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Development In Vitro of Mouse Oocytes from Primordial Follicles[1]

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ABSTRACT

The objective of these studies was to achieve complete oocyte development in vitro beginning with the oocytes in the primordial follicles of newborn mouse ovaries. A two-step strategy was developed: first the ovaries of newborn mice were grown in organ culture for 8 days, and then the developing oocyte-granulosa cell complexes were isolated from the organ-cultured ovaries and cultured for an additional 14 days. The oocytes of primordial follicles are approximately 4190 μm^3 in volume (20 μm in diameter), and this volume increased by approximately 53 810 μm^3 to a final size of 58 000 μm^3—a 13.8-fold increase—during the 8 days of organ culture. In the first experiment the oocyte-granulosa cell complexes were grown in control medium or in medium supplemented with FSH (0.5 ng/ml), epidermal growth factor (EGF; 1.0 ng/ml), or EGF plus FSH. Only 50–60% of the complexes cultured in control medium or in medium supplemented with FSH were recovered at the end of the 14-day culture period. In contrast, more than 90% of the complexes cultured in medium supplemented with EGF were recovered. The median size of the oocytes grown in control medium was 176 800 μm^3 (69-μm diameter), while the median size of those grown in medium supplemented with EGF was slightly smaller (136 400-μm^3 volume; 63-μm diameter), due to the survival of more smaller-size oocytes in EGF-containing medium. Thirty percent of the oocytes recovered after development in FSH-containing medium were competent to undergo germinal vesicle breakdown (GVB). In the second set of experiments, oocyte-granulosa cell complexes isolated from organ-cultured ovaries were cultured in medium supplemented with either 0.5 or 5.0 ng/ml FSH or with these same concentrations of FSH plus 1.0 ng/ml EGF. Again, increased oocyte recovery was observed in the groups cultured with EGF. There was no difference among the groups in the percentage of the oocytes that acquired competence to undergo GVB (32%) or in the percentage of GVB oocytes that produced a polar body, thus indicating progression of meiosis to metaphase II (22%). When the mature oocytes were inseminated, 21% underwent fertilization and cleavage to the 2-cell stage in the groups without EGF during oocyte development, while 42% underwent fertilization and cleavage to the 2-cell stage in the groups cultured with EGF. Less than 2% of the 2-cell-stage embryos developed to the blastocyst stage in any of the groups. One hundred and ninety 2-cell-stage embryos were transferred to the oviducts of pseudopregnant females; two females produced one pup each; one was living and the other had apparently died recently. The results reported here clearly show that complete development of oocytes in vitro from the primordial follicle stage is possible and establish the framework for further studies using oocytes from laboratory animals as model systems for the development of oocytes from humans as well as from animals of agricultural and zoological importance.

INTRODUCTION

Primordial follicles are the earliest form of ovarian follicle and are composed of a primordial, or quiescent, oocyte surrounded by a single layer of flattened somatic cells that are presumably the progenitors of follicular granulosa cells. The oocytes of primordial follicles, hereafter referred to as primordial oocytes, are arrested in prophase of meiosis I and have not entered the phase of dramatic growth typified by oocytes in primary (preantral) follicles. There are thousands of primordial follicles present in the ovaries of neonatal mammals. Primordial follicles can be considered the storage form of the ovarian follicle, though many of them undergo degeneration. In fact, very few primordial follicles ever develop to the graafian follicle stage when they can ovulate and provide an oocyte for fertilization. Thus, the primordial follicles are a large and potentially valuable source of oocytes that could be used for clinical, agricultural, and zoological purposes. Indeed, it has already been shown that primordial follicles harvested by enzyme digestion can restore fertility when transplanted within plasma clots to the ovarian bursa of sterilized animals [1]. Moreover, isolated primordial follicles can be cryopreserved before transplantation by this method [2].

Until now, conditions for complete development of primordial oocytes in vitro have not been established. Nevertheless, in classic experiments by Blandau and colleagues involving organ cultures of fetal mouse ovaries, oocytes grew to 46–53 μm in diameter in 14–20 days [3, 4]. Zonae pellucidae formed around the oocytes. Culture systems have been described for preantral follicle development [5–7] as well as for development of oocyte-granulosa cell complexes prepared from preantral follicles [8–10]. Nevertheless, success with these methods has been restricted to large preantral follicles isolated shortly before the formation of the antrum. The rapidly growing oocytes in these follicles are surrounded by 2–4 layers of granulosa cells, and many of these studies have focused primarily on the development and function of follicular somatic cells rather than on oocyte development [5–7, 11–14]. Thus, the maturational or embryonic developmental competence of the oocytes in these studies was generally not determined at either the beginning or the end of the culture periods. Nevertheless, these studies have demonstrated dramatic development of an antrum-like structure in these follicles, and in one case [15] the

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fertility of the resultant oocytes was clearly demonstrated. Studies using oocyte-granulosa cell complexes from preantral follicles have focused specifically on the development of the oocyte. In this culture system, the acquisition of oocyte competence to undergo not only maturation but also fertilization and embryonic development is well documented [8–10].

The objective of the studies presented here was to achieve complete development in vitro of the primordial oocytes present in the ovaries of newborn mice. The only follicles present in the ovaries of newborn mice are primordial follicles [16]. The strategy was first to establish the earliest stage of preantral follicle development at which our oocyte-granulosa cell culture system could support development of oocytes competent to undergo fertilization and preimplantation embryogenesis. Then a two-step system was devised. The first step was to use ovarian organ culture for the initial processes of preantral follicle development and oocyte growth. In the second step in this system, oocyte-granulosa cell complexes were isolated from the organ cultures and cultured further to complete oocyte development. The development of the oocytes with this two-step culture system is described in terms of both oocyte growth and oocyte competence to undergo maturation, fertilization, and embryogenesis. This is the first documentation of the development in vitro of mammalian primordial oocytes.

MATERIALS AND METHODS

Culture of Oocyte-Granulosa Cell Complexes

All experiments were conducted with (C57BL/6J × SJL) F_1 mice. Oocyte-granulosa cell complexes were isolated from the ovaries of 6-, 8-, and 12-day-old mice as described previously [9, 10] using 0.1% crude collagenase (type CLS I; Worthington Biochemical Corp., Freehold, NJ) and 0.02% deoxyribonuclease I (DNase; Sigma Chemical Co., St. Louis, MO). This procedure yields oocyte-granulosa cell complexes consisting of an oocyte surrounded by one or more layers of granulosa cells; the basal lamina that normally surrounds this complex is at least partly degraded by the enzyme treatment. It does not appear that any layers of granulosa cells become detached. The complexes were cultured on Costar Transwell-COL membrane inserts (3.0-μm pore size, 24-mm diameter) in Costar six-well cluster dishes (Costar Corp., Cambridge, MA), each well containing 4 ml of medium. The culture medium was Waymouth medium MB752/1 supplemented with 0.23 mM pyruvic acid, 50 mg/L streptomycin sulfate, 75 mg/L penicillin G (Sigma), 3 mg/ml BSA (crystallized; ICN Biochemicals, Aurora, OH), ITS (insulin, 5 μg/ml; transferrin, 5 μg/ml; selenium, 5 ng/ml; Collaborative Research, Inc., Bedford, MA), and 1 mg/ml fetuin [17]. The fetuin was purified according to the method of Spiro [18] by the Microchemistry Service of The Jackson Laboratory using fetal bovine serum purchased from Sigma. The final preparations of fetuin were dialyzed extensively against embryo culture-grade water prior to lyophilization. The cultures were incubated at 37°C in modular incubation chambers (Billups Rothenberg, Del Mar, CA) thoroughly infused with a gas mixture composed of 5% O_2:5% CO_2:90% N_2. Cultures were fed every 2 days by replacement of approximately half of the medium in the compartment below the membrane.

Organ Culture of Ovaries from Newborn Mice

Newborn mice were collected on the day of birth (Day 0). Ovaries were excised from the ovarian bursa and transferred with a Pasteur pipette and a drop of medium to a Costar Transwell membrane (non-tissue culture treated, Nucleopore polycarbonate membrane, 3.0-μm pore size, 24-mm diameter). Approximately 1.5 ml of medium had been placed in the compartment below the membrane insert so that when the drop of medium was added to the surface of the membrane, excess medium was drawn into the compartment below the membrane, leaving the ovary covered by only a thin film of medium. The medium was the same as that described above except that it was supplemented with 10% fetal bovine serum (HyClone, Logan, UT) and not ITS or fetuin. Up to 7 ovaries were placed on each membrane. The ovaries from 20–25 mice were used in each experiment. The cultures were incubated at 37°C in modular incubation chambers thoroughly infused with a gas mixture composed of 5% CO_2:95% air. The cultures were fed every 2 days by adding approximately 2 ml of medium to the chamber below the membrane and then withdrawing the same volume.

In some experiments, FSH, EGF, or both were added to the culture medium. FSH was used at a concentration of either 0.5 or 5.0 ng/ml, and EGF was used at 1 ng/ml. The highly purified FSH (ovine [o]FSH-20) was obtained from the National Hormone and Pituitary Program of NIDDK (Baltimore, MD). It is important to note that this is a highly potent preparation of FSH. According to the specifications provided by NIDDK, it is 175-strength NIH-oFSH-S1, or 4463 IU/mg. The expansion of the mouse cumulus oophorus is stimulated by 1–5 ng/ml of this preparation (unpublished data). Mouse receptor-grade EGF, purchased from Upstate Biotechnology Incorporated (Lake Placid, NY), was used at 1 ng/ml, a concentration that stimulates the expansion of mouse cumulus oophorus ([19] and our unpublished results) and is the concentration estimated to be present in mouse plasma [20].

Oocyte Maturation and Fertilization In Vitro

After oocyte growth and development in vitro, the oocyte-granulosa complexes were dislodged from the mem-

brane by sharply jolting the membrane insert with a snap of a finger against the side of the membrane. The complexes were collected and washed three times in fresh medium and allowed to mature for 15–18 h in 2.5 ml of medium supplemented with 100 ng/ml FSH (NIDDK-oFSH-20). The maturation medium and the culture conditions were the same as those used for the culture of isolated oocyte-granulosa cell complexes as described above except it did not contain ITS.

After the incubation for oocyte maturation, the granulosa cells were removed from oocytes by drawing the complexes in and out of a Pasteur pipette. The granulosa cell-free oocytes that had undergone germinal vesicle breakdown (GVB) were collected and washed three times in fertilization medium. The oocytes that did not undergo GVB were cultured for an additional 24 h to assess whether or not further culture without granulosa cells would allow additional oocytes to undergo GVB. The number of oocytes that underwent GVB with granulosa cells was added to the number that subsequently underwent GVB without granulosa cells, and the total was the number of oocytes that had developed competence to undergo GVB during the 10-day culture period.

Ova were fertilized and preimplantation embryos were cultured as described previously [21–23]. Mature ova were fertilized in vitro using epididymal sperm from 3–6-mo-old males that had been housed individually since weaning. Fertilization was carried out for 4 h in Minimum Essential Medium (MEM) prepared with Earle's balanced salt solution, both essential and nonessential amino acids (Gibco, Grand Island, NY), 0.23 mM pyruvic acid, 75 mg/L penicillin G, 50 mg/L streptomycin sulfate, 0.01 mM tetrasodium EDTA (Sigma), and 3 mg/ml BSA (crystallized; ICN Immunochemicals, Lisle, IL). The vitamins and the essential and nonessential amino acids were added from 100-strength or 50-strength concentrates. After incubation for 4 h, the eggs were washed twice in fresh medium and then cultured overnight in 1 ml of the same medium. Embryos that cleaved to the 2-cell stage were collected and washed twice in KSOM [24] supplemented with 0.5-strength essential and nonessential MEM amino acids [22, 23]; they were then cultured until the blastocyst stage in this medium. Embryos were transferred to 3–6-mo-old pseudopregnant (C57BL/6J × SJL) F_1 females that had been mated to sterility-tested vasectomized BALB/cByJ males as described previously [25, 26] except that 2-cell-stage embryos, rather than morula- to blastocyst-stage embryos, were transferred.

Histology

Ovaries were fixed for 3–5 h in 2.5% glutaraldehyde and 2.5% paraformaldehyde in 0.083 M sodium cacodylate buffer, pH 7.2, at 4°C. They were then washed in 0.1 M sodium cacodylate buffer, pH 7.2, for 24 h before embedding in JB-4 (glycol methacrylate) plastic (Polysciences, Inc., Warrington, PA). Sections, 2 μm thick, were stained with periodic acid-Schiff reagent and hematoxylin.

Measurement of Oocyte Size, Presentation of Data, and Statistical Analysis

The size of cultured oocytes was measured without their having been allowed to undergo maturation in vitro. The germinal vesicle (GV)-stage oocytes were denuded of their companion granulosa cells as described above and were measured using the Image Explorer analysis system from Signal Analytics Corp. (Vienna, VA). The IP Lab Spectrum software of this system measured the long and short diameter of the oocytes exclusive of the zona pellucida. The average diameter and oocyte volume were then calculated via StatView for Macintosh (Abacus Concepts, Inc., Berkeley, CA). The size of uncultured oocytes isolated from 6-, 8-, 12-, 18-, and 22-day-old mice was also determined. To obtain the oocytes from the 6-, 8-, and 12-day-old mice, the oocyte-granulosa cell complexes were first isolated as described above; then the oocytes were denuded by incubation in 1 mg/ml collagenase and 0.2 mg/ml DNase in calcium- and magnesium-free Dulbecco's PBS (Gibco-BRL, Grand Island, NY), and the material was repeatedly drawn in and out of a Pasteur pipette. The oocytes were then equilibrated in oocyte culture medium for 1–2 h before the size measurements were obtained. To obtain oocytes from 18- and 22-day-old mice, the antral follicles were punctured with a 25-gauge needle, both cumulus-enclosed and denuded GV-stage oocytes were collected, and the cumulus cell-enclosed oocytes were denuded by being repeatedly drawn in and out of a Pasteur pipette. Thus, the population of oocytes measured included both groups—those that were cumulus-enclosed and those that were denuded upon initial isolation. Oocyte size is presented as notched box and whisker plots prepared using the StatView software specified above.

Percentages, such as those of oocytes at various stages of maturation and those of embryos that cleaved to the 2-cell stage, are presented as the mean percentage of at least three independent experimental replicates; variation between experiments is illustrated using the standard error of the mean. For evaluation of the differences between groups, data were subjected to arcsin transformation and ANOVA. When a significant F ratio was defined by ANOVA, groups were compared via Fisher's Protected Least Significant Difference post hoc test using StatView software; when $p \leq 0.05$, the difference was considered significant.

RESULTS

Developmental Competence of Oocytes Grown In Vitro after Isolation from Preantral Follicles

Oocyte-granulosa cell complexes were isolated from the preantral follicles of 6-, 8-, and 12-day-old mice. Since a

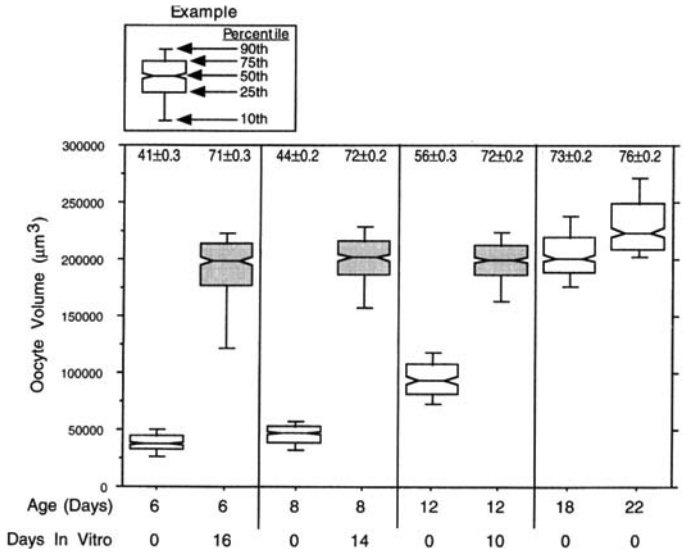


FIG. 1. Growth of oocytes in vitro in oocyte-granulosa cell complexes isolated from the ovaries of 6-, 8-, and 12-day-old mice. The shaded box plots indicate the volume of oocytes grown in vitro, and the unfilled box plots illustrate the size of oocytes isolated from 6-, 8-, 12-, 18-, and 22-day-old mice. The oocytes from 6-, 8-, and 12-day-old mice were cultured for 16, 14, and 10 days, respectively, so that the total chronological age of all groups after culture was 22 days. Lines of the boxes delineate the 25th, 50th, and 75th percentiles and the whiskers depict the 10th and 90th percentiles of a population as illustrated in the example. The notch defines the 95% confidence interval around the median (50th percentile); thus groups that display nonoverlapping notches can be considered different ($p < 0.05$) [39, 40]. The numbers above the box plots indicate the mean ± SEM diameter (μm) of the oocytes in that group. Approximately 225 oocytes were measured for each group.

wave of follicular development begins shortly after birth in mice, discrete populations of preantral follicles of increasing size are present in the ovaries of mice of increasing postnatal age. Thus, the diameters of intact oocyte-granulosa cell complexes isolated at 6, 8, and 12 days after birth

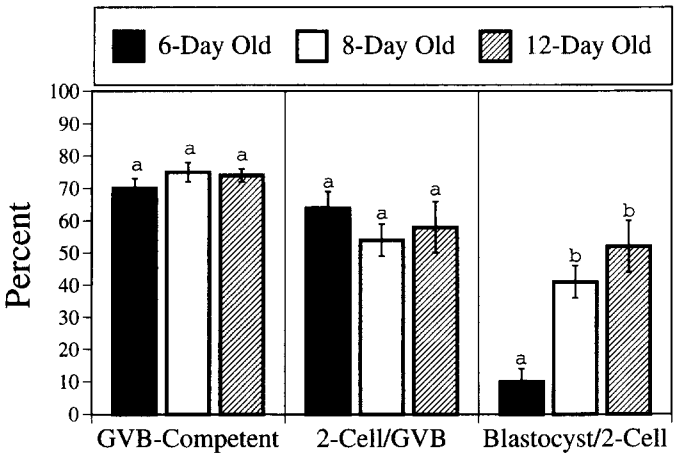


FIG. 2. The developmental competence of oocytes grown in vitro after isolation from 6-, 8-, and 12-day-old mice. The oocyte-granulosa cell complexes were cultured for 16, 14, and 10 days, respectively, so that the total chronological age for all groups was 22 days. The bars indicate the mean and SEM of four independent experiments. Means with different letters are significantly different ($p < 0.05$).

were 37 ± 1.4, 101 ± 2.0, and 134 ± 2.4 μm (mean ± SEM; n = 45, 171, and 104), respectively. In preliminary studies (not shown), we were unable to sustain cultures of oocyte-granulosa cell complexes from mice younger than 6 days of age. Oocytes from the complexes isolated from these mice became denuded of granulosa cells during culture and failed to develop, since the maintenance of gap junctional communication between granulosa cells and the oocytes is essential for oocyte growth and development [8, 27–29]. The complexes isolated from 6- and 8-day-old mice were from follicles that were much smaller than those used in previous studies on the developmental competence of in vitro-grown oocytes. In previous studies on the developmental competence of oocytes from preantral follicles grown in vitro, oocyte-granulosa cell complexes isolated from 12-day-old mice were used [9, 10]. The objective of the present study was to examine the developmental competence of oocytes from the smaller preantral follicles after they were grown in vitro to the same total chronological age (22 days) as those from the larger preantral follicles of 12-day-old mice. The complexes from 6-day-old mice were therefore cultured for 16 days, those from 8-day-old mice for 14 days, and those from 12-day-old mice for 10 days. After culture, complexes were dislodged from the membranes and allowed to undergo maturation.

Oocytes from all groups underwent extensive growth in vitro (Fig. 1). The oocytes from 6-, 8-, and 12-day-old mice grew to approximately the same median volume (~200 000 μm^3) in 16, 14, and 10 days of culture, respectively. To achieve this, the oocytes from the younger mice acquired much more volume during the culture period. For example, the volume of the 6-day-old group increased by 160 000 μm^3 (median volume) as compared to an increase of 106 000 μm^3 by the oocytes from the 12-day-old group. Thus, the size of the 6-day-old group increased 5.3-fold whereas that of the 12-day-old group increased 2.1-fold. None of the cultured oocytes achieved the size of oocytes grown in vivo to the same chronological age (22 days), but rather were about the same size as oocytes of the antral follicles of 18-day-old mice (Fig. 1).

There was no difference in the percentages of oocytes that were competent to undergo GVB; 70–75% were GVB-competent in all groups (Fig. 2). Moreover, there was no difference in the percentages of oocytes undergoing GVB that subsequently underwent fertilization and cleavage to the 2-cell stage: these values were 64 ± 5%, 54 ± 5%, and 58 ± 8% in the 6-, 8-, and 12-day-old groups, respectively. In contrast, there was a remarkable failure of the 2-cell-stage embryos from the 6-day-old group to develop to the blastocyst stage compared with those from the 8- and 12-day-old groups; 10 ± 4%, 41 ± 5%, and 52 ± 8% from the 6-, 8-, and 12-day-old groups, respectively, reached this stage. There was no significant difference in the percentages of 2-cell-stage embryos that developed to the blastocyst stage in the 8- and 12-

day-old groups (Fig. 2). Thus, the culture system supports the development of oocytes from 8-day-old mice to an extent that appears qualitatively similar to that for oocytes from 12-day-old mice with respect to competence to undergo maturation, fertilization, and preimplantation embryogenesis; this similarity was evident despite the fact that oocytes from 8-day-old mice were cultured for 4 days longer than those in the 12-day-old group. However, the oocytes from the 6-day-old mice were qualitatively inferior to those of the 8- and 12-day-old group in their competence to complete the transition from the 2-cell stage to the blastocyst stage. Nevertheless, oocytes from the 6-day-old group were able to undergo fertilization and cleavage to the 2-cell stage with a frequency equivalent to that seen in the 8- and 12-day-old groups. It is not known why the difference of 2 days of postnatal oocyte/follicular development in vivo has such an impact on subsequent development in vitro. Subjective observations of the complexes in vitro indicated that they were more fragile and that the granulosa cells were less tightly associated with the oocytes. On the basis of these experiments, it was decided to maintain the ovaries from newborn mice in organ culture for 8 days prior to culture of isolated oocyte-granulosa cell complexes.

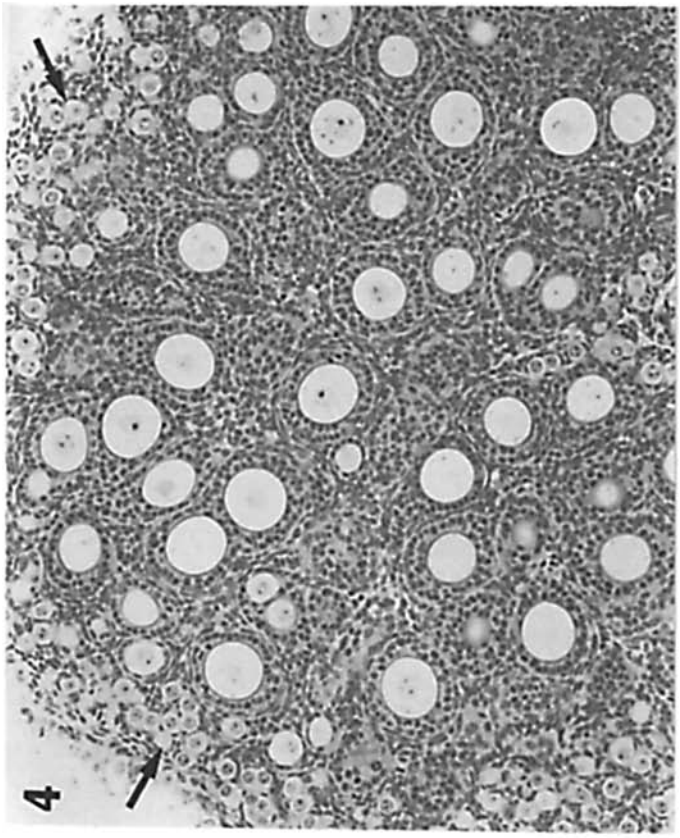
Organ Culture of Newborn Ovaries

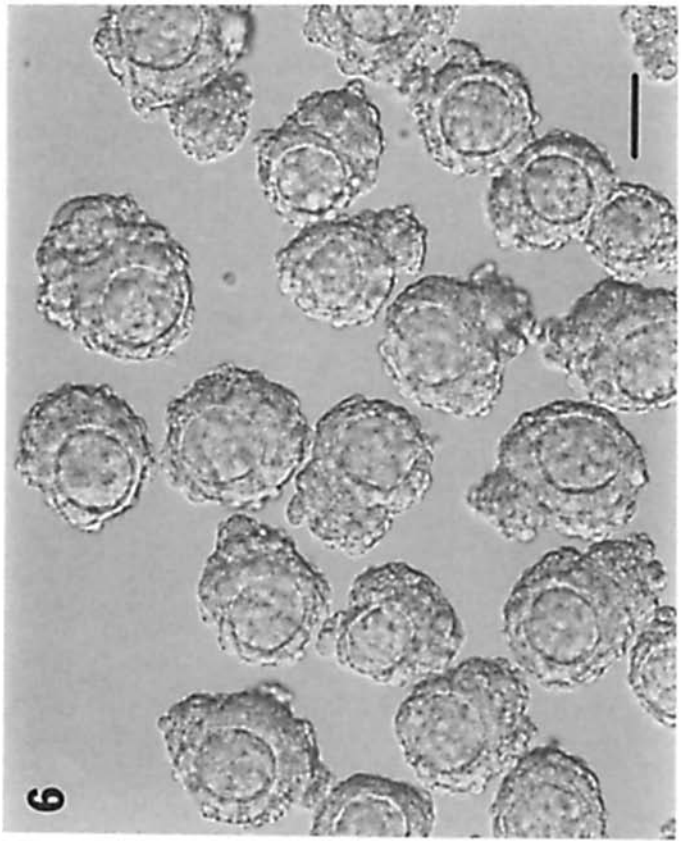
Since oocyte-granulosa cell complexes from mice younger than 6 days old could not be successfully cultured, it was obvious that complete development in vitro of primordial oocytes would require a strategy in which the initial stages of folliculogenesis would progress until an adequate number of granulosa cells accumulated around the oocyte to sustain communication with the oocyte after the isolation of oocyte-granulosa cell complexes and thus support oocyte growth and development. Thus, the strategy emerged for organ culture as the first step in the development in vitro of primordial oocytes. On the basis of the findings described above, the ovaries of newborn mice were organ-cultured for 8 days to determine whether the oocyte-granulosa cell complexes might be equivalent to the complexes isolated from 8-day-old mice. The only follicles present in the ovaries of newborn mice are primordial follicles (Fig. 3). After 8 days of organ culture, many oocytes had grown, and these growing oocytes were enclosed by 1–3 layers of granulosa cell-like somatic cells (Fig. 4). These cells will hereafter be referred to as granulosa cells although they may not be the equivalent of the granulosa cells that develop in vivo for the same period of time. Not all of the primordial follicles initiated growth in the organ cultures. It was primarily follicles in the medullary region that underwent development while those in the cortical areas remained as primordial follicles. This pattern is similar to that observed for the first wave of follicular development in mice in vivo [30], and thus a similar population of primordial follicles may initiate development both in vivo and in vitro. Sometimes the oocyte-granulosa cell complexes were not circumscribed completely by basal lamina in the organ-cultured ovaries, and the primitive theca cells appeared even less defined than in the ovaries of 8-day-old mice (Fig. 5).

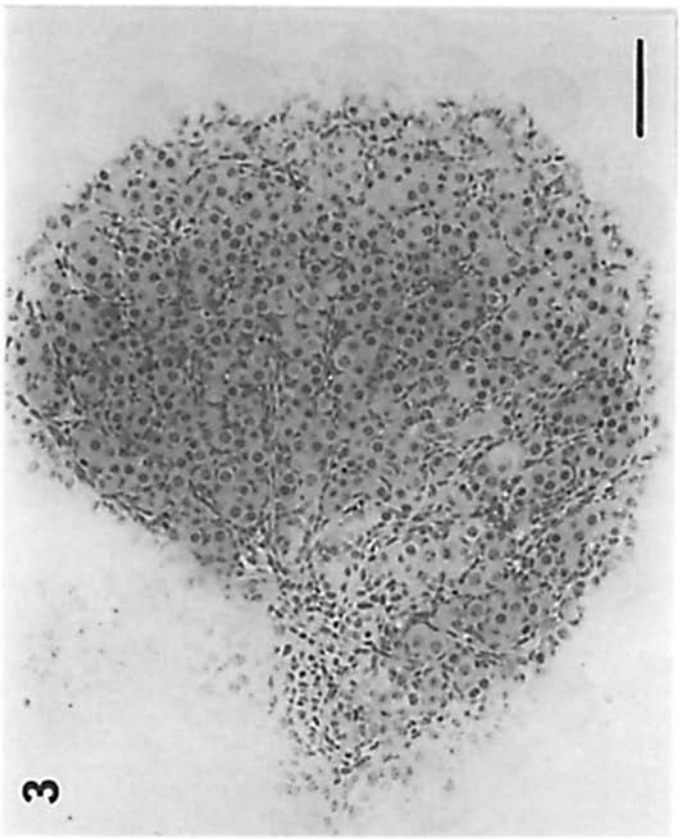
The organ-cultured ovaries were dissociated using collagenase and DNase in the same manner as the ovaries that had developed in vivo. Only oocytes enclosed completely by granulosa cells were selected; to collect these, they were picked up with a micropipette and washed three times with the culture medium used for oocyte-granulosa cell complexes. The appearance of these complexes is shown in Figure 6. To assess the size of the oocytes in these complexes, the complexes were further digested with collagenase and DNase in calcium- and magnesium-free PBS as described above. The size of the oocytes was determined after they were equilibrated with culture medium for 1 h. The morphology of these oocytes was generally normal and included the presence of a zona pellucida, though some oocytes contained some coarse granular material (Fig. 7). The oocytes grown in organ culture were actually larger than those grown in vitro—58 000 μm^3 (median volume) vs. 45 000 μm^3 (Fig. 8). This difference, however, may be attributable to the selection of the oocyte-granulosa cell complexes with the intention of further culture using the oocyte-granulosa cell culture system; only complexes completely enclosed by granulosa cells were selected. Smaller complexes containing smaller oocytes grown in vitro were probably eliminated from selection because they were more delicate than in vivo-grown oocytes and underwent partial denuding during the isolation procedure. Nevertheless, it is clear that the oocytes grew in the organ cultures to a size similar to that of in vivo-grown oocytes. Since primordial oocytes are approximately 4190 μm^3 in volume (20 μm in diameter), the oocytes increased by approximately 53 810 μm^3 to a final size of 58 000 μm^3—a 13.8-fold increase—during the 8 days of organ culture.

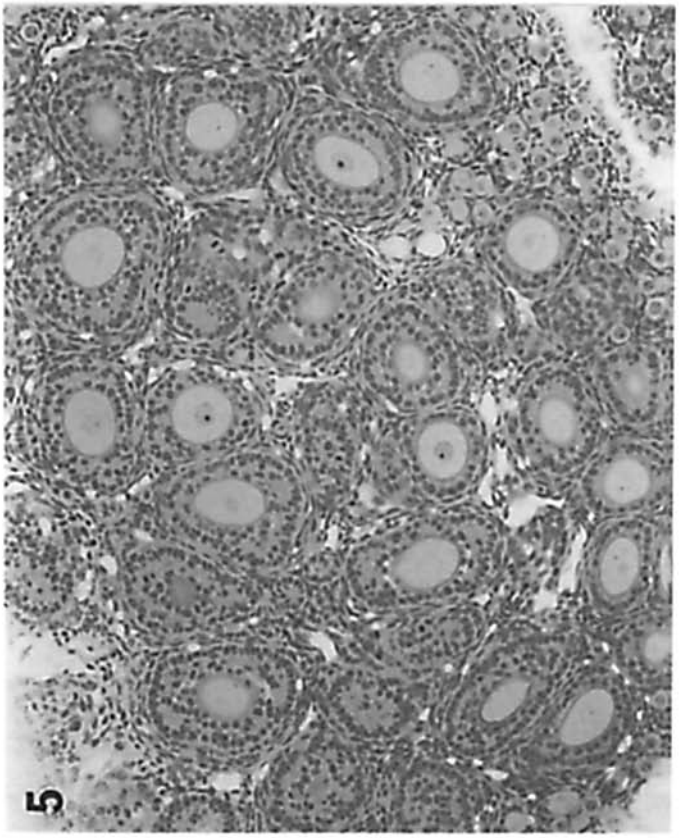
Growth, Maturation, and Fertilization of Oocytes after Isolation from Ovarian Organ Cultures

Oocyte-granulosa cell complexes isolated from the 8-day organ cultures of newborn ovaries were cultured further using the same system that had been employed for the complexes isolated from 6-, 8-, and 12-day-old mice. The complexes were divided into four groups: 1) control, i.e., no added hormones or growth factors; 2) 0.5 ng/ml FSH; 3) 1 ng/ml EGF; and 4) FSH + EGF. Two hundred complexes were cultured in each group. The complexes were cultured for 14 days. The total time in culture, therefore, was 22 days (8 days in organ culture and 14 days in culture of complexes). At this time, complexes in the control cultures appeared very fragile and generally had fewer layers of granulosa cells around the oocytes (not shown). In contrast, the









complexes cultured with either FSH or EGF, or with both, appeared similar to the those cultured after isolation from 6- or 8-day-old mice. Most of the oocytes were completely surrounded by granulosa cells, and other granulosa cells were growing radially away from the complex (Fig. 9). At the end of the 14-day culture period, when the complexes were removed from the membrane and denuded, only about half of the oocytes originally placed in culture were recovered in either the control or the FSH groups (Fig. 10, top). In contrast, approximately 90% of the oocytes in the EGF or EGF + FSH groups were recovered. The appearance of the oocytes isolated from these complexes is illustrated in Figure 11. The oocytes display a well-developed zona pellucida and a granularity that varies from light dispersed granules to a coarse granularity.

Among the oocytes recovered, the percentage (32 ± 10%) of oocytes grown in FSH-supplemented medium that were competent to undergo GVB was higher than that of oocytes grown in control medium or in medium supplemented with EGF alone (13 ± 1% and 10 ± 4%, respectively [Fig. 10, bottom]). However, there was no difference between the groups if the percentage GVB was calculated using the number of oocytes originally placed in culture rather than the number recovered at the end of culture: range, 7 ± 6% (control) to 21 ± 6% (FSH + EGF). It appears, therefore, that neither FSH nor EGF functioned to increase the total number of GVB-competent oocytes. Nevertheless, EGF clearly improved the percentage of recovered oocytes.

The aim of the next experiment was to determine whether increasing the concentration of FSH would improve the percentage of oocytes that acquire competence to undergo GVB. After isolation from the organ-cultured ovaries, the oocyte-granulosa cell complexes were cultured for 14 days in medium supplemented with 0.5 ng/ml FSH; 0.5 ng/ml FSH plus 1 ng/ml EGF; 5.0 ng/ml FSH; or 5.0 ng/ml FSH plus 1 ng/ml EGF. The oocytes grown in medium containing FSH + EGF were slightly smaller than those grown in the medium supplemented with FSH alone because of the recovery of more smaller oocytes from the cultures supplemented with EGF (Fig. 12). Oocytes underwent extensive growth in all groups. For example, the median volume of oocytes in the 0.5 ng/ml FSH plus EGF group grew 3-fold, from 57 000 μm^3 (the size of the oocytes after the 8-day organ culture) to 174 000 μm^3 during the subsequent 14-day culture—an increase of 117 000 μm^3 (Fig. 12). Raising the concentration of FSH from 0.5 to 5.0 ng/ml did not increase the percentage of oocytes recovered after the 14-day culture period. Nevertheless, addition of EGF to either concentration of FSH did increase the percentage recovered (Fig. 13). Increasing the concentration of FSH did not increase the percentage of GVB-competent oocytes; in all cases, approximately 32% of the recovered oocytes were GVB-competent. Moreover, in all cases approximately 22% of the oocytes that underwent GVB produced a polar body, indicating the progression of meiosis to metaphase II (data not shown).

A separate set of experiments, using these same concentrations of FSH and EGF, was conducted to determine whether the oocytes grown in vitro from primordial follicles could undergo fertilization and embryonic development. Fetuin (1 mg/ml) was added to the cultures of oocyte-granulosa cell complexes for the last 7 days to prevent zona pellucida hardening [10]. In this case, the oocytes were removed from the membranes after 14 days of culture and matured in medium containing 100 ng/ml FSH and then inseminated as described in *Materials and Methods*. Remarkably, after the complexes were grown in medium supplemented with 5.0 ng/ml FSH, 21 ± 2% of the oocytes that had undergone GVB cleaved to the 2-cell stage (Fig. 13). Moreover, this percentage increased to 42 ± 9% when the complexes were cultured in the same concentration of FSH plus EGF (Figs. 13 and 14). Development of the 2-cell-stage embryos to the blastocyst stage was rare: < 2% for any group. Nevertheless, 190 two-cell-stage embryos, from additional groups of complexes cultured in medium containing 0.5 ng/ml FSH plus EGF, were transferred to the oviducts of pseudopregnant females, 10 embryos per oviduct. The females were killed on Day 19 of gestation. Two females were found to have one pup each; one was living and one had apparently died recently. The living pup was fostered to a strain LT/SvEi mother and appears to be a normal fertile male.

FIG. 3. Histological section of the ovary of a newborn (C57BL/6J × SJL/J) F_1 mouse. Note that the only follicles present are primordial follicles. Bar indicates 100 μm. Figures 3, 4, and 5 are the same magnification.

FIG. 4. Histological section illustrating the appearance of an ovary, isolated from a newborn mouse, that was organ-cultured for 8 days. Note the presence of many growing oocytes in the medullary region of the ovary. The growing oocytes are enclosed within 1–2 layers of granulosa cells. As in the case of ovaries developing in vivo, the remaining primordial follicles (arrows) are mostly located in the ovarian cortex. Magnification same as Figure 3.

FIG. 5. Histological section of the ovary from an 8-day-old mouse. Magnification same as Figure 3.

FIG. 6. Oocyte-granulosa cell complexes isolated from 8-day organ-cultured ovaries such as those shown in Figure 4. The complexes were selected on the basis of the oocyte's being completely enclosed by granulosa cells. Nomarski interference optics. Bar indicates 50 μm.

DISCUSSION

The development in vitro of oocytes from the primordial stage to mature oocytes has been achieved for the first time. This was accomplished using a two-step system. The first step was an organ culture of newborn ovaries, containing only primordial follicles, for 8 days to allow the development of growing oocytes enclosed by 1–3 layers of granulosa cells. The second step was to isolate the oocyte-granulosa cell complexes and culture them for 14 additional

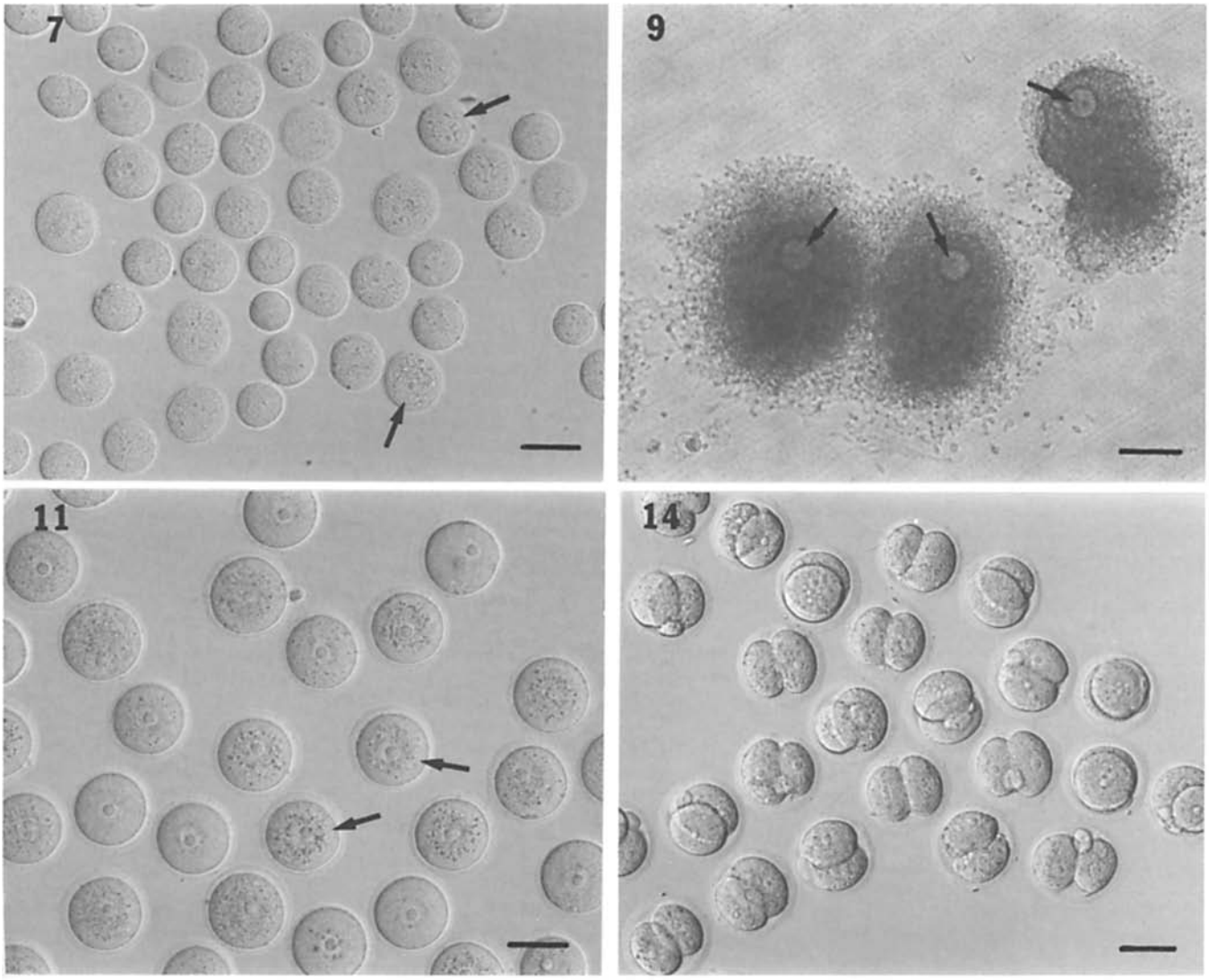


FIG. 7. Oocytes isolated from the complexes shown in Figure 6. Most displayed essentially normal morphology including a developing zona pellucida, except that many oocytes contained some fine granules (arrows) of unknown composition that are not usually observed in growing oocytes. Nomarski interference optics. Bar indicates 50 μm.

FIG. 8. Comparison of oocyte volume after isolation from oocyte-granulosa cell complexes from 8-day-old mice (unfilled box plot) or complexes from 8-day organ cultures of newborn mouse ovaries (shaded box plot). The nonoverlapping notches indicate that the volume of in vitro-grown oocytes (n = 201) was slightly larger ($p < 0.05$) than that of the in vivo-grown oocytes (n = 197). The numbers above the box plots indicate the mean ± SEM diameter (μm) of the oocytes in that group.

FIG. 9. Oocyte-granulosa cell complexes cultured for 14 days after isolation from 8-day organ-cultured ovaries as illustrated in Figure 6. The medium for culture of the complexes was supplemented with 0.5 ng/ml FSH plus 1 ng/ml EGF. Note the apparent proliferation of the granulosa cells around the oocytes (arrows). Nomarski interference optics. Bar indicates 100 μm.

FIG. 10. **Top**) Percentages of the initially seeded 200 oocyte-granulosa cell complexes, isolated from 8-day organ-cultured ovaries, that were recovered after further culture for 14 days. Means with different letters are significantly different ($p < 0.05$). **Bottom**) Percentages of the recovered oocytes that underwent GVB after removal of the granulosa cells and overnight culture. The concentration of FSH was 0.5 ng/ml, and that of EGF was 1 ng/ml. The bars indicate the mean and SEM of three independent experiments.

FIG. 11. Oocytes after the removal of granulosa cells after 14-day culture of oocyte-granulosa cell complexes isolated from 8-day organ-cultured ovaries. The complexes were grown in medium supplemented with 0.5 ng/ml FSH and 1 ng/ml EGF. Note that this figure and Figure 7 are the same magnification. The granularity of some of these oocytes (arrows) is not typical of in vivo-grown oocytes. Nomarski interference optics. Bar indicates 50 μm.

FIG. 12. The volume of oocytes grown in vitro with the two-step system. The stripe-filled box plot on the left indicates the volume of oocytes in oocyte-granulosa cell complexes isolated from 8-day organ-cultured ovaries (step 1 of the two-step system) of newborn mice and illustrates the volume of the oocytes (n = 201) selected for subsequent 14-day culture. The unfilled box plot on the right illustrates the volume of oocytes (n = 227) isolated from the antral follicles of 22-day-old mice; these oocytes were of the same total chronological age as the oocytes in the shaded box plots, which had been first organ-cultured for 8 days and then cultured for an additional 14 days in oocyte-granulosa complexes (step 2 of the two-step system). Although these oocytes underwent extensive growth in vitro, they did not achieve the size of oocytes grown in vivo. Note that the 10th and 25th (arrows) percentiles of the populations of oocytes cultured with EGF are smaller in size than those seen in the populations cultured with FSH alone. In contrast, there were only slight, if any, differences in the size of oocytes at the 50th, 75th, and 90th percentiles. This shows that EGF promoted the survival of small oocytes that were not recovered from the groups cultured without EGF as shown in Figure 13. The numbers above the box plots indicate the mean ± SEM diameter (μm) of the oocytes in that group. Approximately 450 oocytes were measured for each of the groups of cultured oocytes.

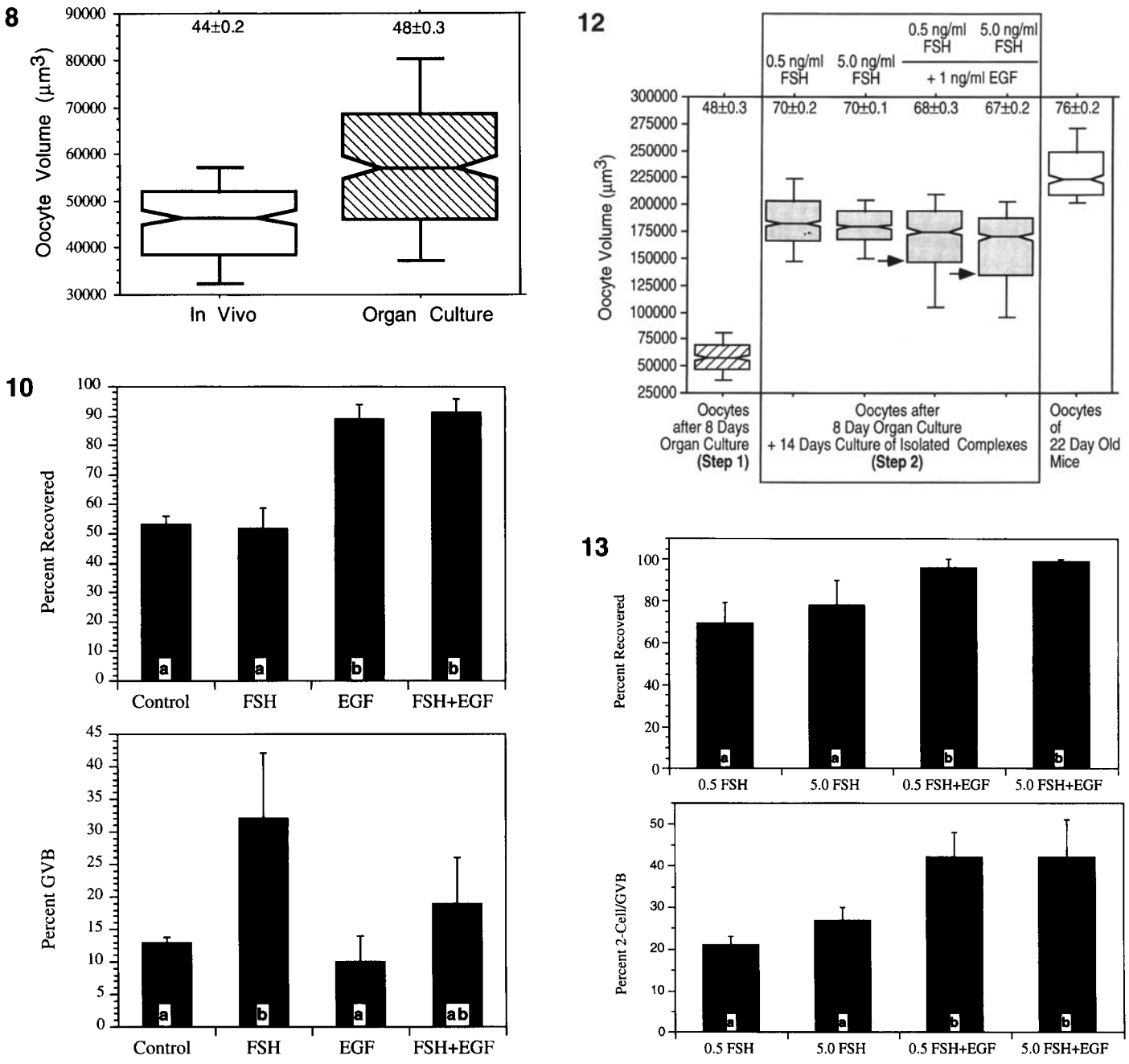


FIG. 13. **Top**) Percentages of the initially seeded 200 oocyte-granulosa cell complexes, isolated from 8-day organ-cultured ovaries, that were recovered after further culture for 14 days. **Bottom**) Percentages of the GVB-oocytes that underwent fertilization and cleavage to the 2-cell stage. The bars indicate the mean and SEM of three independent experiments. Means with different letters are significantly different ($p < 0.05$).

FIG. 14. Two-cell-stage embryos derived from oocytes grown completely in vitro and subsequently matured and fertilized in vitro. The oocytes were grown first in organ cultures of newborn mouse ovaries for 8 days. Then the oocyte-granulosa cell complexes were isolated from the organ-cultured ovaries and cultured for an additional 14 days in medium supplemented with 0.5 ng/ml FSH and 1 ng/ml EGF. The oocyte-granulosa cell complexes were then matured in medium supplemented with 100 ng/ml FSH. Mature oocytes were fertilized in vitro and cultured to the 2-cell stage. Less than 2% of these 2-cell-stage embryos developed to the blastocyst stage in vitro. Nevertheless, two fetuses (one surviving) were produced after the transfer of 190 2-cell-stage embryos to the oviducts of pseudopregnant females. Nomarski interference optics. Bar indicates 50 μm.

days using a system previously described for oocyte-granulosa cell complexes from preantral follicles [9, 10]. Although oocytes grew to a final size of approximately 183 000 μm^3 (70-μm diameter), this was clearly smaller than the size of oocytes grown in vivo (231 000 μm^3 in volume, 76 μm in diameter). Under the best conditions used here, i.e., when the medium for culture of oocyte-granulosa cell complexes was supplemented with both FSH and EGF, 32% of the recovered oocytes were GVB-competent and 22% of the oocytes that underwent GVB produced a polar body

indicating the progression of meiosis to metaphase II. Moreover, 42% of the mature oocytes underwent fertilization and cleavage to the 2-cell stage. Subsequent development occurred only at a very low frequency, with less than 2% of the 2-cell-stage embryos progressing to the blastocyst stage. Even so, transfer of 2-cell-stage embryos resulted in the production of one live pup and another that reached near term before its death.

The primordial oocytes grown with the two-step system were significantly restricted in their competence to complete the 2-cell stage to blastocyst transition. Mouse embryos that fail to activate the zygotic genome do not progress beyond the 2-cell stage [31, 32]. However, embryos derived from in vitro-grown primordial follicles did not all arrest at the 2-cell stage. Rather, development appeared to progress very slowly in all embryos, and arrest did not seem to occur at any specific stage (data not shown). Thus, the developmental deficiency of the in vitro-grown primordial oocytes was not simply a complete failure to activate the zygotic genome. More likely, there were both qualitative and quantitative deficiencies in the deposition of myriad maternal factors during oocyte development in vitro resulting in the frequent failure to drive normal embryogenesis, particularly beyond the 2-cell stage. Previous studies have shown that oocytes isolated from small antral follicles and matured in vitro are often incompetent to complete the transition from the 2-cell to the blastocyst stage despite the apparently normal completion of nuclear maturation, fertilization, and cleavage. Further development of the GV-stage oocyte during the enlargement of the antral follicles is required for the acquisition of competence to complete the 2-cell stage to blastocyst transition [33]. Thus, the role of maternal factors does not end when zygotic genes become activated. It appears that the primordial oocytes grown in vitro most often fail to undergo this final stage of oocyte development. One of the most significant aspects of the establishment of oocyte culture systems is to provide the experimental framework for identification of the molecular events of oocyte growth and maturation and for resolving the complex interactions of hormones, growth factors, and cell-to-cell communication that lead to the formation of oocytes fully competent to complete embryonic development.

The establishment of protocols for the development of oocytes in vitro beginning with primordial oocytes, or earlier, has important applications besides providing an experimental paradigm for understanding the basic mechanisms that govern oocyte and granulosa cell development. Primordial follicles are, by far, the most abundant follicular stage in the ovary, and thus they can be a rich source of oocytes for expanding the populations of valuable agricultural or endangered species. Moreover, cultured oocytes could be used for testing the effects on oocyte development of toxins that might affect fertility or competence of the oocyte to undergo normal embryogenesis.

Only 50–60% of the oocyte-granulosa cell complexes isolated from the 8-day organ cultures of newborn ovaries were recovered after subsequent 14-day culture of the complexes in either control medium or medium supplemented with FSH. In striking contrast, more than 90% of the complexes were recovered when the medium was supplemented with EGF. This seemed to be attributable mainly to the survival of smaller oocytes in the EGF cultures. This is perhaps not surprising in view of the stimulatory effect of EGF on cell proliferation in even the smallest classes of preantral follicles isolated from hamster and pig ovaries [34, 35]. EGF had no beneficial effect on oocyte development when complexes were isolated from 6-day-old mice rather than after 8-day organ culture (data not shown). Thus, it is not clear whether the EGF effect has any physiological significance or whether EGF is simply correcting a culture system-induced artifact.

The primordial oocytes grown with the two-step system described grew to the same size as oocytes grown after isolation from 12-day-old mice, but neither group of oocytes achieved the size of oocytes grown in vivo. This deficiency in oocyte growth in vitro could be attributable to nutritional inadequacies of the culture medium or to a failure to promote the same level of oocyte-granulosa cell communication existing in vivo, or both. This communication may depend upon appropriate signaling by endocrine, autocrine, and paracrine growth factors. The paracrine factors may originate in the oocyte itself [36]. Although maintenance of adequate communication between the granulosa cells and the developing oocyte is necessary for normal oocyte development, it is probably insufficient. The function of the granulosa cells in regulating oocyte development is dependent upon the stage of differentiation of the granulosa cells [37, 38]. Thus, normal oocyte development in vitro will probably depend upon the appropriate regulation of granulosa cell differentiation in vitro. Clearly, this is a complex problem.

Certainly, the system described here requires improvement at both culture steps to improve oocyte development. Even after the system is optimized for the mouse, modification will be required for use with other species. Nevertheless, the basic two-step strategy will probably serve as the essential framework for application to other species, for several reasons. First, it will be very difficult to isolate and culture individual intact primordial follicles. Second, the mechanism involved in the recruitment of primordial follicles into the pool of preantral follicles is unknown and may require the presence of ovarian factors external to the follicle itself. Third, the development of intact follicles from the preantral stage to the stage of large antral follicles will be very difficult to achieve with species having large antral follicles because of problems in nutrient, gas, and waste product exchange. The use of cultured oocyte-granulosa cell complexes eliminates this problem, since the nutrients,

gases, and waste products have more easy access to the oocyte without the mass of the intact follicle as a barrier around it. The results reported here clearly show that complete development of oocytes in vitro from the primordial stage is possible, and establish the framework for further studies using oocytes from laboratory animals as model systems for development of human oocytes as well as oocytes from animals of agricultural and zoological importance.

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