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Tissue-Engineered Follicles Produce Live, Fertile Offspring

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Abstract

Oocytes grown *in vitro* are of low quality and yield few live births, thus limiting the ability to store or bank the ova of women wishing to preserve their fertility. We applied tissue engineering principles to the culture of immature mouse follicles by designing an alginate hydrogel matrix to maintain the oocyte's 3-dimensional (3D) architecture and cell-cell interactions *in vitro*. A 3D culture mimics the *in vivo* follicle environment, and hydrogel-encapsulated follicles develop mature oocytes within the capacity for fertilization similar to that of oocytes matured *in vivo*. Embryos derived from cultured oocytes fertilized *in vitro* and transferred to pseudopregnant female mice were viable, and both male and female offspring were fertile. Our results demonstrate that alginate hydrogel-based 3D *in vitro* culture of follicles permits normal growth and development of follicles and oocytes. This system creates new opportunities for discovery in follicle biology and establishes a core technology for human egg banks for preservation of fertility.

INTRODUCTION

Few Options Currently Exist for women who face the life-preserving but follicle-threatening chemotherapy or radiation therapy for cancer.[1] Emergency *in vitro* fertilization is available to women who can delay treatment for 6–8 weeks and have a partner or sperm bank material for the generation of embryos.[2] Ovarian tissue cryopreservation and autotransplantation is an option to restore fertility, [3,4] but it may increase the risk of reintroduction of hematologic or disseminated malignancies. Methods to grow immature follicles *in vitro* eliminate the potential reintroduction of nascent cancer cells, provide autonomous genetic material for women wishing to begin their own family, and eliminates the need to cryopreserve embryos. Traditional culture systems have placed follicles on 2-dimensional (2D) substrates;[5] however, 3-dimensional (3D) systems more effectively simulate physiologic conditions because many cellular processes in organogenesis occur exclusively in 3 dimensions. Follicle encapsulation within alginate hydrogels, a common tissue engineering scaffold, mimics the ovary by providing the appropriate 3D context while supporting somatic cell and egg interactions to optimize oocyte development.[6,7]

Oocytes grown *in vitro* are of low quality and yield few live births, limiting the development of egg banks for women wishing to preserve their fertility.[1,8] The primary obstacle to preservation of fertility through long-term storage of human ova is the complexity of the

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intercellular interactions essential for normal somatic and germ cell communication and oocyte growth and maturation. These factors then contribute to successful fertilization and, ultimately, the production of healthy offspring. Many aspects of oocyte growth and development are regulated by intercellular communication through gap junctions with adjacent granulosa cells and via paracrine signals received from both granulosa and theca cells.[9–12] In turn, oocyte-derived growth factors stimulate proliferation and differentiation of the surrounding granulosa and theca cells.[13–15] In coordination with endocrine signals from the pituitary, oocyte-somatic cell communication within the follicle is essential for progression of immature follicles from the primary to secondary stages, selection of preantral follicles into the growing pool, development of the fluid-filled antrum, and final maturation into a Graafian follicle poised for ovulation and fertilization. Therefore, maintenance of the 3D architecture of the ovarian follicle is essential for follicle development, oocyte growth and maturation, and ovulation of an oocyte capable of being fertilized.

We have developed a 3D *in vitro* follicle culture system that uses an alginate hydrogel matrix as a scaffold for follicle growth. With this system, we have demonstrated that a single follicle can be encapsulated into an alginate bead and cultured *in vitro*,[6] and that the culture system can be adapted to mimic the milieu of growth factors and hormones necessary for normal oocyte growth and maturation. [7,16] Importantly, the alginate matrix provides the structural support necessary to maintain essential oocyte-somatic cell interactions *in vitro* within the growing follicle. The ultimate test of the utility of the alginate hydrogel for *in vitro* follicle culture is whether oocytes cultured in this system undergo normal growth and maturation, such that they acquire full capacity for fertilization and produce viable and fertile offspring. Oocytes grown and matured within the alginate culture system should be able to progress to metaphase II, undergo successful *in vitro* fertilization (IVF), and produce implantable zygotes that develop into viable, fertile offspring.

MATERIALS AND METHODS

Animals

Immature follicles were isolated from prepubertal, 16-day-old female F1 hybrids (C57BL/6j×CBA/Ca), and sperm was prepared from proven CD1 male breeders. Eight- to 10-week-old CD1 female mice that had been mated to vasectomized CD1 male mice served as pseudopregnant mice for IVF. Animals were housed in a temperature- and light-controlled environment (12 h of light:12 h of dark) and provided with food and water ad libitum. Animals were fed Teklad Global (Madison, WI) irradiated 2919 chow, which does not contain soybean or alfalfa meal and therefore contains minimal phytoestrogens. Animals were treated in accordance with the National Institutes of Health Guide for the Care and Use of Laboratory Animals and the established institutional animal use and care protocol at Northwestern University.

Alginate hydrogel preparation

Sodium alginate (55–65% guluronic acid) was provided by FMC BioPolymer (Philadelphia, PA). Alginate was dissolved in deionized water to a concentration of 1% (w/v) and then purified with activated charcoal (0.5 g charcoal/g alginate) to remove organic impurities and improve the purity of the alginate.[17] Following charcoal treatment, alginate solution was sterile-filtered through 0.22-μM filters, lyophilized within Steriflip conical tubes (Millipore, Billerica, MA), and sterile-aliquoted. Aliquots of charcoal-stripped and sterilized sodium alginate were reconstituted with sterile 1×phosphate-buffered saline (PBS) to a concentration of 1.5% (w/v) for each experiment.

Follicle isolation, encapsulation, and culture

Multilayered secondary follicles (150–180 μm, type 5b) were isolated from 16-day-old female mice and encapsulated into a sterile 1.5% (w/v) alginate bead as described previously[7] with slight modifications. Ovaries were incubated in αMEM (Invitrogen, Carlsbad, CA) containing 1% fetal calf serum (FCS) (Invitrogen), 0.1% type I collagenase, and 0.02% DNase I (Worthington Biochemical, Lakewood, NJ) at 37°C and 5% carbon dioxide (CO_2) for 30 min. Follicles were mechanically isolated using insulin-gauge needles in L15 media (Invitrogen) containing 1% FCS. Individual follicles were maintained in αMEM/1% FCS at 37°C, 5% CO_2 for 2 h before encapsulation. Only follicles displaying the following characteristics during the 2-h preincubation period were selected for encapsulation and culture: (1) diameter of 150–180 μm; (2) intact with some attached, fibroblast-like theca cells; and (3) visible, immature oocyte that was round and centrally located within the follicle.

After washing through 1.5% alginate twice, single follicles were pipetted into the middle of each alginate droplet (2–3 μL) suspended on a polypropylene mesh (0.1-mm opening; McMaster-Carr, Atlanta, GA). The mesh was immediately immersed in sterile 50 mM calcium chloride for 2 min to crosslink the alginate; it was then rinsed in culture media (αMEM, 10 mIU/mL recombinant follicle-stimulating hormone [A.F. Parlow, National Hormone and Pituitary Program, National Institute of Diabetes and Digestive and Kidney Diseases, Bethesda, Maryland], 3 mg/mL bovine serum albumin [BSA], 1 mg/mL bovine fetuin [Sigma-Aldrich, St. Louis, MO], 5 μg/mL insulin, 5 μg/mL transferrin, and 5 ng/mL selenium). Alginate beads containing a single follicle were plated at 1 follicle per well in 96-well plates in 100 μL of culture media. Fetuin, dialyzed extensively against embryo culture-grade water and lyophilized, was added to prevent zona pellucida hardening.[18] Throughout isolation, encapsulation, and plating, follicles were maintained at 37°C and a pH of 7.0.

Encapsulated follicles were cultured at 37°C in 5% CO_2 for 8 days. Every other day, half of the media (50 μL) was exchanged and stored at −80°C. Follicle survival and diameter were assessed using an inverted Leica DM IRB microscope with transmitted light and phase objectives (Leica, Bannockburn, IL). Follicles were designated dead if the oocyte was no longer surrounded by a granulosa cell layer or if the granulosa cells had become dark and fragmented. After 8 days, the culture media was replaced by 100 μL L15 medium containing 10 mIU/mL alginate lyase for 30 min at 37°C. Follicles were removed from the degraded alginate bead, and all remaining alginate was removed using a new IVF dish containing L15 medium with 1% FCS.

Follicle and oocyte measurement

Pictures of encapsulated follicles were taken on culture days 0, 4, and 8 using an inverted Leica DM IRB microscope. The diameter of follicles containing oocytes that had not yet matured was measured in duplicate from the outer layer of theca cells using ImageJ 1.33U (National Institutes of Health, Bethesda, Maryland) and was based on a calibrated ocular micrometer. The diameters of oocytes from follicles cultured *in vitro* were obtained on day 0 and day 8 and were compared with those of control *in vivo* oocytes collected from unexpanded cumulus oocyte complexes of antral follicles from superovulated 24-day-old mice (primed with 5IU equine chorionic gonadotropin (eCG) [Sigma-Aldrich] for 46 h). Control oocytes were denuded by gentle aspiration through glass pipettes. The oocyte diameter was measured without the zona pellucida.

Oocyte maturation

After follicles were retrieved from the alginate bead, they were transferred to maturation media composed of αMEM, 10% FCS, 1.5 IU/mL human chorionic gonadotropin (HCG), and 5 ng/mL epidermal growth factor (Sigma-Aldrich) for 16 h at 37°C, 5% CO_2.[19] Oocytes were then

denuded from the surrounding cumulus cells by treatment with 0.3% hyaluronidase and gentle aspiration through a polished drawn glass pipette. The oocytes were considered to be in metaphase I if neither the germinal vesicle nor the first polar body was visible. If a polar body was present in the perivitelline space, the oocytes were classified as metaphase II. Fragmented or shrunken oocytes were classified as degenerated and were discarded. Control *in vivo* oocytes were collected from 24-day-old mice primed with eCG for 46 h, placed in maturation media, denuded, and classified as described previously.

IVF and embryo transfer

Two hours before IVF, motile sperm was prepared from a sperm suspension collected from the cauda epididymis of proven CD1 male breeder mice using Percoll gradient-centrifugation (PGC) as described elsewhere.[20] PGC sperm was capacitated in IVF medium (KSOM [Specialty Media, Phillipsburg, NJ] supplemented with 3 mg/mL BSA, 5.36 mM D-glucose) for 30 min. Fifteen to 20 metaphase II oocytes were placed in 50 μL IVF medium microdrops containing 1×10^6 sperm/mL and incubated under mineral oil for 7–8 h at 37°C, 5% CO_2. Oocytes were then washed 3 times in fresh KSOM to remove all bound sperm. Fertilized oocytes were identified by the presence of 2 pronuclei (2PN). As a control, CD1 oocytes were obtained from day-24 mice primed with 5 IU of eCG for 48 h and 5 IU of HCG for 14 h before collection. The 2PN zygotes were transferred to the oviducts of 8- to 10-week-old pseudopregnant CD1 female rats 0.5 days postcoitum.

Histology and theca cell staining

Follicles cultured for 8 days were removed from the alginate bead as described previously and fixed for 2 h at 4°C in 4% paraformaldehyde in 1×PBS. Follicles were dehydrated in ascending concentrations of ethanol (10–100%), and embedded in paraffin by an automated tissue processor (Leica, Mannheim, Germany). Serial 4-μm sections were cut and stained with hematoxylin and eosin. To verify the presence of an intact theca cell layer, follicles were stained with 3β-hydroxysteroid dehydrogenase (3βHSD) solution containing 0.12 mg/mL nitroblue tetrazolium chloride, 0.25 mg/mL beta-nicotinamide adenine dinucleotide hydrate (β-NAD+), and 0.025 mg/mL epiandrosterone (Sigma-Aldrich) in 1×PBS for 30 min at room temperature in the dark.[21]

Hormone assays

Androstenedione, 17β-estradiol, and progesterone were measured in conditioned media collected on follicle culture days 2, 4, 6, and 8 using commercially available radioimmunoassay kits (androstenedione and 17β-estradiol, Diagnostic Systems Laboratories, Inc., Webster, TX; progesterone, Diagnostic Products Corp., Los Angeles, CA). Media collected from wells containing no follicle was used as the assay control.

Statistical analysis

Oocyte survival rate, size, and steroid productions were obtained from 6 independent cultures. Two cultures were used to measure oocyte size. The other 4 cultures are for *in vitro* maturation, IVF, and embryo transfers. Follicle size and steroid hormone concentrations were analyzed by 1-way analysis of variance (ANOVA). Oocyte size, *in vitro* maturation rate, and IVF rate were analyzed using a 1-way ANOVA followed by a paired *t*-test. A *p*-value less than 0.05 was considered statistically significant. All statistical calculations were done with GraphPad Prism software, version 4.00 (San Diego, CA).

RESULTS

Evaluation of *in vitro* cultured follicle growth

Alginate hydrogel-embedded follicles (*n*=129) maintained their 3D structures and had a survival rate of 93.3% ± 1.6% through an 8-day culture period (Fig. 1A–C). The average follicle diameter increased from 156.1 ± 6.0 μm on day 0 to 348.8 ± 44.8 μm on day 8 (Fig. 1D). Follicles grown *in vitro* maintained structures that phenocopied those of *in vivo* control follicles: a spherical shape with a central fluid-filled antral cavity containing an oocyte surrounded by cumulus cells (Fig. 2A). Embedded follicles also had an intact theca cell layer, as revealed by 3βHSD staining (Fig. 2B).

Steroid production *in vitro*

Secretion of androstenedione, estradiol, and progesterone from follicles cultured *in vitro* is depicted in Fig. 3A. Steroid levels were undetectable on day 2 of culture but began to increase by day 4. Mean androstenedione levels rose to 1.27 ± 0.27 ng/mL at day 6 and 2.12 ± 0.52 ng/mL at day 8, indicating that theca cells were exhibiting normal physiologic function in culture. Average progesterone concentrations remained under 1 ng/mL up to day 4 of culture, then increased to approximately 2 ng/mL as luteinization of granulosa cells occurred. Average estradiol production increased quickly from a mean 0.19 ± 0.09 ng/mL on day 4 to 4.29 ± 0.96 ng/mL on day 8 of culture.

Oocyte growth and meiotic competence

In vivo, immature oocytes grow in size while remaining in prophase I, and must undergo a process of maturation in which the germ cell progresses from prophase I to metaphase II in response to increasing concentrations of gonadotropins in order to become competent for fertilization. [22] Similarly, oocytes cultured *in vitro* must mature and progress to metaphase II in response to exogenous gonadotropins, a process termed *in vitro* maturation. Throughout the culture period, oocytes underwent extensive growth and maintained meiotic arrest (Fig. 2C). The average size of oocytes increased from 61.78 ± 2.67 μm on day 1 to 68.57 ± 2.77 μm on day 8 of culture ($n = 30$; $p < .05$). The diameter of oocytes grown *in vitro* approached that of *in vivo* control oocytes of the same chronologic age (69.58 ± 1.50 μm); this difference was not statistically significant ($n = 30$; $p = .078$) (Fig. 3B).

After retrieval of the follicles from the alginate hydrogel matrix on day 8, *in vitro* maturation was induced by exposing the follicles to exogenous HCG, and the granulosa cells were removed. Of 99 fully grown, denuded oocytes retrieved from the alginate culture system, a mean of 82.3% ± 8.8% resumed meiosis and underwent germinal vesicle breakdown, 70.9% ± 9.9% extruded the first polar body (Fig. 2D) and matured to metaphase II, and 11.4% ± 5.3% remained in metaphase I (Fig. 3C). Notably, the maturation rate of cultured oocytes was lower than that of control oocytes that developed *in vivo*, with a mean of 96.7% ± 0.5% undergoing germinal vesicle breakdown and 91.9% ± 2.9% reaching metaphase II (Fig. 3C).

IVF and embryo development

Subsequent IVF of mature oocytes should result in the extrusion of the second polar body and the formation of 2PN. *In vitro*–cultured, denuded oocytes in metaphase II ($n = 86$) and control oocytes collected from superovulated mice (*in vivo* controls, $n = 65$) were fertilized *in vitro* under the same conditions. The development of 2PN zygotes was scored as a successful fertilization (Fig. 2E), and occurred in a mean of 68.2% ± 14.5% of oocytes cultured *in vitro* and 81.7% ± 5.0% of *in vivo* control oocytes (Fig. 3D). Twenty 2PN-stage zygotes derived from oocytes cultured *in vitro* and 16 derived from *in vivo* control oocytes were transferred to the oviducts of pseudopregnant CD1 female rats, 6 zygotes per oviduct. Two female and 2

male brown pups derived from oocytes cultured *in vitro* (from C57BL/6×CBA F1 hybrids) and 4 (2 males/2 females) white pups derived from *in vivo* control oocytes (from superovulated CD1 mice) were successfully delivered after a 19-day gestation (Fig. 2F). All 4 of the mice derived from oocytes cultured *in vitro* developed normally and were fertile.

DISCUSSION

In recent years, *in vitro* culture of ovarian follicles has advanced the study of follicle growth and ovulation and has provided insight into the importance of cell-cell interactions within the follicle and the roles of various endocrine and paracrine factors, including steroid and peptide hormones, growth factors, cytokines, and neuropeptidergic substances. [23] The clinical application of this knowledge has led to the possibility that *in vitro* follicle culture can be used as a tool in the treatment of infertility. It holds great promise as a means to preserve fertility that has been compromised by disease or its treatment (i.e., cancer and chemotherapy or radiation therapy). However, the inherent complexity of oocyte growth and maturation has proven a formidable obstacle to the “banking” of human eggs as is currently possible with human sperm.

Current follicle culture systems use 2D substrates (e.g., tissue culture wells); however, 3D systems may more effectively simulate physiologic conditions. Granulosa cells encapsulated in alginate allowed self-organization into clusters; the luteinization index (P4:E2 ratio) was significantly lower in encapsulated granulosa cells than in monolayer cultures.[24] Our previous studies have demonstrated that an alginate hydrogel matrix can support encapsulated follicle growth and the development of meiotically competent oocytes.[7] Here, we report that oocytes from immature mouse follicles encapsulated in alginate hydrogel were able to grow, mature, and be fertilized, resulting in the live birth of viable, fertile offspring.

Since our first attempt at encapsulation of follicles using the alginate matrix, the method of follicle isolation and culture medium components have been optimized to improve follicle survival rate (93%) and success of oocyte maturation after release from the hydrogel scaffold (70%).[7] We used a combination of enzymatic and mechanical techniques for initial isolation of immature follicles, thereby increasing the number of intact follicles collected and encapsulated. Bovine fetuin, which is included in the culture media to prevent zona pellucida hardening, also has known growth-promoting effects,[25] which may have contributed to the higher follicle survival rates and oocyte growth and maturation rates compared with previous studies.[7,16]

The current study demonstrated that oocytes from alginate-encapsulated follicles grew *in vitro* to a size comparable to that of oocytes grown *in vivo*, consistent with previous studies.[26–28] This study also showed that oocytes grown *in vitro* in an alginate hydrogel scaffold are capable of resuming meiosis, undergoing germinal vesicle breakdown, extrusion of the polar body, and normal fertilization, resulting in offspring that developed to term and were viable and fertile. The 20% live birth rate from transferred mouse embryos reported in this study is a significant improvement over the previously reported rate of 5.7%[29] and 7.4%,[30] and similar to that in another report,[26] all for 2D systems. Nevertheless, fewer *in vitro*–cultured oocytes (68%) were successfully fertilized than *in vivo* control oocytes (82%), suggesting that there may be additional factors *in vivo* that are necessary for optimal oocyte growth and maturation, and that the alginate culture system requires further optimization.

Importantly, the physiologic functions of each cell type within the cultured follicles were retained while encapsulated in the alginate hydrogel, suggesting that cell-cell interactions between the oocyte, granulosa cells, and theca cells remained intact. As expected, granulosa cell production of estradiol increased linearly after day 4 of culture[31] and theca cell function

was normal based on the progressive increase in androstenedione levels over time. Furthermore, the proportion of estradiol to progesterone secreted by the follicles indicated that no significant premature luteinization occurred and the interaction between the oocyte and somatic cells remained intact.[32] Thus, the 3D system permits normal follicle development.

Following our initial reports on culturing of mouse follicles in alginate hydrogels,[6] an independent group verified our results in the short-term culture of rat follicles.[33] Although the rat studies have not yet shown that the hydrogel system can support the development of meiotically competent rat oocytes, as we have previously shown for mouse oocytes,[7,34] it demonstrated that connexin 43 was expressed at similar levels between encapsulated and *in vivo* follicles. On the basis of these results, we assume that suitable conditions can be developed for species other than mouse. The real value of the system may be best exploited in the primate and other species that support large follicle growth. Alginate maintains integral connections between the oocyte and somatic cells,[6] and these connections will be critical to the management of oocyte and follicle development during the extended time that is necessary for oocyte maturation.

In conclusion, alginate-based 3D culture systems have been applied to the *in vitro* culture of mouse ovarian follicles. Morphological and functional analysis of follicle growth and hormone secretion patterns suggested that follicles cultured *in vitro* in the alginate system mimic that of follicles *in vivo*. Fertilization of oocytes and implantation of embryos derived from follicles cultured *in vitro* resulted in live birth rates comparable to those reported in other culture systems. The alginate culture system provides a novel opportunity for the *in vitro* storage and maturation of ovarian follicles, and may allow the establishment of oocyte banks for use in *in vitro* fertilization procedures. Further studies will be needed to refine the alginate culture system for application to human follicles and to determine the safety and efficacy of this approach for fertility preservation.

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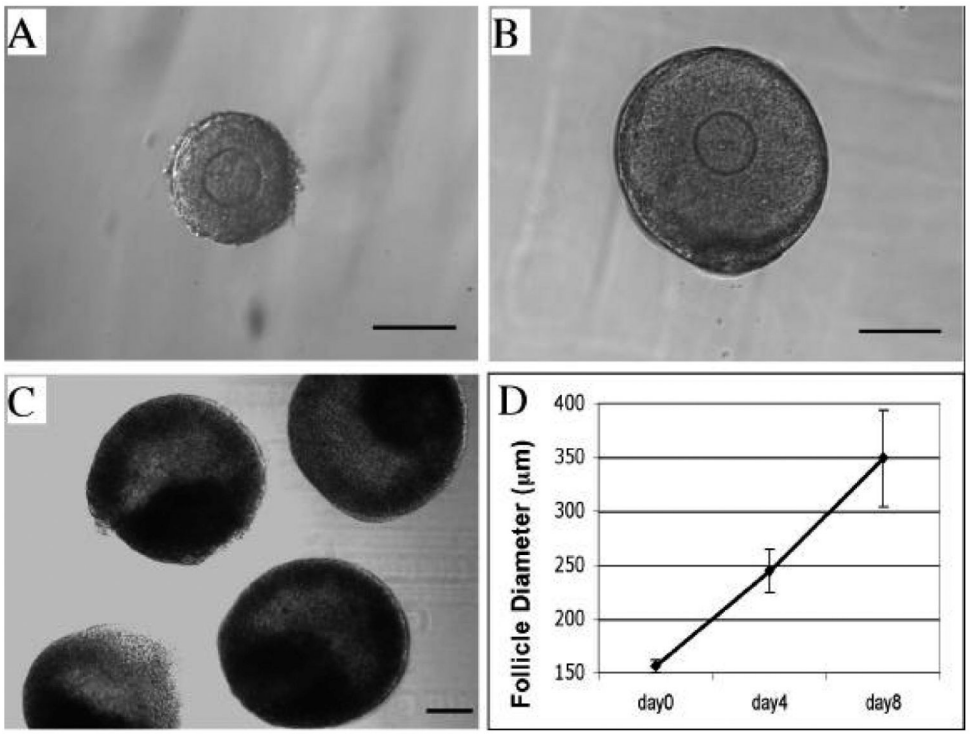

FIG. 1.

Development and differentiation of a representative multilayered secondary follicle for 8 days in alginate scaffold. (**A**) At day 0, a multilayered secondary follicle with a centrally located immature oocyte and some attached theca cells around was isolated and encapsulated in alginate gel. (**B**) Granulosa cells clearly proliferated after 4 days culture. (**C**) Follicle maintained their 3-dimensional structure and formed antrums at day 8 (follicles have been removed from alginate gel). (**D**) The average size of follicles increased 123% from day 0 to day 8. Bar = 100 μm. Color images available online at www.liebertpub.com/ten.

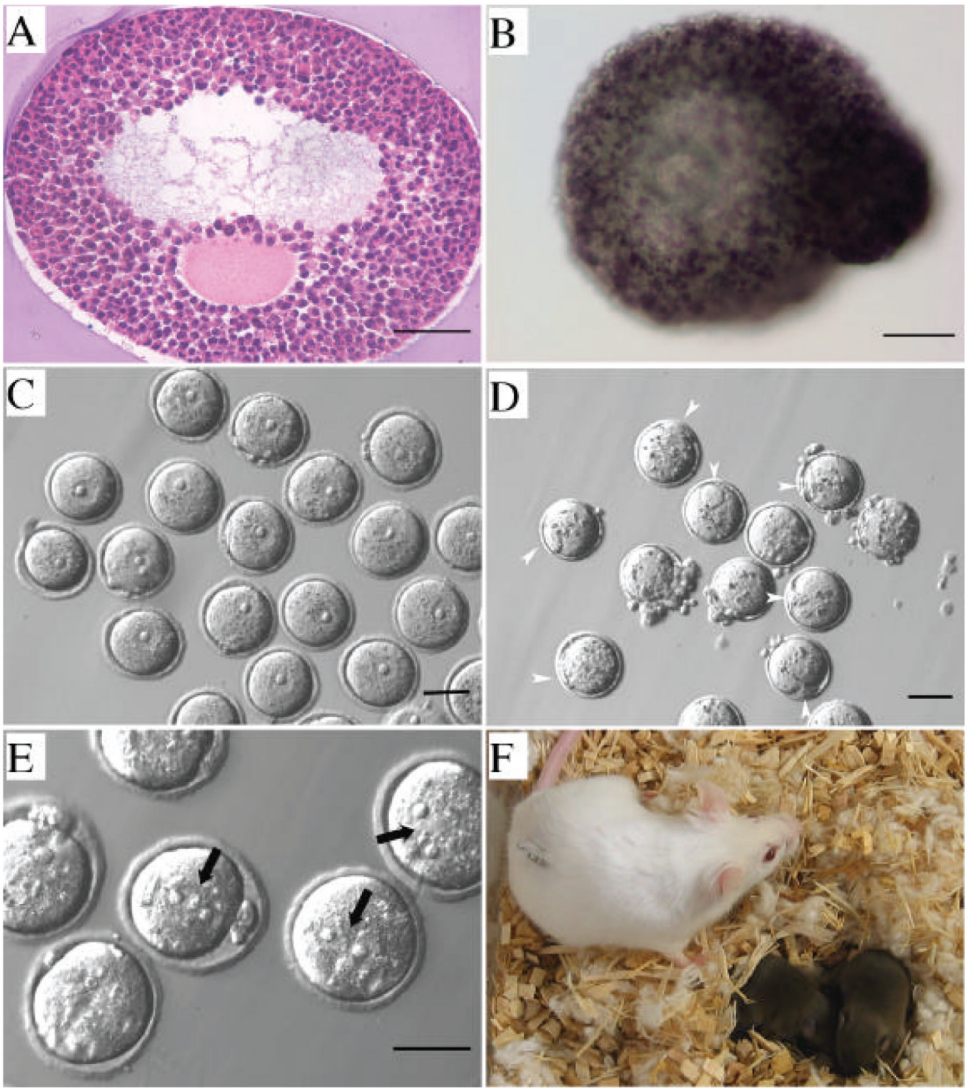

FIG. 2.

(**A**) After 8 days of culture, follicle displayed an *in vivo* preovulatory phenotype, spherical shape with a central fluid-filled antral cavity, an oocyte within tightly compacted cumulus cells, layers of mural granulosa cells outside, and (**B**) an intact outer theca cell layer shown by 3β-hydroxysteroid dehydrogenase stain. (**C**) Oocytes maintained their meiotic arrest during culture (granulosa cells have been removed), and (**D**) resumed meiosis and extruded first polar body (white arrow) after exogenous human chorionic gonadotropin stimulation. (**E**) Metaphase II oocytes can be fertilized normally *in vitro* (black arrow indicates 2 pronuclei), and (**F**) resulted in the live birth after being transferred back into the oviduct of pseudopregnant mouse. Bar = 100 μm (A, B), 50 μm (C–E).

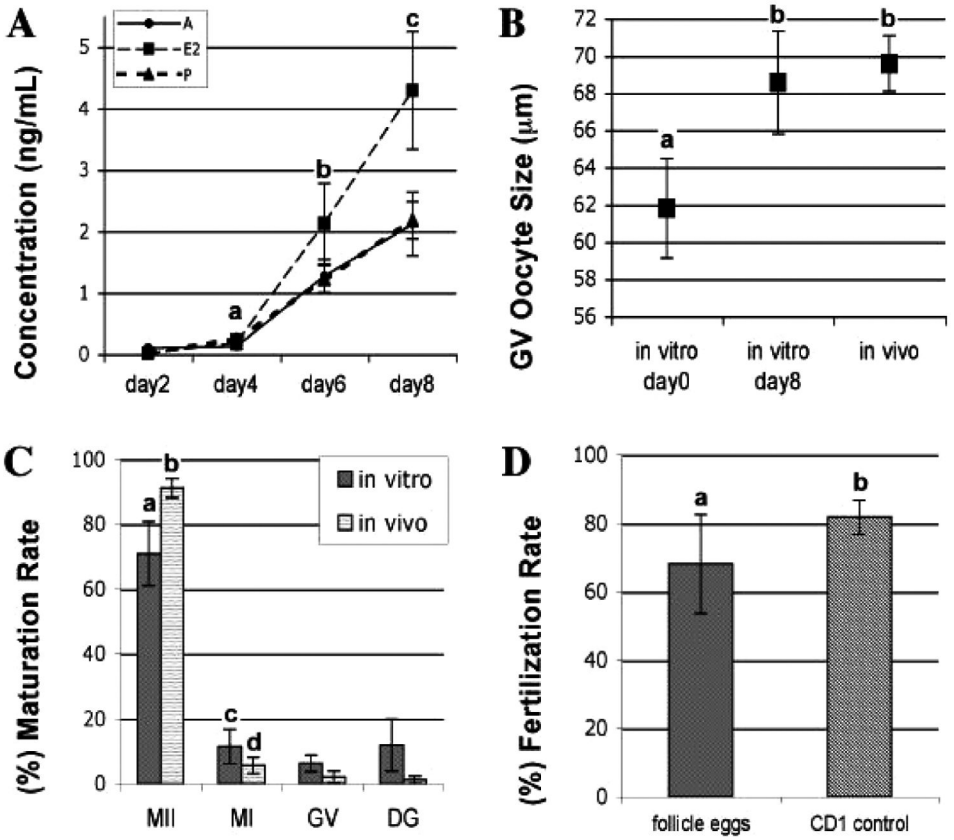

FIG. 3.

(**A**) Basal steroid concentrations were measured from the condition media collected every other day (A: androstenedione, E2: estradiol, P: progesterone). Data represented as mean±SD (n = 4). Androstenedione increases represent theca cells maintaining normal physiologic function. Estradiol and progesterone both increased, and the ratio of P to E2 was less than 0.5. (**B**) Oocytes from *in vitro* cultured multilayered secondary follicles increased significantly from day 0 to day 8 (n = 30; p <.05). In addition, the final size of *in vitro* growth oocytes was not significantly different in size from oocytes grown *in vivo* (n = 30; p =.078). (**C**) Most fully grown oocytes (n = 99) underwent germinal vesicle breakdown and progressed to metaphase II, although at slightly lower rates than oocytes developed *in vivo* (n = 93). (**D**) The fertilization rate, determined by the appearance of 2 pronuclei, was 68.2%± 14.5% for metaphase II oocytes (n = 86) from *in vitro* culture follicles, and 81.7%±5.0% for metaphase II oocytes (n = 65) from *in vivo* control. Statistical significance was noted between groups with different letters. DG = degeneration; GV = germinal vesicle.