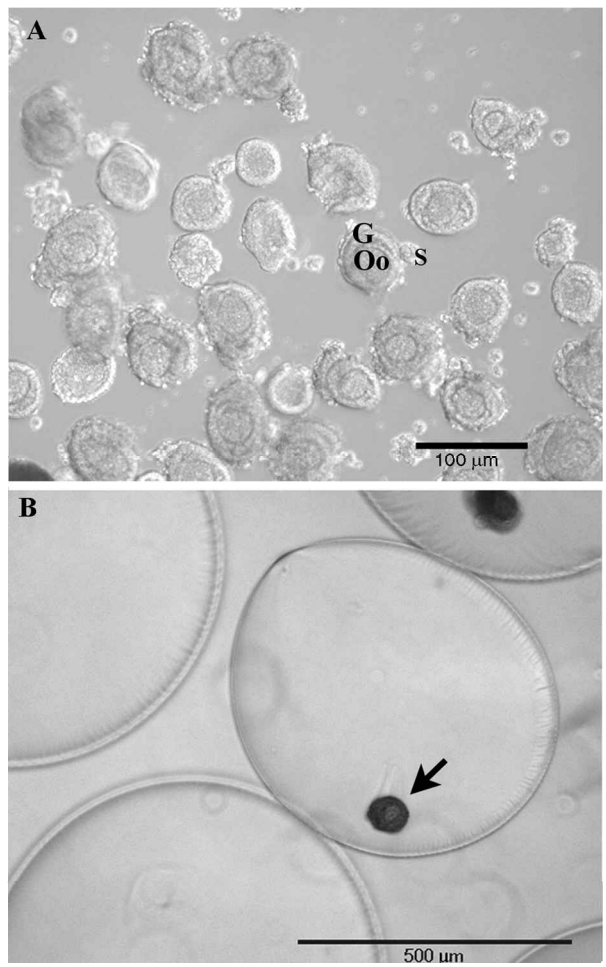

FIG. 1. Encapsulation of mouse granulosa cell–oocyte complexes (GOCs). (**A**) GOCs freed by collagenase digestion of ovaries from 12-day-old immature mice. Oocytes are immature, averaging 47 ± 6 μm with few granulosa cell layers. G, Granulosa cell; Oo, oocyte; S, stroma. Scale bar: 100 μm. (**B**) GOC (arrow) after incorporation into the alginate "bead." Single (shown, arrow) or multiple GOCs can be encapsulated. Scale bar: 500 μm.

Table 1. Incorporation of Granulosa Cell–Oocyte Complexes into Alginate Beads

Incorporation efficiency[a]	*Percentage (*n)[b]
GOC: Isolated (4 females)	$n = 700$
GOC: Incorporated	50% (350)
Bead–GOC distribution[c]	
0 GOC	61% (327)
1 GOC	22% (118)
2 GOC	8% (41)
3+GOC	9% (50)

[a]Incorporation efficiency is the number of GOCs within beads divided by the starting number of GOCs obtained by collagenase digestion.

[b]Numbers in parentheses are numbers of GOCs.

[c]Bead–GOC distribution is the number of GOCs incorporated into each alginate bead.

tion values for one experiment are shown in Table 1. Variations in the number of GOCs incorporated into each bead are possible: larger volumes of alginate gel yield more beads with single GOCs.

Transmission electron microscopy of GOCs after 6 days in culture

On incorporation into alginate beads, GOCs were cultured over a total of 10 days in serum-free medium. The GOCs grew as intact spheres of cells within the alginate bead. To determine whether the dynamics of granulosa cell and oocyte growth were normal, cells were assessed morphologically by transmission electron microscopy (TEM) during and after the culture period (Fig. 2). At this stage, the oocytes showed no signs of degeneration, had cortical granules around the periphery, and contained an intact zona pellucida with numerous microvilli projecting from the oocyte (Fig. 2A and 2B). The granulosa cells surrounding the oocyte were organized, spherical and had few vacuoles (Fig. 2). No morphological evidence of cellular apoptosis[21] was noted in intact GOCs containing an oocyte.

Ultrastructure of GOCs after 10 days in culture

At the end of the 10-day culture period, GOCs within alginate beads were analyzed by TEM for ultrastructure changes in the granulosa cells and oocytes. Semithin sections through an intact GOC grown for 10 days in an alginate bead are shown in Fig. 3. Gross examination of this GOC revealed a 60-μm oocyte within several layers of granulosa cells, indicating that neither oocyte nor granulosa cell growth was retarded by the alginate matrix. These sizes of oocyte were never recovered in GOCs collected from 12-day-old (immature) mice before culture. The granulosa cells closest to the oocyte were more ordered with fewer vacuoles than those toward the periphery; however, the granulosa cells show more vacuoles than at 6 days in culture. On the upper perimeter of the GOC, a thin layer of cells with elongated nuclei was noticeable (Fig. 3A, arrows). These cells may represent the formation of a "theca"-like layer surrounding the granulosa cells, perhaps derived from the originally isolated stroma. The same GOC is shown during TEM in Fig. 4. The oocyte maintained an intact zona pellucida with shorter microvilli projecting from the oocyte surface. The granulosa cells had numerous mitochondria and abundant lipid droplets. The appearance of lipid droplets in the cytoplasm of the granulosa cells at the end of culture, but not at 6 days, would be consistent with the idea that these cells became steroidogenic at some stage late in the culture period.

Oocyte retrieval and maturation from GOCs grown in alginate beads

Oocytes grown within alginate beads can be retrieved and matured and are capable of resuming meiosis. After 10 days in culture, GOCs were released from alginate beads and analyzed for their ability to undergo *in vitro* maturation. The results of *in vitro* maturation of oocytes from bead-cultured GOCs are presented in Table 2. On average, 40% of oocytes retrieved from *in vitro* growth in alginate beads underwent germinal vesicle breakdown (GVBD) and proceeded to meiosis II as indicated by the formation of a polar body. This number was comparable to oocytes collected from concomitant GOC cultures grown on collagen-coated membranes (data not shown). Thus, not only did the alginate beads allow for oocyte growth and survival, but the oocytes were capable of resuming meiosis, a necessary step for subsequent fertilization and proper development.

DISCUSSION

This report presents a three-dimensional system for the encapsulation and culture of granulosa cell–oocyte complexes. *In vitro* culture of ovarian follicles has applications in the study of normal ovarian physiology and in the treatment of infertility or germline preservation. For the study of normal ovarian physiology and development, this system can be used in the examination of paracrine communication between the oocyte and granulosa cell and of dual influences on the maturation process. A defined system is required such that experimental variables can be added and analyzed with ease. For clinical applications in the treatment of infertility or preservation of the germline, culture of GOCs or immature follicles could yield far more oocytes than conventional *in vitro* fertilization, as the majority of mammalian oocytes are con-

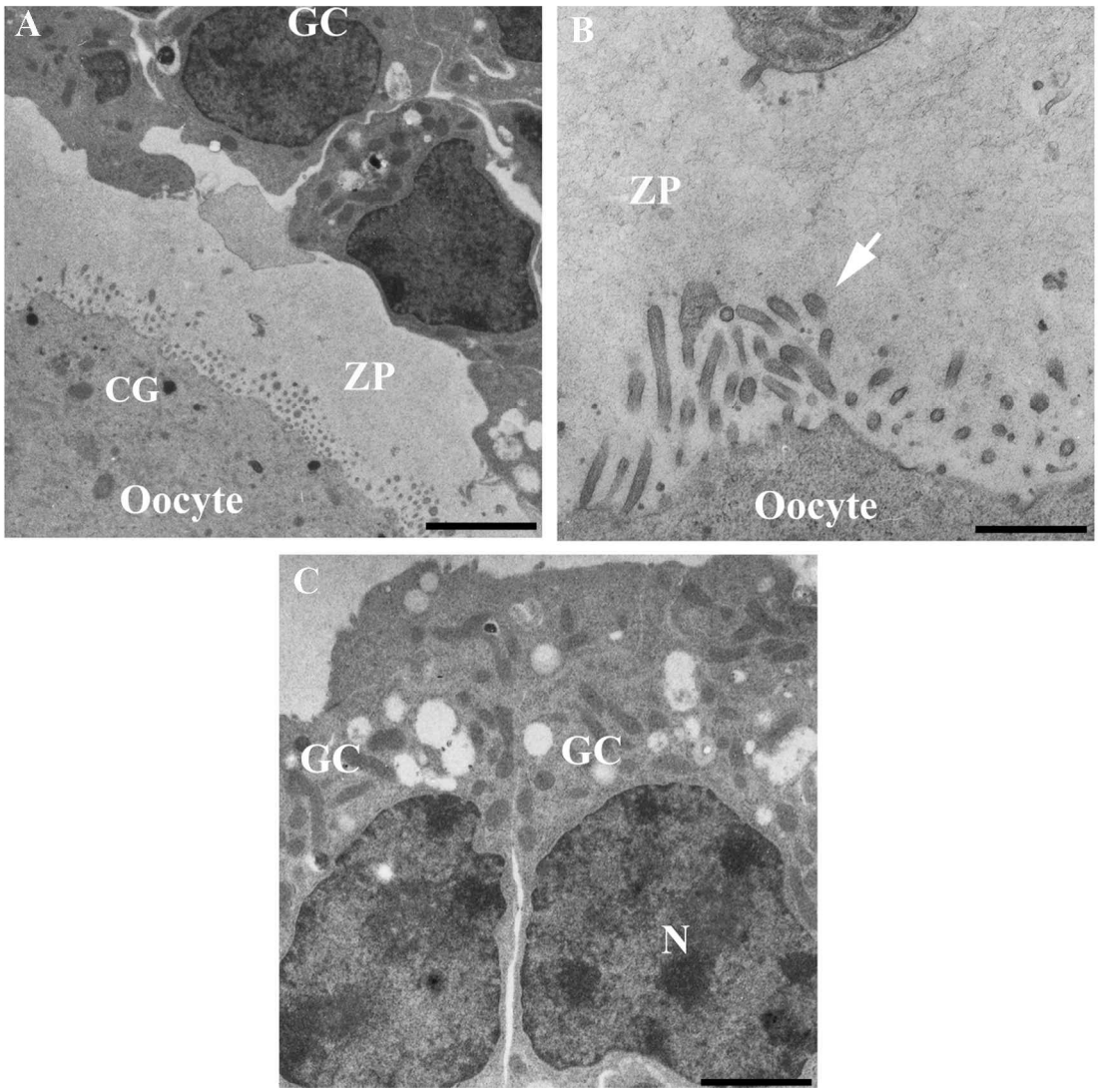

FIG. 2. Transmission electron microscopy after 6 days of *in vitro* culture. (**A**) Granulosa cell and oocytes from an alginate-enclosed GOC. Note the well-developed zona pellucida (ZP) and cortical granules (CG) in the cytoplasm of the oocyte. Scale bar: 10 μm. Original magnification, $\times$1400. GC, Granulosa cell. (**B**) Higher magnification of (**A**). Microvilli (arrow) from the oocyte project into the zona pellucida. Scale bar: 1 μm. Original magnification, $\times$14,000. (**C**) Granulosa cells at the periphery of the GOC are spherical and maintain lateral cell–cell contacts. Nucleus, N. Scale bar: 8 μm. Original magnification, $\times$1400.

tained in immature and quiescent follicles. In these studies, GOCs isolated from the ovaries of 12-day-old mice were encapsulated within alginate beads with an incorporation efficiency of 50%. The encapsulation procedure does not disrupt the connections between the granulosa cells and the oocyte. Immature GOCs are grown within an alginate hydrogel matrix, under serum-free conditions, in either group or individual cultures. The granulosa cells proliferated, and the oocytes also grew in volume. Structural features characteristic of mature oocytes were observed, including cortical granule formation, a well-developed zona pellucida with microvilli, normal mitochondria, and lattice-like structures in the cytoplasm.

The ovarian follicle is a three-dimensional structure and current culture methods on treated membranes or tissue culture plates do not maintain the physiologically spatial arrangement of cells. Enzymatically isolated GOCs develop on "stalks" above the collagen-coated membrane surfaces on which they are grown.[10] During enzymatic harvesting, the basal lamina surrounding the follicle is disrupted. Subsequently, when these GOCs are cultured, the granulosa cells migrate away from the oocyte and onto available surfaces. To solve this problem, some three-dimensional systems based on collagen have been developed for culturing GOCs both *in vitro* and for implantation into kidney capsules.[16,22,23] However, colla-

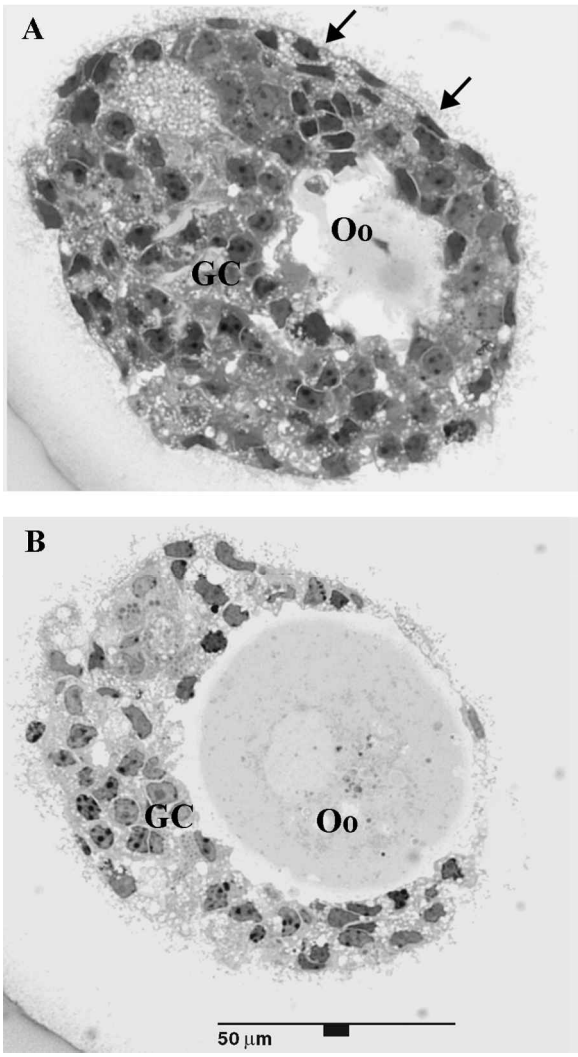

FIG. 3. Semithin sections through a GOC within an alginate bead grown for 10 days *in vitro*. (**A**) An early section shows the beginning of the oocyte within the layers of granulosa cells. A layer of "theca-like" cells is noticeable on the periphery of the GOC (arrows). (**B**) A central section of the GOC shows an oocyte measuring 60 μm. Scale bar: 50 μm. GC, Granulosa cells; Oo, oocyte. Original magnification, ×400.

gen gels are not readily manipulated for studying individual complexes on a large scale and removal of the complex after culture is not easy.[16] An alginate-based system can maintain the spatial arrangement for GOCs. Importantly, GOCs in alginate have the added benefit of being readily removed at the end of the culture period by dissolution of the gel. In these studies, alginate exhibits minimal cellular interactions with mammalian cells, and thus likely provides only mechanical support. However, cell adhesion ligands can be incorporated onto the alginate backbone,[24] which are known to influence granulosa cell morphology, differentiation, and signaling.[25]

Alginate hydrogels have a number of advantages as cell scaffolds and have been developed as a system for growth of a number of cell types including mouse embryos, chondrocytes, myoblasts, and pancreatic islet cells.[24,26–28] Alginate is a nontoxic polysaccharide composed of repeating units of mannuronic and guluronic acids that may be reversibly linked by exposure to divalent cations such as calcium. The matrix is easy to use and commercially available. Cells are incorporated into the alginate solution before gelation.[28] Chelation of the calcium results in release of the cells or tissue from the matrix. The reversible nature of the matrix makes alginate hydrogels an ideal system for developing and studying GOCs. Oocytes grown *in vitro* can be readily retrieved from the matrix for further uses, which could include fertilization. In addition, pore size of the alginate can be regulated so that proteins may freely diffuse into and out of the bead.[29,30] Thus, protein factors that influence follicle and oocyte development including growth and differentiation factor 9, Kit ligand, activin, and inhibin, could be added to the culture medium and their effects on individual follicle development analyzed.

During follicle development, the oocyte and the surrounding (cumulus) granulosa cells maintain contact through gap junctions that are required for growth and maturation of both cell types. Removal of an oocyte from an *in vitro*-cultured GOC results in premature luteinization of the granulosa cells[31] whereas the addition of oocytes to granulosa cell cultures prevents it.[32] Follicle development does not occur in mouse "knockout" models in which the junctions are disrupted by deletion of the connexin 43 gene, a necessary component of gap junctions in the ovary.[33] These mice are defective in multiple aspects of oocyte development including a poorly developed zona pellucida, a lack of cortical granules, the presence of vacuoles in the cytoplasm, and an inability to undergo meiotic maturation.[33] Long-term growth in current systems is complicated by loose cell–cell contacts that are readily lost in culture.[34] None of these features are present in our *in vitro* culture system, suggesting that the interaction between the granulosa cells and the oocyte is maintained. A surrounding alginate matrix acts as a structural mimic for the ovary by maintaining the positions of the granulosa cells with the oocyte. Grown within the alginate matrix, the granulosa cells and oocytes appear structurally intact at both 6 and 10 days in culture. The granulosa cells proliferated, and the oocytes also grew in volume and obtained the structural characteristics[35] of mature oocytes including cortical granule formation, a well-developed zona pellucida with microvilli, oval mitochondria, and lattice-like structures in the cytoplasm. In some instances, a morphologically distinct layer of cells surrounding the GOC was noticeable at the end of the culture period. This may indicate retention of a thecal cell layer during GOC preparation or perhaps de-

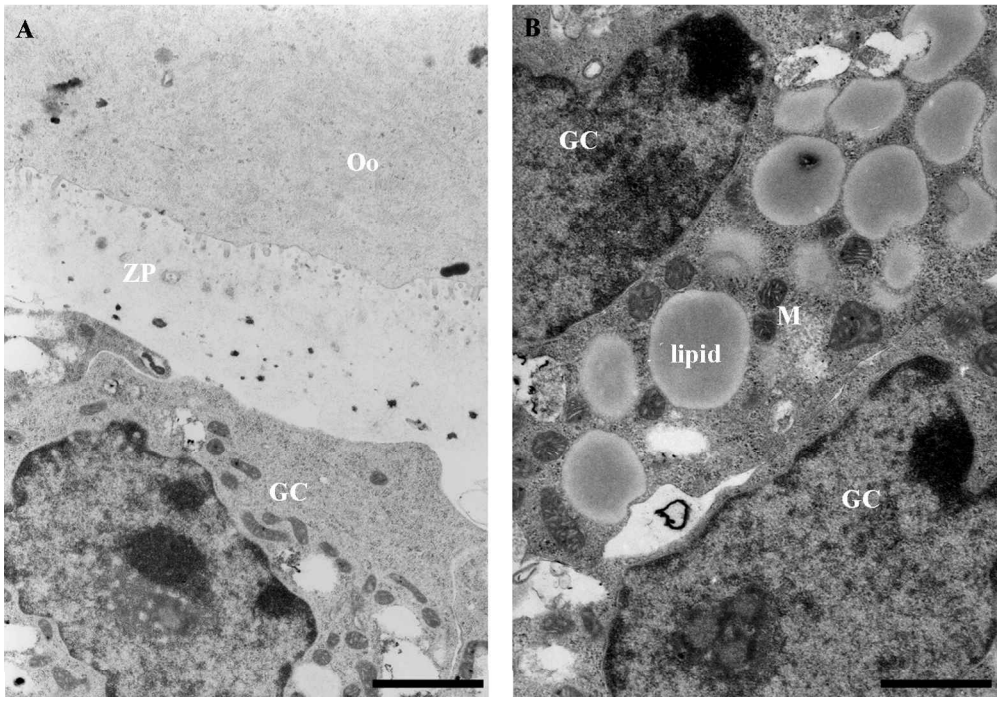

FIG. 4. Transmission electron microscopy of GOCs grown for 10 days in alginate "beads." (**A**) The oocyte (Oo) and granulosa cells (GC) are separated by the zona pellucida (ZP). Scale bar: 5 μm. Original magnification, $\times$7200. (**B**) Higher magnification of the granulosa cells in (**A**). Note the abundant lipid droplets and mitochondria (M) in the cytoplasm. Scale bar: 2.5 μm. Original magnification, $\times$10,000.

velopment of that layer from the initially attached stroma. Further experiments will be required to determine the cellular constituency as well as the derivation of this layer.

This system can be used to examine environmental effects on the growth and maturation of individual or multiple GOCs. GOCs derived by enzymatic digestion of ovaries from immature 12-day-old mice are embedded and cultured in single alginate beads. During encapsulation, one or multiple GOCs are incorporated. The initial volume of alginate solution mixed with the GOCs affects the mean number of GOCs found within each bead. For this study, we chose only those beads that contained an individual GOC. Previous work had shown that physical contact of one follicle with another results in one growing larger and obtaining dominance over the other.[36] Note, however, that it is unknown whether GOCs will interact in the same way as intact follicles under similar conditions. Future studies could examine follicle–follicle interactions and whether grouping of GOCs within the beads can produce a "dominant follicle."

This alginate-based culture system may provide the flexibility with which to examine the components that promote oocyte maturation. Growth factors and or hormones may prove necessary for follicle maturation, which cannot be readily studied in two-dimensional systems. Addition of FSH to two-dimensional cultures of GOCs results in granulosa cell proliferation, migration from the oocyte, and subsequent flattening of the oocyte. The alginate system can maintain the granulosa cell–oocyte interactions in the presence of growth and differentiation factors. This system can be used in studies of follicles at different stages of development. For ex-

TABLE 2. MATURATION OF *in Vitro*-GROWN OOCYTES

Experiment	*Total oocytes*	*GVBD*[a]	*GV*[a]	*Degenerating*[a]
1	25	44% (11)	20% (5)	36% (9)
2	80	30% (24)	34% (27)	36% (29)
3	84	41% (34)	33% (28)	26% (22)

Abbreviations: GV, germinal vesicles; GVBD, germinal vesicle breakdown.

[a]Entries represent percentage relative to total oocytes and actual number in parentheses.

ample, a commonly used model for maturation involves the manual dissection of ovaries, which results in the isolation of follicles with diameters of at least 150 μm that retain all cell types, including the stromal and thecal cells layers. The integrity of these manually dissected follicles can be maintained if grown under conditions that do not allow attachment to the culture surface.[8,11,12,18,37,38] Thus manually dissected follicles may grow within the beads, eliminating the daily plating changes necessary in other systems to keep them from adhering to the culture surface.[8,15,37,38] Therefore, alginate culture may improve these systems, as the alginate provides structural support. Another candidate arena for the use of alginate cultures is during *in vitro* growth of GOCs for animal species with large follicle sizes (such as bovids). With simple changes in the bead fabrication process (i.e., flow rate, needle diameter), the diameter of the beads can be adjusted to match the size requirements of the enclosed GOCs.

In conclusion, we have developed a novel three-dimensional culture system that allows immature GOCs to be maintained in culture. This technology improves on and expands current culture methodologies. Future experiments will be directed to determining whether *in vitro*-grown oocytes display normal nuclear and cytoplasmic maturation. Defects in cytoplasmic maturation may lead to improper development of the embryos and possibly abnormalities in the offspring. The demonstration of normal embryo production by subsequent *in vitro* maturation and fertilization in this mouse model could justify the application of this system for subsequent use with *in vitro* fertilization.

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