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Identification of a Stage-Specific Permissive In Vitro Culture Environment for Follicle Growth and Oocyte Development[1]

Min Xu,[3,4] Erin West,[5] Lonnie D. Shea,[5,6] and Teresa K. Woodruff[2,4,5,6,7]

Center for Reproductive Science[3] and Departments of Neurobiology and Physiology[4] and Chemical and Biological Engineering,[5] Northwestern University, Evanston, Illinois 60208
Robert H. Lurie Comprehensive Cancer Center[6] and Department of Medicine,[7] Feinberg School of Medicine, Northwestern University, Chicago, Illinois 60611

ABSTRACT

The availability of viable oocytes is the limiting factor in the development of new reproductive techniques. Many attempts have been made to grow immature oocytes in vitro during recent decades. Recently, a modified alginate-based three-dimensional culture system was designed to support the growth and maturation of multilayered secondary follicles. This system was able to produce oocytes that successfully completed meiosis, fertilization, and development to the blastocyst stage. Subsequent attempts to culture two-layered secondary follicles were unsuccessful under the original conditions. Herein, we investigated the effect of alginate consistency on two-layered follicle growth and oocyte developmental competence by encapsulating follicles into alginate scaffolds of various concentrations. Although there were no significant differences in survival rates, 0.25% and 0.5% alginate supported more rapid growth of follicles and antrum formation compared with 1.5% and 1.0% alginate after 8 days of culture. Alginate scaffold concentration also affected the proliferation and differentiation of somatic cells (theca and granulosa cells), measured in terms of morphological changes, steroid profiles (androstenedione, estradiol, and progesterone), and specific molecular markers (*Fshr*, *Lhcgr*, and *Gja1*). Theca cell proliferation and steroid production were hindered in follicles cultured in 1.5% alginate. In vitro fertilization and embryo culture revealed that oocytes obtained from 0.25% alginate retained the highest developmental competence. Overall, the present study showed that the alginate scaffold consistency affects folliculogenesis and oocyte development in vitro and that the alginate culture system can and should be tailored to maximally support follicle growth depending on the size and stage of the follicles selected for culture.

follicular development, oocyte development

INTRODUCTION

In vitro follicle culture techniques not only provide an important model for understanding the mechanism of follicle growth and oocyte maturation but also may have major clinical applications, in combination with successful cryopreservation of ovarian tissue, in restoring or preserving fertility. Several follicle culture systems that support oocyte development in vitro have been developed, and the appropriate use of these approaches, alone or in combination, could permit the growth of any stage of follicle. Thus far, live births have been achieved using follicles grown in vitro from fresh [1–4] or cryopreserved [5, 6] ovarian tissues only in mice. Nevertheless, recent reports that successful transplantation of cryopreserved ovarian tissue in women led to pregnancy and birth of healthy offspring [7–9] raise the possibility that in vitro follicle culture techniques may become another option for preserving fertility.

Two types of in vitro culture systems, termed nonspherical (two-dimensional) and spherical (three-dimensional), have been developed to support oocyte maturation in cultured preantral follicles. Nonspherical culture systems were developed to culture enzymatically isolated granulosa-oocyte complexes (GOCs) on a collagen membrane [1, 3, 10] and to grow attached primary and secondary follicles mechanically isolated from mice [11, 12]. Although both of these nonspherical culture systems were able to support the growth of meiotically competent eggs that could be fertilized and resulted in live birth in mice, such systems have been unable to support normal follicle development in larger mammalian species, including cows [13], sheep [14], and humans [15, 16].

Ovarian folliculogenesis is a complex developmental process that is regulated by various endocrine, paracrine, and autocrine factors [17, 18], as well as intraovarian cell-cell and cell-matrix interactions [19, 20]. Loss of follicle architecture and the critical cellular interactions between adjacent somatic cells and between somatic and germ cells may lead to uncoordinated growth and differentiation of granulosa and thecal cells and the oocyte. To address these issues, spherical culture systems that are capable of supporting and maintaining follicle structure in vitro were developed.

The first spherical culture system was developed by Nayudu and Osborn [21] and was later modified by other groups in an effort to optimize preservation of follicle integrity [2, 22–25]. Most researchers used later-stage preantral follicles (~170–240 μm) that were only able to remain viable in culture for short periods of time (4–6 days). Other studies have reported follicle culture using the inverted drop [25] or rotating wall vessel [26] suspension system to maintain spherical structure. However, it is unclear whether oocyte development and maturation were supported in these systems, as only somatic cell growth was measured. Recently, a three-dimensional in vitro follicle culture system was developed that uses an alginate hydrogel matrix as a scaffold for follicle growth [27]. This culture system mimics the stromal microenvironment of the ovary to support the growth and maturation of multilayered secondary follicles (150–180 μm, comprising several layers of granulosa cells surrounding the oocyte) in vitro [28, 29]. The alginate culture system was able to produce meiotically competent

[1]Supported by grant U54-HD41857 from the National Institutes of Health.
[2]Correspondence: Teresa K. Woodruff, Department of Neurobiology and Physiology, Northwestern University, 2205 Tech Drive, Hogan 4–150, Evanston, IL 60208. FAX: 847 491 2225;
e-mail: tkw@northwestern.edu

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oocytes that were able to be fertilized and result in live birth of viable offspring [30]. The primary objective of the present study was to design a similar culture system to support the growth of earlier-stage secondary follicles (100–130 μm, comprising two layers of granulosa cells surrounding the oocyte). Specifically, the physiological effects of alginate consistency on follicle and oocyte development were studied by encapsulating follicles into varying concentrations of alginate scaffolding.

MATERIALS AND METHODS

Animals and Materials

Immature follicles were isolated from prepubertal 12-day-old female F1 hybrids (C57BL/6j × CBA/Ca), and sperm was prepared from proven CD-1 male breeders. Animals were housed in a temperature- and light-controlled environment (12L:12D) and were provided with food and water ad libidum. Animals were fed Teklad Global irradiated 2919 chow, which does not contain soybean or alfalfa meal and contains minimal phytoestrogens. Animals were treated in accord with the National Institutes of Health Guide for the Care and Use of Laboratory Animals and the established Institutional Animal Care and Use Committee protocol at Northwestern University.

Alginate Hydrogel Preparation

Sodium alginate (55%–65% glucuronic acid) was provided by FMC BioPolymers (Philadelphia, PA). Alginate was dissolved in deionized water to a concentration of 1% (w/v) and was then purified with activated charcoal (0.5 g of charcoal per gram of alginate) to remove organic impurities and to improve the purity of the alginate. Following charcoal treatment, alginate solution was sterile filtered through 0.22-μM filters, lyophilized within Steriflip conical tubes (Millipore, Billerica, MA), and sterilely aliquoted. Aliquots of charcoal-stripped and sterilized sodium alginate were reconstituted with sterile 1× PBS to concentrations of 1.5%, 1.0%, 0.5%, and 0.25% (w/v) for each experiment.

Follicle Isolation, Encapsulation, and Culture

Two-layered secondary follicles (100–130 μm, type 4) were mechanically isolated from 12-day-old female mice by using insulin-gauge needles in L15 media (Invitrogen, Carlsbad, CA) containing 1% fetal calf serum (FCS). Individual follicles were maintained in α minimal essential medium (αMEM)/1% FCS at 37°C, 5% CO_2 for 2 h before encapsulation. Only those follicles displaying the following characteristics during the 2-h preincubation period were selected for encapsulation and culture: 1) diameter of 100–130 μm, 2) intact nature with some attached fibroblast-like theca cells, and 3) a visible immature oocyte that was round and centrally located within the follicle.

Selected follicles were then encapsulated into alginate beads prepared at various concentrations (1.5%, 1.0%, 0.5%, and 0.25%) (w/v) as described previously with slight modifications [28]. 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). When encapsulating follicles into the 1.5% and 1.0% alginate beads, the mesh was immediately immersed in sterile encapsulation solution (50 mM $CaCl_2$ and 140 mM NaCl). When encapsulating follicles into the 0.5% and 0.25% alginate beads, the mesh was turned over after follicle placement and was then flipped into the encapsulation solution by shaking the mesh quickly. Alginate beads were left in the encapsulation solution for 2 min to cross-link the alginate and were then rinsed in culture media (αMEM with 10 mIU/ml of recombinant FSH [A. F. Parlow, National Hormone and Peptide Program, National Institute of Diabetes and Digestive and Kidney Diseases], 3 mg/ml of BSA, 1 mg/ml of bovine fetuin [Sigma-Aldrich, St. Louis, MO], 5 μg/ml of insulin, 5 μg/ml of transferrin, and 5 ng/ml of selenium). Alginate beads containing a single follicle were plated one 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 (ZP) hardening. Throughout isolation, encapsulation, and plating, follicles were maintained at 37°C and at pH 7.

Encapsulated follicles were cultured at 37°C in 5% CO_2 for 8 days (for RNA extraction and oocyte size measurement) or for 12 days (for in vitro maturation/in vitro fertilization [IVF] experiment and oocyte size measurement). 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 considered dead if the oocyte was no longer surrounded by a granulosa cell layer or if the granulosa cells had become dark and fragmented and the follicle had decreased in size. After 8 or 12 days of culture, the culture media was replaced by 100 μl of L15 medium containing 10 units/ml of alginate lyase (Sigma-Aldrich) for 30 min at 37°C. Follicles were removed from the degraded alginate bead, and all remaining alginate was removed in a separate IVF dish containing L15 medium with 1% FCS.

Follicle and Oocyte Measurement

Photographs of each individual follicle and measurements using a calibrated micrometer (Fisher Micromaster, 1 mm with 0.01-mm division) were taken under inverted microscope with the same setup parameters. All photographs were imported into ImageJ 1.33U (National Institutes of Health) for measurement. Diameter was measured in units of pixels and was then converted to micrometers based on the pixel number to micrometer conversion determined by measuring the calibrated micrometer. The diameter of follicles containing oocytes was measured in duplicate from the outer layer of theca cells. The diameters of oocytes from in vitro cultured follicles were obtained on Days 8 and 12. Immature oocytes were denuded by gentle aspiration through glass pipettes. The oocyte diameter was measured without the ZP as already described.

Oocyte Maturation, Fertilization, and Embryo Culture

After 12 days of culture, follicles were retrieved from the alginate bead and were transferred to maturation media composed of αMEM, 10% FCS, 1.5 IU/ml of hCG, and 5 ng/ml of epidermal growth factor (EGF) (Sigma-Aldrich) for 16 h at 37°C, 5% CO_2. 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 have undergone germinal vesicle breakdown (GVBD) if a germinal vesicle was not visible. If a polar body was present in the perivitelline space, the oocytes were classified as metaphase II (MII). Fragmented or shrunken oocytes were classified as degenerated.

Motile sperm was prepared from a sperm suspension collected from the cauda epididymis of proven CD-1 male breeder mice using Percoll gradient centrifugation (PGC) as described previously [31]. The PGC sperm was capacitated in IVF medium (potassium simplex optimized medium [KSOM] [Specialty Media, Phillipsburg, NJ] supplemented with 3 mg/ml of BSA and 5.36 mM D-glucose) for 30 min. Fifteen to 20 MII oocytes were placed in 50 μl of 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 three times in fresh KSOM to remove all bound sperm and were transferred into a 20-μl fresh KSOM drop overnight. Embryos that cleaved to the two-cell stage were characterized as fertilized. Embryos were washed in KSOM and were cultured until the blastocyst stage. The blastocyst formation rate was scored at Day 5 of culture.

Characterization of Follicle Functionality

After 8 days of culture, follicles were isolated from the alginate beads as already described. Immature denuded oocytes were separated from the surrounding somatic cells by gentle aspiration through glass pipettes in L15 media. Oocytes and somatic cells were separately transferred into two clean tubes with a minimal amount of media. The tRNA was purified from oocytes and somatic cells using the Absolutely RNA Microprep Kit (Stratagene, Cedar Creek, TX) according to the manufacturer's procedure. The tRNA was reverse transcribed into first-strand cDNA (SuperScript First-Stand Kit; Invitrogen) using random hexamer primers and was stored at −20°C. Real-time PCR was used to compare the mRNA expression levels of FSH receptor (*Fshr*), LH/choriogonadotropin receptor (*Lhcgr*), and Gap junction protein (alpha 1, 43 kDa) (*Gja1*) (also known as connexin 43) in somatic cells, as well as of growth differentiation factor 9 (*Gdf9*) and NACHT (leucine-rich repeat and PYD-containing 5) (*Nalp5*) (also known as *MATER*) in denuded oocytes. Glyceraldehyde-3-phosphate dehydrogenase (*Gapdh)* was used as an endogenous control. All real-time PCR experiments were performed using Taqman probes. RT reactions run in the absence of reverse transcriptase served as a negative control.

Hormone Assays

Androstenedione, 17β-estradiol, and progesterone were measured in conditioned media collected on Follicle Culture Days 4, 6, 8, 10, and 12 using commercially available radioimmunoassay kits (Diagnostic Systems Laboratories, Inc, Webster, TX [androstenedione and 17β-estradiol]; and Diagnostic Products Corporation, Los Angeles, CA [progesterone]). In each independent experiment, condition media obtained from each time point (different culture day) were pooled together. Four independent measurements (duplicates per measure) for each steroid at each time point were performed.

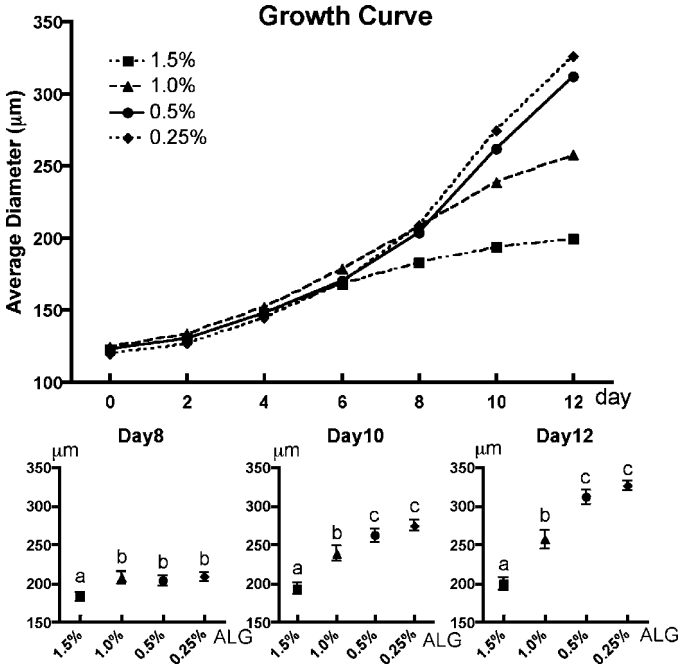

FIG. 1. Two-layered secondary follicle growth in alginate scaffolds of different concentrations. During the first 6 days of culture, no significant difference in follicle size was observed among the four groups. At Day 8, follicles encapsulated in 1.5% alginate demonstrated significantly less growth than those in the other three alginate concentrations. At Days 10 and 12, follicles in 0.5%, 0.25%, and 0.5% alginates were significantly larger than follicles grown in 1.5% and 1.0% alginate. Statistical significance was observed between groups with different letters ($P < 0.05$).

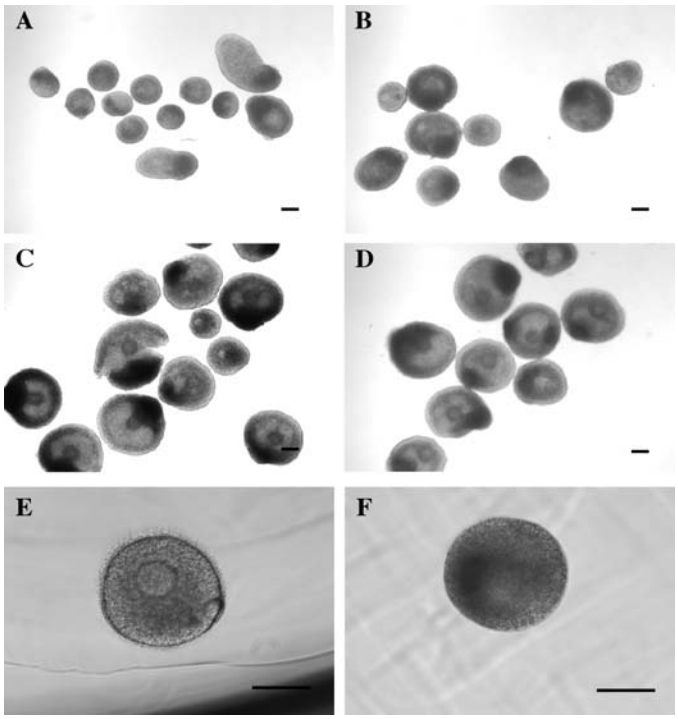

FIG. 2. Development and differentiation of two-layered secondary follicles in alginate scaffold. At Day 12, fewer follicles encapsulated in 1.5% and 1.0% alginate (**A** and **B**) developed to the antrum stage compared with those in 0.5% and 0.25% alginate (**C** and **D**). Follicles encapsulated in 0.25% alginate began to form multiple laminar-like theca cell layers at Day 8 (**E**), while no such layer was observed in follicles encapsulated in 1.5% alginate (**F**). Bar = 200 μm.

The sensitivities for the androstenedione, estradiol, and progesterone assays are 0.1 ng/ml, 10 pg/ml, and 0.1 ng/ml, respectively. Media collected from wells containing no follicles was used as the assay control.

Statistical Analysis

Follicle size, survival rate, antral and theca growth rate, steroid production, and IVF and embryo culture were conducted using four independent cultures of 18–24 follicles each time for each alginate concentration. Three independent cultures of 12–18 follicles each time for each alginate concentration were used for measurement of denuded oocyte size and RNA preparation. Data were analyzed using a one-way ANOVA, followed by a paired Student *t*-test. $P < 0.05$ was considered statistically significant. All statistical calculations were performed using the software GraphPad Prism version 4.0.

RESULTS

Folliculogenesis

Evaluation of in vitro cultured follicle growth. Follicles maintained their three-dimensional structures in all alginate bead concentrations tested. Survival rates did not differ significantly among the different groups. During the first 6 days of culture, follicle sizes among the four groups were not significantly different; however, after 8 days of culture, follicle growth was negatively correlated with alginate concentration (Fig. 1 and Table 1). During the last 4 days of culture, follicles embedded in 0.5% and 0.25% alginate had linear growth curves and were significantly larger than follicles grown in 1.5% and 1.0% alginate. Few antral follicles developed in the 1.5% alginate cultures, whereas 73.2% and 86.2% of follicles grown in 0.5% and 0.25% alginate, respectively, developed an antrum (Fig. 2 and Table 1). In addition, multiple laminar-like theca cell layers were observed after Day 8 among most of the follicles cultured in 0.5% and 0.25% alginate.

Steroid production in vitro. Secretion patterns of androstenedione, 17β-estradiol, and progesterone from each group of in vitro cultured follicles were consistent with the observed changes in follicle morphology and differentiation (Fig. 3). All

TABLE 1. Survival rates, follicle size measurement, antrum and theca cell layer observed rates from two-layered secondary follicles cultured in alginate scaffold in vitro.

Alginate concentration	N[a]	Survival (%)[b]	Follicle diameter (μm)[b]			Antrum (%)[b]	Theca layer (%)[b]	
			Day 8	Day 10	Day 12		Day 8	Day 10
1.50%	101	73.6 ± 9.3	182.7 ± 6.3	193.5 ± 6.9	199.4 ± 8.1	5.0 ± 1.8[c]	0[c]	0[c]
1.00%	108	84.0 ± 5.3	207.9 ± 8.3	238.5 ± 10.1	257.3 ± 11.9	63.9 ± 1.7[d]	14.0 ± 4.1[d]	18.9 ± 4.1[d]
0.50%	118	84.8 ± 5.8	203.7 ± 6.7	261.6 ± 8.3	311.5 ± 8.7	73.2 ± 8.5[e]	66.4 ± 5.7[e]	79.2 ± 2.1[e,f]
0.25%	96	78.0 ± 3.8	208.5 ± 6.0	274.7 ± 7.0	326.2 ± 6.3	86.2 ± 8.0[e]	72.2 ± 3.5[e]	79.3 ± 4.8[e]

[a] N = starting follicle number.
[b] Values are the average ± SEM of multiple follicles from four independent cultures.
[c-f] Different superscripts within each column indicate statistically significant differences ($P < 0.05$).

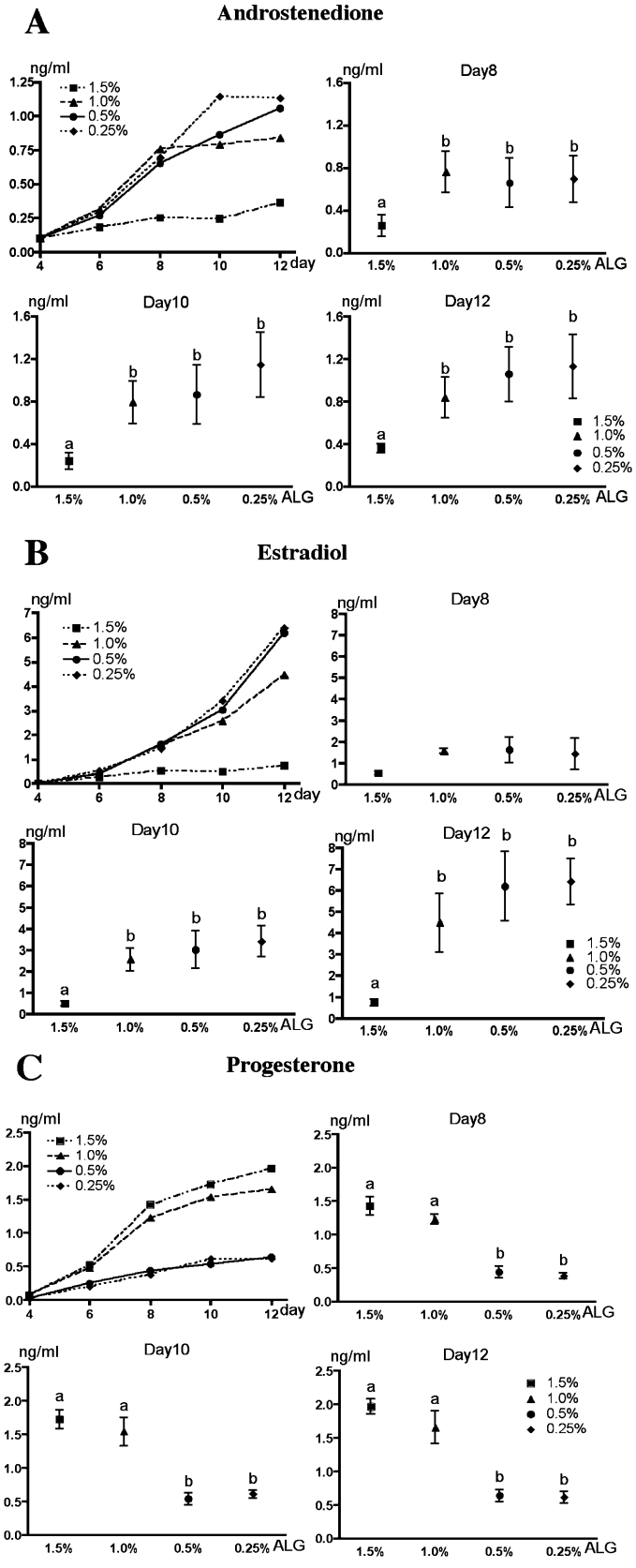

FIG. 3. Steroid secretion profiles of two-layered secondary follicle cultured in alginates of different concentrations. All steroid levels increased significantly from baseline over time. From Day 8 to Day 12, follicles grown in 1.5% alginate showed significantly decreased androstenedione secretion (**A**). No significant changes in estradiol secretion between alginate conditions was observed at Day 8, but from Day 10 to Day 12, estradiol secretion by follicles cultured in 1.5% alginate was significantly less than that secreted by follicles in the other alginate conditions (**B**). Progesterone secretion was significantly lower in follicles encapsulated in 0.5% and 0.25% alginate compared with those grown in 1.5% and 1.0% alginate through the last 6 days of culture (**C**). Data are mean ± SEM, n = 4. Statistical significance was observed between groups with different letters ($P < 0.05$).

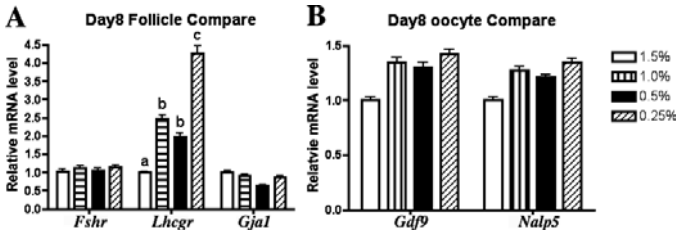

FIG. 4. Relative gene expression levels characterized by real-time PCR. *Fshr* and *Gja1* mRNA expression was not significantly different in response to change of alginate concentration. *Lhcgr* mRNA expression was significantly upregulated in follicles encapsulated in 0.25% alginate. *Lhcgr* mRNA expression in 0.25% alginate was two-fold higher than that in 0.5% and 1.0% alginate and four-fold higher than that in 1.5% alginate (**A**). *Gdf9* and *Nalp5* mRNA expression was slightly lower in the 1.5% alginate group compared with the other groups, but the difference was not significant (**B**). Statistical significance was observed between groups with different letters ($P < 0.05$).

steroid levels increased significantly from baseline over time. At Day 8, follicles grown in lower alginate concentrations showed significant increases in androstenedione (Fig. 3A) and estradiol (Fig. 3B) secretion, although estradiol levels rose more slowly than androstenedione levels during the culture period. Significant increases in estradiol production by follicles grown in lower-concentration alginate were not observed until Days 10 and 12. In contrast, progesterone secretion was significantly lower in follicles encapsulated in 0.5% and 0.25% alginate compared with those grown in 1.5% and 1.0% alginate (Fig. 3C).

Characterization of differential gene expression by RT-PCR. At each culture time point, 10–12 follicles were collected from each alginate concentration. Three independent cultures were used for RNA preparation. The differential mRNA expression levels of three genes (*Fshr*, *Lhcgr*, and *Gja1*) in each alginate group at Day 8 of culture were compared using real-time PCR. Day 8 cultured follicles were selected for these experiments because growth and morphology differences among the test groups had developed by this time point (Figs. 1 and 2). There was no significant difference in *Fshr* and *Gja1* expression in the different alginate groups (Fig. 4A); *Lhcgr* expression was significantly upregulated in follicles at lower alginate concentrations, with follicles encapsulated in 0.25% alginate having approximately four times higher *Lhcgr* expression than those grown in 1.5% alginate.

Oogenesis

Oocyte growth. To compare the oocyte sizes among groups in different alginate concentrations, germinal vesicle oocytes were denuded by gentle aspiration through glass pipettes after 8 and 12 days of culture. On Day 8, the mean diameter of oocytes cultured in 0.25% alginate was smaller than that in those cultured in the other alginate concentrations (Fig. 5A). However, by Day 12, the mean diameter of oocytes cultured in the lower concentrations of alginate had increased,

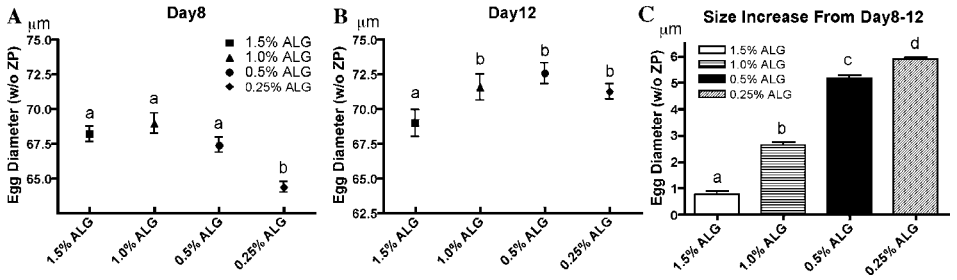

FIG. 5. Oocyte size from different groups of alginate concentrations. On Day 8, the mean diameter of oocytes cultured in 0.25% alginate was smaller than that of those cultured in the other alginate concentrations (**A**). However, on Day 12, the mean diameter of oocytes cultured in 1.5% alginate was smallest (**B**). Decreasing alginate concentration increased overall oocyte growth between Day 8 and Day 12 (**C**). Statistical significance was observed between groups with different letters ($P < 0.05$).

while the oocyte size in 1.5% alginate remained unchanged (Fig. 5B). At the end of 12 days, oocytes in 0.25% alginate were not significantly different from those cultured in 1.0% or 0.5% alginate and were significantly larger than those cultured in 1.5% alginate. The overall increase in oocyte size between Day 8 and Day 12 was highest in follicles grown in 0.25% alginate (Fig. 5C).

Meiotic competence, IVF, and embryo development. After 12 days of culture, follicles were separated from alginate beads and were stimulated with hCG and EGF for 16 h. Mucification was observed for follicles if they had formed an antrum by the end of culture (data not shown). No significant differences of GVBD rates were found among the alginate concentration groups (Table 2). However, more oocytes cultured in 0.5% and 0.25% alginate extruded the first polar body compared with those cultured in 1.5% and 1.0% alginate (Fig. 6D and Table 2).

Subsequent IVF of mature oocytes resulted in two-cell embryos after 24 h (Fig. 6E). The fertilization rates of oocytes cultured in 0.25% alginate were significantly higher than those cultured in 0.5%, 1.0%, and 1.5% alginate. After 5 days, 29.4% embryos from the 0.25% alginate group developed to expanded blastocysts, whereas no blastocysts developed from embryos from the other alginate concentration groups (Fig. 6F and Table 2).

Characterization of differential gene expression by RT-PCR. The differential mRNA expression levels of two oocyte-specific genes (*Gdf9* and *Nalp5*) in oocytes grown in each alginate concentration group were compared by real-time PCR. To eliminate the effect of somatic cells, denuded oocytes were used for tRNA extraction and PCR amplification. Although *Gdf9* and *Nalp5* expression were slightly lower in the 1.5% alginate group compared with the other groups, the difference was not statistically significant (Fig. 4B).

DISCUSSION

In vitro culture of whole-mouse follicles in alginate requires that the scaffold provide follicle three-dimensional structural support yet allow substantial follicle growth and development. It was previously shown that mouse secondary follicles (multilayered) can be encapsulated in 1.5% alginate scaffold matrices and cultured in vitro for 8 days [30]. Follicles not only maintained their spherical architecture but also matured in vitro and developed meiotically competent oocytes. In addition, mature oocytes obtained from these cultured follicles could be fertilized, resulting in offspring that developed to term and were viable and fertile [30]. However, when slightly earlier-stage two-layered secondary follicles were encapsulated in 1.5% alginate, they had a slower growth rate and failed to provide mature oocytes after 8 days of culture [28, 29]. Two possible reasons could explain these results: (1) the in vitro culture time was too short to allow for follicle development or (2) the physical properties of the alginate bead limited the growth of these earlier follicles.

Extension of the culture time of two-layered secondary follicles encapsulated in 1.5% alginate resulted in a significant improvement in oocyte meiotic competence. The mean rates of GVBD and MII oocyte development after 12 days were 84.2% and 56.3%, respectively, in the present study (Table 2), which were significantly higher than those reported in previous investigations (27.3% and 0%, respectively) [28]. Nonetheless, the mean follicle size increase from Day 8 to Day 12 was only 9.1% (from 182.7 μm to 199.4 μm), with the final follicle size much smaller than would be expected based on in vivo follicle development [32]. Furthermore, few follicles grown in 1.5% alginate formed an antrum (Table 2). These results indicate that two-layered secondary follicle cultures likely require longer culture times to achieve maturation but that growth was still suboptimal at the 1.5% alginate concentration. Although 1.5% alginate beads were "soft" enough for multilayered secondary follicle expansion, it is possible that they impeded two-layered secondary follicle growth, which in turn negatively affected oocyte developmental competence.

Decreasing the concentration of alginate to increase follicle growth within the hydrogel can result from changes in the hydrogel mechanics and in the transport of media components through the hydrogel. The mechanical properties of a hydrogel

TABLE 2. Meiotic competence, fertilization rate, and developmental competence of oocytes from two-layered secondary follicles cultured in alginate.[a]

Alginate concentration	N[b]	MII[c]	GVBD	GV	DG	Two-cell embryos[d]	Blastocysts[e]
1.50%	76	56.3%[f]	84.2%	5.3%	10.5%	5.6%[f]	0.0%[f]
1.00%	92	58.3%[f]	78.3%	10.9%	10.9%	14.3%[g]	0.0%[f]
0.50%	95	67.1%[g]	86.3%	6.3%	7.4%	11.5%[g]	0.0%[f]
0.25%	76	67.2%[g]	88.2%	3.9%	9.2%	41.5%[h]	29.4%[g]

[a] MII, metaphase II; GVBD, germinal vesicle breakdown; GV, germinal vesicle; DG, degenerate.
[b] N = surviving follicle number
[c] The percentage of MII oocytes was calculated as a proportion of oocytes undergoing GVBD.
[d] Two-cell embryos/MII oocytes.
[e] Day 5 blastocysts/two-cell.
[f-h] Different superscripts within each column indicate statistically significant differences ($P < 0.05$).

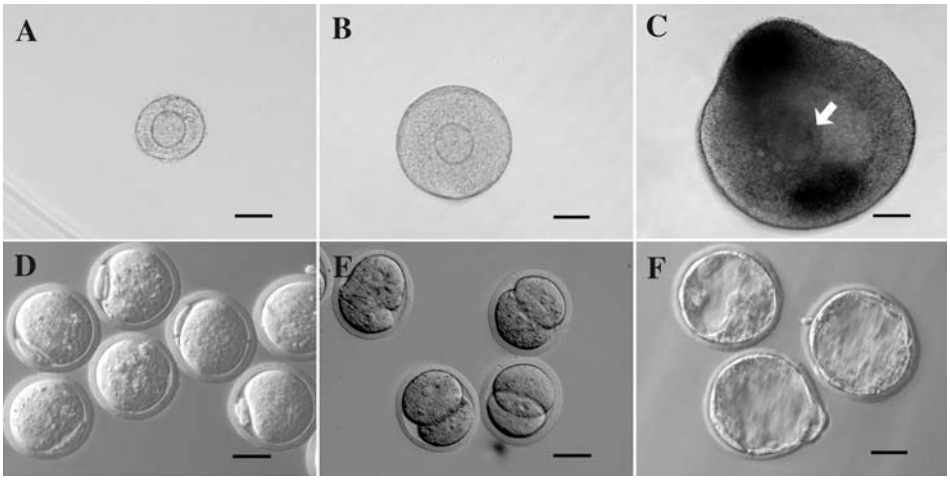

FIG. 6. Representative photographs of in vitro growth of two-layered secondary follicles encapsulated in 0.25% alginate and preimplantation embryo culture. A two-layered secondary follicle (119 μm in diameter) encapsulated in 0.25% alginate (**A**) increased to 212 μm in diameter after 6 days of culture (**B**) and maintained spherical structure and finally reached 392 μm in diameter with an antral cavity containing a cumulus-oocyte-complex (arrow) inside at Day 12 (**C**). After induction by hCG on Day 12, oocytes resumed meiosis and extruded the first polar body (**D**). Two-cell embryos (**E**) were obtained from IVF of MII oocytes and developed to fully expanded blastocysts at Day 5 after fertilization (**F**). Bar = 100 μm (**A**–**C**) and 50 μm (**D**–**F**).

are dependent on several factors, such as the mass of polymer and the extent of cross-linking [33]. Decreasing the percentage of alginate reduces the extent of cross-linking within the gel, thereby decreasing the modulus for the material [34]. Less rigid hydrogels deform more readily, which would create space as the follicle increases in size. The other mechanism by which the alginate percentage may affect follicle growth is the extracellular transport of macromolecules [35, 36]. The culture media contains macromolecules necessary for follicle growth, and cells within the follicle are producing and secreting factors that affect the maturation process. Decreasing the percentage of alginate increases the mean mesh size within the hydrogel, which may enhance macromolecular transport through the hydrogel. However, natural and synthetic matrices, such as the negative-charged polysaccharide alginate, can also bind macromolecules to serve as reservoirs for growth factors [36]. Decreasing the percentage of alginate would reduce binding sites for these macromolecules and increase transport. Therefore, factors added to the culture media would be transported through the gel more easily with a reduction in the alginate percentage. In addition, factors produced by the follicle, which may affect growth positively and negatively, would more effectively escape from the follicle. Identifying the contribution of each factor to follicle growth is a focus of ongoing studies in our laboratory.

In the present study, the effect of alginate rigidity on two-layer secondary follicle growth and oocyte developmental competence was studied by encapsulating individual follicles into alginate beads of varying concentrations. The overall aim of the present study was to optimize the in vitro growth conditions of two-layered secondary follicles from 12-day-old mice such that their three-dimensional structure is maintained and the follicles are capable of producing high-quality mature oocytes that are competent for fertilization and development.

Four different concentrations of alginate were chosen for the present study. Slight modifications in the encapsulation method were made to improve the encapsulation rate into 0.5% and 0.25% alginate matrices. Bead shape and size were not different among the four alginate concentration groups, indicating that there were no apparent differences between the two encapsulating methods used.

Among the four alginate concentrations tested, there were no significant differences in survival rates, which were comparable to those previously reported [3, 11]. Follicle growth did not differ among the four groups until Day 8 of culture (Fig. 1). However, by Day 12, follicles encapsulated in 0.5% and 0.25% alginate had increased to more than 300 μm, significantly larger than those cultured in 1.5% alginate (Table 1 and Fig. 1). The rapid size increase in the last 4 days of culture was accompanied by a high rate of antrum formation: 86.2% and 73.2% of follicles formed an antrum in the 0.25% and 0.5% alginate groups, respectively. Antrum formation appeared to occur once the follicles reached ~200 μm in diameter (Fig. 2), the same size at which early follicles typically develop an antrum in vivo [37, 38]. Although antrum development is not mandatory for development of mature oocytes in vitro [1], the antrum is a clear marker of normal follicle growth and differentiation [39].

In the present study, varying concentrations of alginate differentially affected proliferation and differentiation of theca cells, which in turn affected follicle growth and oocyte maturation. In our culture system, alginate at lower concentrations appeared to provide a permissive environment that allows theca cells to proliferate and differentiate, as evidenced by development of multiple laminar-like theca cell layers at Day 8 (Fig. 2E). According to the two-cell/two-gonadotropin theory, FSH-stimulated estrogen synthesis in granulosa cells is dependent on a supply of LH-stimulated androgens from theca cells [40]. Because LH is not supplied in the current system, we propose that the higher outputs of androstenedione in lower-consistency alginate groups could be due not only to the more proliferated and better differentiated theca cells but also to changes in regulation of the follicle's autocrine and paracrine factors. FSH has been shown to be necessary for normal antrum formation in preantral follicles cultured in vitro [21, 41]. Findings from our study further suggest that the presence of differentiated theca cells and the secretion of androstenedione are also required for antral development in culture, because follicles encapsulated in 1.5% alginate (1) were less likely to develop an antrum, (2) did not develop laminar-like thecal cells, and (3) produced lower androstenedione levels.

Normal folliculogenesis requires the coordination of the physiological functions of each cell type within the follicle [18, 42]. In the present study, alginate concentration not only affected theca cell proliferation and differentiation but also regulated granulosa cell proliferation and differentiation. The more rapid increase in follicle size in the 0.5% and 0.25% alginate groups could be attributed to rapid theca and granulosa cell proliferation and the accumulation of follicular fluid [32]. The lower production of estradiol by follicles grown in 1.5% alginate may be due to inadequate production of androgens by theca cells or due to an aromatization deficiency in granulosa cells or decreased proliferation of granulosa cells (Fig. 3B). By RT-PCR, no difference in *Fshr* mRNA expression was

observed among the four test groups (Fig. 4A). Because FSH-stimulated aromatase activity and estradiol production in granulosa cells are highly dependent on FSH receptor density [43], we propose that low estradiol production by follicles grown in 1.5% alginate is not due to downregulated aromatase activity but rather to a lack of androgen provided by theca cells and decreased granulosa cell numbers. In support of this hypothesis, we found that *Lhcgr* mRNA levels were significantly lower in the 1.5% alginate group compared with the 0.25% alginate group (Fig. 4B). *Lhcgr* mRNA has been localized exclusively to the theca cells of immature follicles and to theca and granulosa cells of mature antral follicles [44]. The induction of *Lhcgr* on granulosa cells by FSH has been confirmed in vivo [45] and in vitro [46]. Upregulation of LHCGR is a prerequisite for ovulation, cumulus cell mucification, and mural granulosa cell luteinization triggered by the LH surge in vivo. In our study, the upregulation of *Lhcgr* mRNA expression correlated with higher antrum formation, oocyte fertilization, and blastocyst formation rates. In addition, increased *Lhcgr* mRNA correlated with lower progesterone production in follicles encapsulated in 0.25% and 0.5% alginate compared with those grown in 1.0% and 1.5% alginate (Fig. 3C), which indicated that the elevation of *Lhcgr* did not cause premature luteinization. Our results differ from those reported in a previous study that showed that FSH-induced elevation of *Lhcgr* in granulosa cells was associated with abnormal oocyte development [47]. This difference may be due to our use of intact follicle culture rather than GOC culture. Taken together, *Lhcgr* expression differences in the present study reflected not only granulosa cells' but also theca cells' function within an architecturally intact follicle structure in vitro.

The rates of oocyte development and competence were also affected by different alginate concentration conditions. Although oocytes in 0.25% alginate were smaller than those in the other alginate groups at Day 8 (Fig. 5A), the size at the end of 12 days was not significantly different from that of those cultured in 1.0% or 0.5% alginate (Fig. 5B), and they grew significantly faster than oocytes in the other groups during the final 4 days of culture (Fig. 5C). These oocytes also had the highest fertilization and blastocyst formation rates among the four groups (Table 2). Conversely, oocytes in the 1.5% alginate group grew less than 1 μm in the last 4 days of culture and displayed deficient developmental competence, with lower maturation and fertilization rates and no blastocyst formation. The transition from the preantral to antral stages of follicular development is complex, requiring precisely coordinated signaling within the follicle unit. In the three-dimensional organization of the follicle, early antral stages are particularly crucial for oocyte development, as it is during this time that the accelerated rate of growth of the oocyte takes place, and RNA and proteins are accumulated for subsequent oocyte maturation and preimplantation development [48, 49]. Previous studies have documented that GDF9-deficient mice had a larger oocyte size relative to that of controls in the preantral follicle stage but that follicles exhibited limited overall growth [50, 51]. Similarly, oocytes grown in 1.5% alginate developed more rapidly during the first 8 days of culture and then arrested development, somewhat mimicking the phenotype of GDF9-deficient mice. RT-PCR demonstrated slightly lower expression of *Gdf9* mRNA in the 1.5% alginate group, although this was not significantly different from the other groups. This result suggests that the deceleration of oocyte development in follicles grown in 1.5% alginate may be due to a lack of sustained nutrition and support from surrounding somatic cells [52] rather than a deficiency in GDF9.

The physical environment has been implicated in directing cellular processes such as migration and differentiation in a variety of cells and tissues (e.g., stem cells and blood vessels) [53]. To our knowledge, these studies are the first to suggest a role for the physical environment in ovarian follicle maturation. This concept may provide a unique perspective on normal follicle recruitment and on disorders in which follicles are unable to advance beyond an immature stage and the associated oocytes are of poor quality, such as in polycystic ovary syndrome.

In conclusion, two-layered secondary follicles were grown in vitro in an alginate-based three-dimensional scaffold to produce mature oocytes capable of fertilization and blastocyst formation (Fig. 6). Follicle and oocyte developmental competencies were affected by the concentration of surrounding alginate matrix. Follicles encapsulated in 0.25% alginate displayed the highest developmental capacity and produced a greater number of mature oocytes. Our study also supports a role for alginate in regulating theca and granulosa cell proliferation and differentiation by some mechanism, which could be a mechanically based signal that activates other independent proliferation-related pathways. This study also confirms the previous hypothesis that alginate artificially mimics, at least partially, in vivo stroma function by providing a permissive environment. The overall blastocyst formation rate for in vitro cultured oocytes was still lower than the rate for in vivo grown oocytes (data not shown). The decreased oocyte quality could result from the lack of another important stroma component, the extracellular matrix. Although the alginate in vitro culture system requires further refinement in terms of culturing conditions and media components, these data indicate that the system can be adapted to optimally support the development of follicles at various developmental stages.

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