BIOLOGY OF REPRODUCTION 41, 268–276 (1989)

Capacity of Mouse Oocytes from Preantral Follicles to Undergo Embryogenesis and Development to Live Young after Growth, Maturation, and Fertilization in Vitro[1]

JOHN J. EPPIG[2] and ALLEN C. SCHROEDER[3]

The Jackson Laboratory
Bar Harbor, Maine 04609

ABSTRACT

A system is described here by which live mice can be produced from oocytes isolated from 12-day-old mice, be grown, matured, and fertilized in vitro, and then be transferred to pseudopregnant females. These oocytes were, at the time of isolation from preantral follicles, in about mid-growth phase and incompetent of undergoing germinal vesicle breakdown (GVB) without further development. The developmental competence of mouse oocytes that grew and underwent maturation in vitro was compared to oocytes that grew in vivo and underwent maturation in vitro. After isolation from mice 16 through 28 days old, oocytes were found to increase in size and to sequentially acquire the ability to undergo GVB, produce a polar body, cleave to the 2-cell stage after insemination, and develop to the blastocyst stage. Moreover, the number of cells per blastocyst increased with the age of the mice from which the immature oocytes were isolated. Oocyte-granulosa cell complexes isolated from 12-day-old mice were cultured for 10 days. At the end of the culture period, the oocytes had grown to a size equivalent to oocytes isolated from 16-day-old mice, and 87% of the in-vitro-grown (IVG) oocytes underwent GVB; 79% of these produced a clearly visible polar body when maturation occurred in the presence of follicle-stimulating hormone (FSH). The IVG oocytes cleaved to the 2-cell stage after insemination in vitro with a frequency equivalent to superovulated ova and ova that matured in vitro after isolation from 22-day-old mice. Twenty-four percent of the 2-cell embryos derived from IVG oocytes developed to the expanded blastocyst stage. This frequency was equivalent to that by 2-cell embryos derived from oocytes isolated from 18-day-old mice. The blastocysts derived from IVG oocytes contained the same number of cells as the blastocysts derived from oocytes isolated from 18- and 20-day-old mice. Live young were produced by the transfer of 2- or 4-cell embryos derived from IVG oocytes, but at a low frequency (5.1%). These results demonstrate for the first time that oocytes from preantral follicles of mice can complete growth and acquire full developmental competence in vitro so that live young can be produced after maturation and fertilization in vitro and transfer to foster mothers.

INTRODUCTION

In the mouse, a group of oocytes, arrested in the dictyate stage of the first meiotic division, begins to grow shortly after birth. As these oocytes grow, the granulosa cells that enclose them proliferate and sustain oocyte growth and development via gap junctions that metabolically couple the two cell types. Oocytes acquire competence to undergo germinal vesicle breakdown (GVB), the first morphological manifestation of oocyte maturation, and complete the first meiotic division as they near completion of their growth phase. Populations of oocytes with increasing mean diameters can be obtained by isolation from juvenile mice of increasing ages. It was found that oocytes isolated from mice 13 days of age or younger, whose mean diameter was less than 60 μm, were unable to undergo spontaneous GVB in culture, but an increasing percentage of larger oocytes isolated from mice 15 days of age and older was able to undergo maturation in culture (Szybec, 1972; Sorensen and Wassarman, 1976). Although many oocytes isolated at 15 days were capable of undergoing GVB in culture, they generally were unable to complete the first meiotic division and reach metaphase II. Nevertheless, as the age of the mice and diameter of the oocyte increased, more of the oocytes

Accepted June 20, 1989.
Received January 20, 1989.
[1]This work was done as part of the National Cooperative Program on Non-Human In Vitro Fertilization and Preimplantation Development and was funded by the National Institute of Child Health and Human Development, NIH, through cooperative agreement HD 21970.
[2]Reprint requests: John J. Eppig, The Jackson Laboratory, Bar Harbor, ME 04609.
[3]Visiting Investigator from the Biology Department, Gettysburg College, Gettysburg, PA 17325.

progressed to metaphase II (Sorensen and Wassarman, 1976). Therefore, competence to undergo GVB is normally achieved when the oocytes reach approximately 60 μm in diameter, and the capacity to mature to metaphase II is acquired subsequently.

Oocyte growth and acquisition of GVB competence in vitro has been demonstrated with oocyte-granulosa cell complexes isolated from 8- to 10-day-old mice (Eppig, 1977; Eppig and Downs, 1987). Oocyte growth in vitro requires metabolic cooperativity with companion granulosa cells (Eppig, 1979; Brower and Schultz, 1982; Herlands and Schultz, 1984). Although the acquisition of competence to undergo GVB is normally correlated with oocyte growth, these two processes are independent of each other. This was demonstrated by culturing oocytes from mice 7–13 days old on a monolayer of fibroblasts that sustains oocyte viability but not metabolic cooperativity or oocyte growth. Many of these oocytes acquired competence to undergo GVB at a time similar to that when GVB competence is acquired in vivo but without achieving a diameter of 60 μm (Canipari et al., 1984).

There was once considerable controversy as to whether oocytes that underwent spontaneous maturation in vitro were normal because there had been little success in fertilizing them (Thibault, 1977; Leibfried and Bavister, 1983). More recently, however, it has been shown that oocytes of mice, rats, sheep, and cattle have a full developmental capacity after maturation in vitro (Newcomb et al., 1978; Shalgi et al., 1979; Schroeder and Eppig, 1984; Staigmiller and Moor, 1984; Goto et al., 1988; Sirard et al., 1988; Vanderhyden and Armstrong, 1989). Although the effect of gonadotropins on the developmental capacity of oocytes undergoing maturation in vitro remains to be fully resolved, follicle-stimulating hormone (FSH) added to the medium for oocyte maturation increased the frequency of preimplantation development of oocytes isolated from gonadotropin-primed mice (Downs et al., 1986; Schroeder et al., 1988). Similarly, addition of luteinizing hormone (LH) to the medium for the maturation of rat oocytes increased their capacity for development (Shalgi et al., 1979).

In the experiments presented here, we first assessed the developmental capacity of oocytes matured in vitro after isolation from juvenile mice at various times after the acquisition of meiotic competence in vivo. With increasing age and mean oocyte diameter, increasing percentage of oocytes acquired the capacity to cleave after maturation and insemination in vitro. The capacity of the oocyte to complete preimplantation development appeared to be acquired by the developing oocytes subsequent to the capacity to cleave to the 2-cell stage. These data were used as a baseline for assessing the developmental capacity of oocytes that acquired meiotic competence during growth in vitro. Secondly, we isolated oocyte-granulosa cell complexes from 12-day-old mice and cultured them for 10 days. The oocytes grew to the size of oocytes isolated from 16-day-old mice. Such in vitro-grown oocytes (IVG) underwent cleavage to the 2-cell stage after insemination in vitro at the same frequency as oocytes isolated from 22-day-old mice. The capacity of the 2-cell embryos derived from oocytes grown in vitro to develop to blastocysts was the same as those derived from 18-day-old mice. Live young were produced from the oocytes grown in vitro after transfer to pseudopregnant foster mothers. These results demonstrate for the first time that oocytes with full developmental capacity can be produced in vitro from oocytes isolated before they have completed their growth phase or acquired meiotic competence.

MATERIALS AND METHODS

Animals

(C57BL/6J × SJL/J)F_1 mice were used for all experiments. In establishing the age of the mice, the day of birth was considered to be Day 1. Males that provided sperm were at least 3 mo old and were caged individually since weaning.

Culture Systems

The medium for oocyte growth and maturation that was used for all experiments was Eagle's Minimum Essential Medium (MEM) supplemented with 5% fetal bovine serum (HyClone, Inc., Logan, UT), 0.23 mM pyruvic acid, 0.01% phenol red (Difco Laboratories, Detroit, MI), 50 mg/liter streptomycin sulfate, and 75 mg/liter penicillin G potassium salt. The MEM was prepared with Earle's salts (components purchased from Sigma Chemical Co., St. Louis, MO), amino acid, and vitamin concentrates (Whittaker, M. A. Bioproducts, Inc., Walkersville, MD), and water distilled once after collection from a spring in Franklin, ME. Fresh medium was prepared weekly and stored in Corning polycarbonate flasks (Corning Glass Works, Corning, NY) under an atmosphere of 5% O_2, 5% CO_2, 90% N_2

(hereafter referred to as the 5-5-90 gas mixture). Medium was filter-sterilized with Corning disposable filter units equipped with a 0.45-μm pore size cellulose acetate membrane.

For the maturation of oocytes from 16- to 28-day-old mice, the ovaries were placed into culture medium and antral follicles were punctured with 30-gauge needles, which had been wiped clean of lubricants with Kimwipes soaked with 70% ethanol, to liberate the oocyte-cumulus cell complexes. Oocytes completely enclosed by cumulus cells were collected with a micropipette and washed by serially transferring them through 4 dishes (Corning, 35-mm tissue culture dishes), each containing 2.5 ml of medium. The oocyte-cumulus cell complexes were transferred to Costar Transwell membrane inserts (3.0-μm pore size, polycarbonate, 24.5 mm diameter, not tissue culture-treated; Costar Corp., Cambridge, MA) in Costar 6-well cluster dishes containing 4 ml medium with 1 μg/ml FSH (ovine FSH-17). The dishes with the oocytes were incubated 15–16 h in modular incubator chambers (Billups-Rothenberg, Del Mar, CA) containing the 5-5-90 gas mixture at 37°C. The ova were then collected and washed in Whitten's medium (Whitten, 1971) and inseminated as described previously (Schroeder and Eppig, 1984). After insemination, the eggs were washed again and cultured in 1 ml of Whitten's medium in silicone-rubber-stoppered borosilicate glass tubes with the 5-5-90 gas atmosphere in a 37°C water bath. The number of eggs that underwent cleavage to the 2-cell stage was determined 20–22 h later. Cleaved eggs were washed and cultured, and the number of 2-cells that continued development to expanded blastocysts was assessed 5 days after insemination. The number of cells in the blastocysts was determined according to the method of Ebert et al. (1985). Oocyte diameter was determined for each group after isolation. Oocytes were denuded of their cumulus cells by repeatedly drawing them in and out of Pasteur pipette while subjecting them to a shearing action on the bottom of a culture dish. Oocytes were allowed to equilibrate for 20 min and then were measured, excluding the zona pellucida, with a Nikon inverted microscope equipped with a calibrated ocular micrometer. At least 40 oocytes were measured in each of at least three different sets of oocytes from mice of different litters.

For oocyte growth and development in vitro, the ovaries of 12-day-old mice were dissociated with collagenase as described previously (Eppig and Downs, 1987). This procedure produces oocyte-granulosa cell complexes without theca cells or basal lamina. There are no antral follicles in the ovaries of 12-day-old mice. The largest follicles are preantral follicles consisting of about 2 layers of granulosa cells around the oocyte. Such oocytes are in about mid-growth phase and are incompetent to undergo spontaneous maturation (0/312) at the time of isolation. The complexes were cultured on Costar Transwell-COL membrane inserts (3.0 μm pore size, 24.5 mm diameter) in Costar 6-well cluster dishes with 5 ml medium per well. These membranes were treated with an equimolar mixture of Types I and III collagen produced from bovine placentae. Preliminary experiments showed that these membranes allowed attachment and maintenance of the complexes with only minimal migration of the granulosa cells from the oocytes compared to other substrates tested. Maintenance of oocyte-granulosa cell interaction is essential for oocyte growth and development. The medium was also supplemented with 0.05 mM 3-isobutyl-1-methylxanthine (IBMX, Aldrich Chemical Co., Milwaukee, WI), which functioned to maintain all of the oocytes in meiotic arrest as meiotic competence was acquired by the oocytes developing in vitro. The oocyte-granulosa cell complexes were cultured for 10 days in modular incubator chambers as described above. Cultures were fed every other day by exchanging approximately 1 ml fresh medium for the same volume of used medium. Care was taken during the culture period to avoid jolting the cultures, which easily dislodges the complexes from the membrane. After 10 days, oocytes that had become dislodged were removed with a Pasteur pipette and discarded. Those that remained attached to the membrane were washed with medium without IBMX; FSH (1 μg/ml) was added, and the oocytes were allowed to mature for 16 h. Cumulus cell-enclosed oocytes were then removed from the membrane, washed in Whitten's medium, and divided into two groups: (1) to determine the number of oocytes that had undergone maturation and extruded a clearly visible polar body, and (2) to assess the developmental capacity of the ova after insemination. For Group 1 oocytes, adherent cumulus cells were removed and the oocytes were examined with a Wild M5A stereomicroscope to determine the percentage that had undergone GVB and produced a polar body. Group 2 ova were inseminated and cultured in Whitten's medium as described previously (Schroeder and Eppig, 1984). The percentage of mature ova that cleaved to the 2-cell stage after insemination (Group 2) was estimated assuming that Group 2 had the same percentage mature as Group 1. This

indirect procedure was used because we could not confirm the presence or absence of a germinal vesicle through the cumulus layer before insemination, and we have found that higher frequencies of fertilization and cleavage occur when the cumulus cells are not removed before insemination and when manipulations are minimized (unpublished observations). Two-cell embryos derived from in vitro-grown oocytes, from oocytes that underwent maturation in vitro after isolation from 18- and 22-day-old mice, or from superovulated ova were cultured in glass tubes as described above. Culture tubes were examined with the aid of an inverted microscope, without removing the embryos from the tubes, to determine the percentage that had progressed beyond the 2-cell stage, developed to morulae, and formed expanded blastocysts. The diameter of oocytes grown in vitro and the number of cells in blastocysts derived from oocytes grown in vitro were determined as described above.

Statistics

The percentile distributions of oocyte diameter and number of cells per blastocyst are presented in notched box-and-whisker plots (Kafadar, 1985) using the Stat View 512+ program (Brainpower, Inc., Calabasas, CA) on a Macintosh II microcomputer. Non-overlapping notches between a pair of samples indicates a significant difference ($p<0.05$) in the populations (Kafadar, 1985). These differences were further tested with the CLR ANOVA Analysis of Variance Program (Clear Lake Research, Houston, TX) on the Macintosh II microcomputer. If one-way analysis of variance indicated significant differences, it was followed by Newman-Keul's multiple range test to make pairwise comparisons. Frequencies of GVB, polar body formation, and development to various stages of embryogenesis were compared with χ^2 analysis, using data pooled from all experiments.

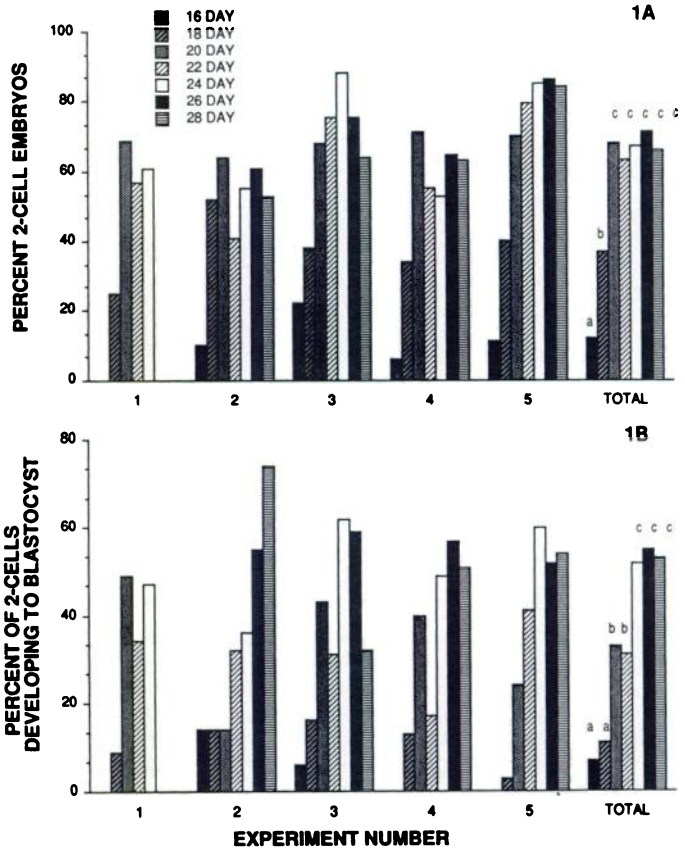

FIG. 1. Preimplantation developmental competence of oocytes that underwent maturation in vitro after isolation from mice 16 to 28 days old. *A)* Percentage of mature ova that developed to the 2-cell stage after insemination in vitro. *B)* Percentage of 2-cell embryos that developed to the expanded blastocyst stage. Results of 5 separate experiments are presented along with the total of those 5 experiments. The number of oocytes or 2-cell embryos included in each group, 16- to 28-day, are

	Fig. 1A	Fig. 1B
Expt. 1	37, 34, 54, 56, 77, 68, 0	0, 33, 35, 32, 49, 0
Expt. 2	68, 85, 69, 76, 85, 78, 66	7, 44, 49, 19, 47, 69, 35
Expt. 3	79, 99, 78, 64, 46, 61, 53	17, 38, 53, 48, 42, 46, 34
Expt. 4	35, 93, 49, 94, 97, 78, 59	2, 32, 35, 52, 51, 51, 37
Expt. 5	19, 82, 77, 77, 94, 76, 68	2, 33, 54, 61, 80, 65, 57
Total	238, 493, 327, 337, 399, 313, 246	28, 180, 221, 212, 269, 222, 163

Groups were compared by χ^2 analysis using the total of the 5 experiments. A different *letter* over the *bar* indicates a difference of at least $p<0.05$.

RESULTS

Capacity of Oocytes Isolated from 16- to 28-Day-Old Mice to Undergo Preimplantation Development

Cumulus cell-enclosed oocytes isolated from mice 16–28 days of age were cultured for 15–16 h to allow spontaneous maturation, and then inseminated in vitro. In preliminary experiments, it was found that 75% of the oocytes from 16-day-old mice underwent GVB, but only 37% of these produced a clearly visible polar body. Almost all of the oocytes (95%) from 18-day-old mice underwent GVB, and 57% of these produced a polar body compared to at least 70% produced by the oocytes from older mice. (Hereafter, oocytes that underwent GVB are referred to as mature, whether or not they produced a clearly visible polar body).

The capacity of the mature ova to cleave to the 2-cell stage after insemination is shown on Figure 1A, and the

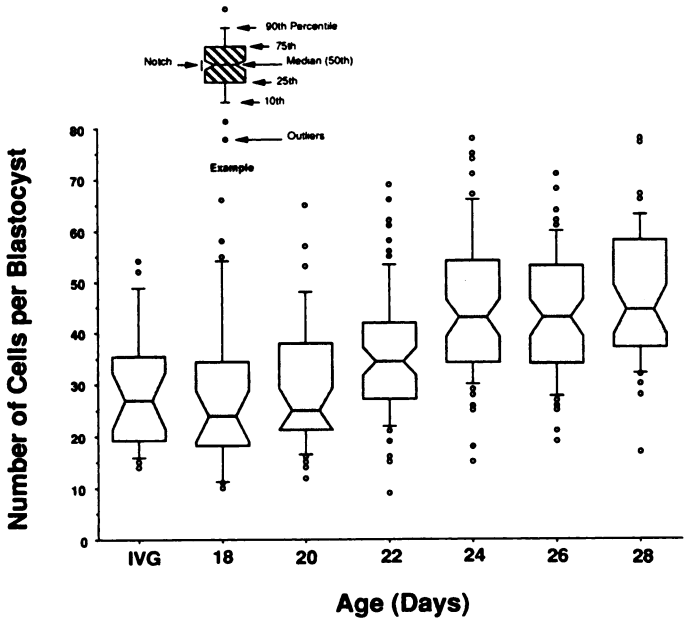

FIG. 2. Effect of the age of the mice from which the oocytes were isolated on the number of cells in the expanded blastocysts after maturation, insemination, and preimplantation development in vitro. Data are presented by using notched box-and-whisker plots as the percentile distribution of blastocysts containing the number of cells indicated on the y-axis. The blastocysts were derived from oocytes that underwent maturation in vitro after isolation from 18- to 28-day-old mice or from oocytes from 12-day-old mice grown in vitro for 10 days (IVG). Non-overlapping notches between a pair of samples indicates a significant difference ($p<0.05$) in the populations (Kafadar, 1985). These differences were verified by one-way analysis of variance followed by the Newman-Keul's Multiple Range Test. The number of blastocysts in each group were as follows: IVG, 23; 18-day-old, 28; 20-day-old, 38; 22-day-old, 74; 24-day-old, 74; 26-day-old, 69; 28-day-old, 46.

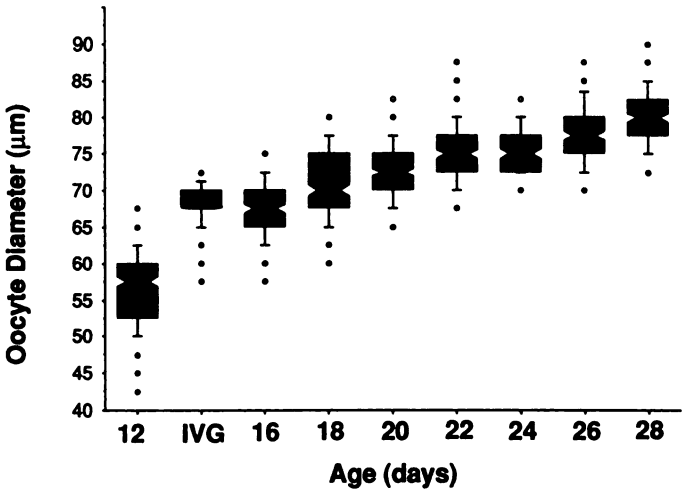

FIG. 3. Diameter of oocytes isolated from mice 12 to 28 days old and of oocytes from mice 12 days old grown in vitro for 10 days (IVG). Data are presented by using notched box-and-whisker plots as the percentile distribution of oocytes, with the diameter indicated on the y-axis. The number of oocytes in each group were as follows: 12-day-old, 246; IVG, 130; 16-day-old, 121; 18-day-old, 178; 20-day-old, 205; 22-day-old, 148; 24-day-old, 113; 26-day-old 141; 28-day-old, 126.

capacity of the 2-cell stage embryos to develop to blastocysts is shown on Figure 1B. Very few (12%) of the eggs derived from 16-day-old mice were able to develop to the 2-cell stage after insemination, and only 7% of these were able to develop to blastocysts. A higher percentage (37%) of the oocytes derived from 18-day-old mice were able to develop to the 2-cell stage after insemination, but the capacity of these to develop to blastocysts was not greater than the capacity of the 2-cells derived from oocytes from 16-day-old mice to continue development. The maximum frequency of cleavage to the 2-cell stage (about 70%) was achieved by oocytes derived from mice 20 through 28 days old, whereas the maximum frequency of development to the blastocyst stage (about 55%) was achieved by mice that were 24 through 28 days old.

The percentile distribution of the number of cells per blastocyst derived from oocytes obtained from mice of different ages and matured in vitro is presented in notched box-and-whisker plots in Figure 2. The blastocysts derived from oocytes matured in vitro after isolation from 18- or 20-day-old mice were composed of only 18.0 ± 2.9 (mean ± SEM) (median = 24) or 30.0 ± 2.1 (median = 25.0) cells, respectively. The mean number of cells per blastocyst increased to 36.0 ± 1.4 (median = 34.4; $p<0.05$) when the oocytes were isolated from 22-day-old mice, and again to 45.0 ± 1.5 (median = 43.0; $p<0.05$) when the oocytes were isolated from 24-day-old mice. No further increases in the number of cells per blastocyst were detected in embryos derived from oocytes matured in vitro after isolation from 26- or 28-day-old mice.

Oocyte Growth in Vitro

The percentile distribution of the diameter of oocytes isolated from 12- to 28-day-old mice and oocytes from 12-day-old mice grown in vitro (IVG) for 10 days is presented in Figure 3. Oocytes isolated from 12-day-old mice were 56.00 ± 0.29 µm (mean ± SEM) (median = 57.50) in diameter, and the mean size increased to 68.00 ± 0.23 µm (median = 67.50) during the 10-day culture period. The diameter of the oocytes after growth in vitro was the same as the diameter of oocytes freshly isolated from 16-day-old mice (68.00 ± 0.33; median = 67.70), but significantly smaller ($p<0.01$) than the oocytes of equivalent chronological age (22 days old; 74.00 ± 0.35 µm; median = 75.00).

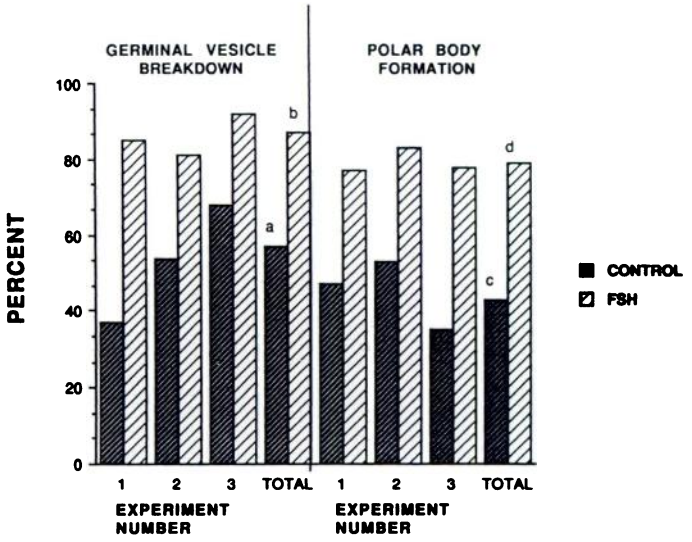

FIG. 4. Effect of follicle-stimulating hormone (FSH) (1 μg/ml) on the percentage of oocytes undergoing germinal vesicle breakdown (GVB) and the percentage of GVB-oocytes that produced a visible polar body after oocytes from 12-day-old mice were grown in vitro for 10 days. Results are shown for 3 independent experiments and for the total of all 3 experiments. Control and FSH totals were compared by χ^2 analysis. The number of oocytes in each experiment were as follows: Expt. 1, C = 51, FSH = 52; Expt. 2, C = 94, FSH = 37; Expt. 3, C = 106, FSH = 83; Total C = 251, FSH = 172.

Maturation of IVG Oocytes

After culturing the oocytes from 12-day-old mice for 10 days, medium containing IBMX was replaced with IBMX-free medium. FSH (1 μg/ml) was added to half of the cultures, and the percent of the oocytes that underwent GVB and polar body production was determined. As shown in Figure 4, 57% of the oocytes in medium without FSH underwent GVB and 43% of these produced a polar body. When the maturation medium contained FSH, 87% of the oocytes underwent GVB, and 79% of these produced a visible polar body. FSH also stimulated the expansion (mucification) of most of the cumuli oophori.

Developmental Capacity of IVG Oocytes

Because of the beneficial effect of FSH on the frequency of GVB and polar body production by IVG oocytes, FSH was included during the maturation period for all subsequent experiments. The following groups of ova were used as controls for the IVG oocytes: (1) oocytes that underwent maturation in vitro in the presence of FSH after isolation from 18- and 22-day-old mice, and (2) oocytes that underwent maturation in vivo and were superovulated in response to an

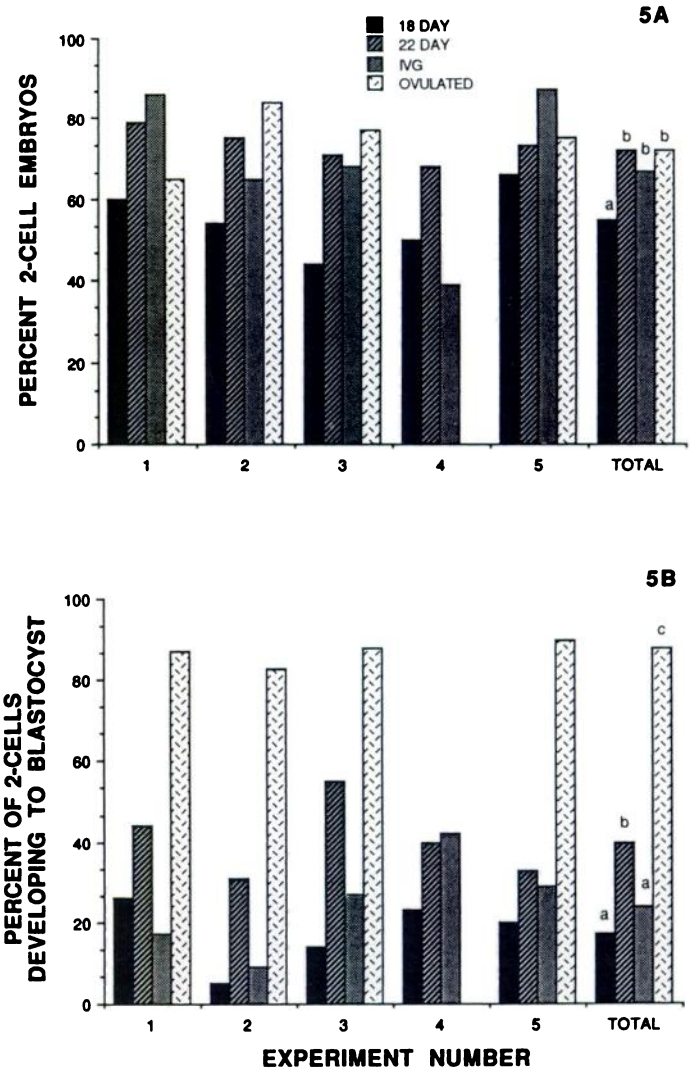

FIG. 5. Comparison of preimplantation development by oocytes grown in vitro (IVG) and in vivo. IVG oocytes were isolated from 12-day-old mice and grown in vitro for 10 days before maturation in vitro. Oocytes grown in vivo were isolated from the ovaries of 18- or 22-day-old mice and underwent maturation in vitro, or were isolated from the oviducts of superovulated 22-day-old mice. Follicle-stimulating hormone (FSH) was added to the medium used for the maturation of all of these groups of oocytes, in contrast to the experiments shown in *Figure 1* wherein FSH was not added. The results of 5 independent experiments are shown along with the total of the 5 experiments. The groups were compared by χ^2 analysis using the total of the experiments. A different *letter* over the *bar* indicates a difference of at least $p<0.05$. The number of oocytes or 2-cell embryos included in each group (18-day, 22-day, IVG, and ovulated) are

	Fig. 5A	Fig. 5B
Expt. 1	81, 43, 132, 165	42, 34, 113, 30
Expt. 2	82, 65, 190, 50	44, 49, 124, 30
Expt. 3	84, 75, 245, 65	37, 53, 167, 50
Expt. 4	52, 88, 193	26, 60, 76
Expt. 5	67, 74, 138, 112	44, 54, 118, 84
Total	366, 345, 898, 392	193, 250, 598, 194

The ovulated sample in Experiment 4 was lost.

injection of 5 IU of human chorionic gonadotropin 48 h after priming 20-day-old mice with pregnant mare's serum gonadotropin. Oocytes in control Group I underwent maturation on Transwell-COL filter inserts, rather than on the uncoated inserts used in the initial experiments described above, so that maturation of IVG oocytes and oocytes grown in vivo would occur under identical conditions. After maturation, the ova were inseminated as described previously (Schroeder and Eppig, 1984). The IVG ova cleaved to the 2-cell stage after insemination, with a frequency equivalent to superovulated ova and ova matured in vitro after isolation from 22-day-old mice (Fig. 5A). Twenty-four percent of the 2-cell embryos derived from IVG oocytes developed to the expanded blastocyst stage. This percentage was equivalent to that by 2-cell embryos derived from oocytes isolated from 18-day-old mice, but significantly less than by embryos derived from oocytes isolated from mice of the equivalent chronological age (22 days old) (Fig. 5B). The blastocysts derived from IVG oocytes contained the same number of cells as the blastocysts derived from oocytes isolated from 18- and 20-day-old mice, but fewer cells than in blastocysts derived from oocytes isolated from 22- to 28-day-old mice (Fig. 2).

Most of the 2-cell stage embryos derived from ovulated ova fertilized in vitro reached the expanded blastocyst stage (Figs. 5 and 6). Transition from the 2-cell stage to the expanded blastocyst stage by embryos derived from oocytes that underwent maturation in vitro, however, was restricted. After maturation in vitro, progressively fewer embryos were able to advance past the 2-cell stage to the morula stage and to the blastocyst stage (Fig. 6). Nevertheless, 16% of the mature ova derived from IVG oocytes developed to the expanded blastocyst stage in vitro.

Capacity of Embryos Derived from IVG Oocytes to Develop to Live Young

After using the protocol described here for growth and maturation of oocytes isolated from 12-day-old mice in vitro, 137 2- to 4-cell stage embryos were transferred to oviducts of 7 pseudopregnant females (10–15 embryos per oviduct), and 3 females became pregnant. Embryos, foster mothers, and vasectomized males were genetically marked at the hemoglobin locus, as described previously (Schroeder and Eppig, 1984), to permit verification that live young were derived from transferred embryos. Seven (5.1%) live, apparently normal, pups were born and 12 resorptions were detected. These results demonstrate that some IVG oocytes have the capacity to develop to live offspring after maturation and fertilization in vitro and transfer to foster mothers.

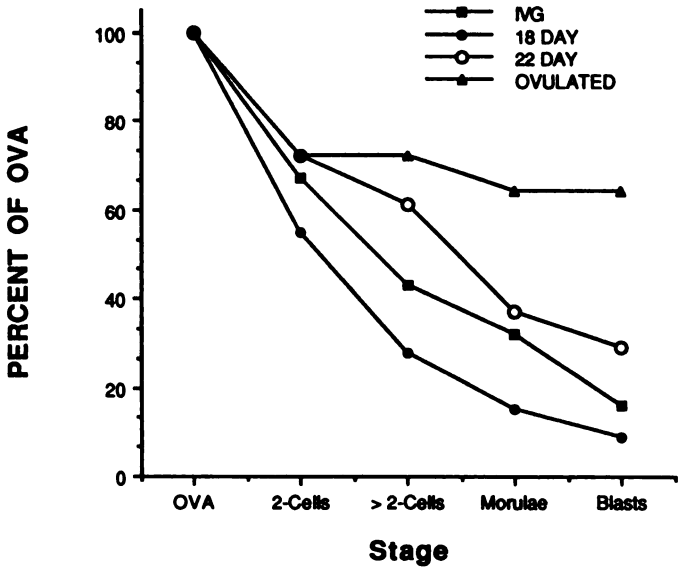

FIG. 6. Progress of embryos through the midstages of preimplantation development after insemination of oocytes grown in vitro (IVG) and oocytes grown in vivo. Data are the total of the same groups shown in *Figure 5*.

DISCUSSION

A system is described here by which live mice can be produced from oocytes isolated from 12-day-old mice, and grown, matured, and fertilized in vitro and transferred to pseudopregnant females. These oocytes were, at the time of isolation from preantral follicles, in about mid-growth phase and incompetent to undergo GVB (0/312) without further development. During a 10-day culture period, the oocytes grew to a mean diameter equivalent to that of oocytes isolated from 16-day-old mice. About 87% of the IVG oocytes were competent to undergo GVB, and 79% of these produced a visible polar body upon removal of IBMX from the culture medium and addition of FSH. The IVG ova developed to the 2-cell stage with a frequency equivalent to that of superovulated ova after insemination and cleavage in vitro. Development to the expanded blastocyst stage by the cleaved eggs derived from IVG oocytes was comparable in frequency and cell number to oocytes that underwent maturation in vitro after isolation from 18-day-old mice. Live offspring were produced after transfer of embryos to pseudopregnant fos-

ter mothers, albeit at a low frequency (5.1%).

Oocytes appear to sequentially acquire competence to undergo GVB, complete the first meiotic division, undergo fertilization and cleavage to the 2-cell stage, and advance to the expanded blastocyst stage. Thus we confirmed the observation first made by Sorensen and Wassarman (1976) that oocytes isolated from juvenile mice of increasing age and increasing in size acquire competence first to undergo GVB, and then to complete the first meiotic division and to produce the first polar body. Additionally, the results presented here indicate that capacity to undergo maturation does not assure competence to cleave to the 2-cell stage after insemination. Likewise, ability to cleave to the 2-cell stage does not assure competence to develop beyond this stage. The number of cells in the expanded blastocyst also appears to increase with the age and diameter of the oocyte, but, just as acquisition of competence to undergo maturation is independent of oocyte size (Canipari et al., 1984), so may be the number of cells in the blastocyst. This is suggested by the observation that the oocytes grown in vitro reached a size equivalent to that of oocytes from 16-day-old mice, but the blastocysts derived from the IVG oocytes contained the same number of cells as blastocysts derived from oocytes isolated from 18- and 20-day-old mice whose oocytes are larger than those isolated from 16-day-old mice. Thus oocytes from the older mice appeared to be qualitatively different from the oocytes of the younger mice in a way that affected their capacity to develop after insemination. It remains to be determined whether the increased developmental capacity resulted from changes intrinsic to the oocyte, or from different signals originating in the somatic cells that communicate with the oocyte, or from both.

Addition of FSH to the medium for maturation of IVG oocytes increased the percentage of oocytes that produced a polar body. FSH also increases the developmental capacity of oocytes undergoing maturation in vitro after growth in vivo (Downs et al., 1986). In contrast, oocyte growth and the acquisition of meiotic competence occur in vitro in the absence of gonadotropins (Eppig, 1977). Gonadotropins were not added to the oocyte culture system used for oocyte growth in the study presented here, although very low concentrations of these hormones may have been present in the serum. These results suggest that gonadotropins may not be required for the acquisition of full development competence by growing oocytes. Gonadotropins, however, may be beneficial for the development of normal oocytes, because oocytes isolated from hypogonadal mice genetically deficient in circulating gonadotropins underwent fertilization and embryogenesis at very low frequency after maturation in vitro. Injecting these mice with pregnant mare's serum gonadotropin greatly increases the number of oocytes that can be fertilized and complete preimplantation development after maturation in vitro (Schroeder and Eppig, 1989).

An essential factor for oocyte growth in vitro is the maintenance of metabolic coupling between the oocyte and its companion granulosa cells (Eppig, 1977, 1979). Granulosa cells were maintained in close association with the oocytes in the culture system described here. Although other cell types can establish metabolic cooperativity with oocytes, the oocytes appear to require a communication specifically with granulosa cells (Buccione et al., 1987). It seems likely that maintenance of granulosa cell differentiation and function contributes to the normal development of the oocytes. Future development of the oocyte culture system should, therefore, focus not only on providing factors that could affect the oocyte directly, but also the development and function of the granulosa cells.

IBMX was an important component of the oocyte culture system presented here. This phosphodiesterase inhibitor functioned to maintain the oocytes in meiotic arrest after they had developed competence to undergo GVB during the 10-day culture period. It is essential that all of the oocytes begin maturation at a specific time because the time of insemination after the initiation of maturation is critical for successful fertilization and embryogenesis (Schroeder et al., 1988). Thus, IBMX serves to synchronize maturation so that insemination can be carried out 15–16 h after the initiation of maturation. IBMX may also have promoted granulosa cell function, because in the absence of IBMX or the naturally occurring phosphodiesterase inhibitor hypoxanthine, many oocytes were released by the granulosa cells and were found denuded in the culture dish (Eppig and Downs, 1987).

Most oocytes found in preantral follicles do not become ovulated because the follicles that contain them are not selected for appropriate preovulatory development and, therefore, they undergo atretic degeneration. These oocytes represent a large source of germ plasm that potentially could be used to expand the populations of rare animals. The results presented here demonstrate that it is feasible to harvest this group of follicles and to grow the oocytes in culture to produce fertilizable eggs with the full developmental potential to produce live

young. Considerable improvement of the culture system will be required, however, to increase the frequency of successful development to term. The rationale for modifications of the culture system must come from an increased understanding of the development and function of granulosa cells in preantral follicles, oocyte-granulosa cell interactions, and oocyte metabolism. This system for oocyte growth and development in vitro could also provide the opportunity for manipulating oocyte gene expression in vitro and assessing the role of these genes in oocyte development or the capacity of the oocyte to undergo fertilization and embryogenesis. Moreover, it is possible that this system could be used for introducing foreign genes into the oocyte and producing transgenic animals.

ACKNOWLEDGMENTS

The technical assistance of Marilyn O'Brien is greatly appreciated. We are grateful to the Nation Institute of Diabetes, Digestive and Kidney Disease and the National Hormone and Pituitary Program of the University of Maryland School of Medicine for generously providing the FSH used in these experiments and to John Biggers for introducing us to notched box plots.

REFERENCES

Brower PT, Schultz RM, 1982. Intercellular communication between granulosa cells and mouse oocytes: existence and possible nutritional role during oocyte growth. Dev Biol 90:144–53

Buccione R, Cecconi S, Tatone C, Mangia F, Colonna R, 1987. Follicle cell regulation of mammalian oocyte growth. J Exp Zool 242:351–54

Canipari R, Palombi F, Riminucci M, Mangia F, 1984. Early programming of maturation competence in mouse oogenesis. Dev Biol 102:519–24

Downs SM, Schroeder AC, Eppig JJ, 1986. Developmental capacity of mouse oocytes following maintenance of meiotic arrest in vitro. Gamete Res 15:305–16

Ebert KM, Hammer RE, Papaioannou VE, 1985. A simple method for counting nuclei in the preimplantation mouse embryo. Experientia 41:1207–09

Eppig JJ, 1977. Mouse oocyte development in vitro with various cultures systems. Dev Biol 60:371–88

Eppig JJ, 1979. A comparison between oocyte growth in coculture with granulosa cells and oocytes with granulosa cell-oocyte junctional contact maintained in vitro. J Exp Zool 209:345–53

Eppig JJ, Downs SM, 1987. The effect of hypoxanthine on mouse oocyte growth and development in vitro: maintenance of meiotic arrest and gonadotropin-induced oocyte maturation. Dev Biol 119:313–21

Goto K, Kajihara Y, Kosaka S, Koba M, Nakanishi Y, Ogawa K, 1988. Pregnancies after coculture of cumulus cells with bovine embryos derived from in vitro fertilization of in vitro matured follicular oocytes. J Reprod Fertil 83:753–58

Herlands RL, Schultz RM, 1984. Regulation of mouse oocyte growth: probable nutritional role for intercellular communication between follicle cells and oocytes in oocyte growth. J Exp Zool 229:317–25

Kafadar K, 1985. Notched box-and-whisker plot. In: Kotz S, Johnson NL (eds.), Encyclopedia of Statistical Sciences, Vol. 6. New York: John Wiley and Sons, pp. 367–70

Leibfried ML, Bavister BD, 1983. Fertilizability of in vitro matured oocytes from golden hamsters. J Exp Zool 226:481–85

Newcomb R, Cristie WB, Rowson LEA, 1978. Birth of calves after in vivo fertilization of oocytes removed from follicles and matured in vitro. Vet Rec 102:461–62

Schroeder AC, Downs SM, Eppig JJ, 1988. Factors affecting the developmental capacity of mouse oocytes undergoing maturation in vitro. Ann NY Acad Sci 541:197–204

Schroeder AC, Eppig JJ, 1984. The developmental capacity of mouse oocytes that matured spontaneously in vitro is normal. Dev Biol 102:493–97

Schroeder AC, Eppig JJ, 1989. Developmental capacity of mouse oocytes that undergo maturation in vitro: effect of the hormonal status of the oocyte donor. Gamete Res 24:1–12

Shalgi R, Dekel N, Kraicer PF, 1979. The effect of LH on the fertilizability and developmental capacity of rat oocytes matured in vitro. J Reprod Fertil 55:429–35

Sirard MA, Parrish JJ, Ware CB, Leibfried-Rutledge ML, First NL, 1988. The culture of bovine oocytes to obtain developmentally competent embryos. Biol Reprod 39:546–52

Sorensen RA, Wassarman PM, 1976. Relationship between growth and meiotic maturation of the mouse oocyte. Dev Biol 50:531–36

Staigmiller RB, Moor RM, 1984. Effect of follicle cells on the maturation and developmental competence of ovine oocytes matured outside the follicle. Gamete Res 9:221–29

Szybek K, 1972. In vitro maturation of oocytes from sexually immature mice. J Endocrinol 54:527–28

Thibault C, 1977. Are follicular maturation and oocyte maturation independent processes? J Reprod Fertil 51:1–15

Vanderhyden BC, Armstrong DT, 1989. Role of cumulus cells and serum on the in vitro maturation, fertilization and subsequent development of rat oocytes. Biol Reprod 40:720–28

Whitten WK, 1971. Nutrient requirements for the culture of preimplantation embryos in vitro. Adv Biosci 6:129–39