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A Revised Protocol for In Vitro Development of Mouse Oocytes from Primordial Follicles Dramatically Improves Their Developmental Competence[1]

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ABSTRACT

The objective of this study was to improve the conditions for oocyte development in vitro beginning with the primordial follicles of newborn mice. Previous studies showed that oocytes competent of meiotic maturation, fertilization, and preimplantation could develop in vitro from primordial follicles. However, the success rates were low and only one live offspring was produced (0.5% of embryos transferred). A revised protocol was compared with the original protocol using oocyte maturation and preimplantation development as end points. The percentage of oocytes maturing to metaphase II and developing to the blastocyst stage was significantly improved using the revised protocol. In addition, we compared the production of offspring from two-cell stage embryos derived from in vitro-grown and in vivo-grown oocytes. Of 1160 transferred two-cell stage embryos derived from in vitro-grown oocytes, 66 (5.7%) developed to term and 7 pups (10.6%) died at birth. The remaining 59 pups (27 females, 32 males) survived to adulthood. By comparison, of 437 transferred two-cell stage embryos derived from in vivo-grown oocytes, 76 (17.4%) developed to term and 4 (5.3%) died at birth. The remaining 72 pups (35 females, 37 males) survived to adulthood. These studies provide proof of the principle that fully competent mammalian oocytes can develop in vitro from primordial follicles and present a significant advance in oocyte culture technology.

gamete biology, oocyte development, ovary, primordial follicles

INTRODUCTION

Technologies to enable oocyte growth and development in culture will provide valuable experimental tools for studies of the mechanisms governing oocyte development as well as practical clinical, agricultural, and zoological applications. A two-step culture protocol was devised to produce oocytes competent enough to undergo maturation, fertilization, and development to live offspring from primordial follicles of newborn mice [1]. The first step was organ culture of intact newborn ovaries, and the second step was isolation and culture of oocyte-granulosa cell complexes isolated from preantral follicles that developed in the organ-cultured ovaries. Unfortunately, the frequency of successful preimplantation development was very low and only one live offspring (0.5% of embryos transferred), known as Eggbert, was produced. Thus, the conditions for oocyte development were obviously not optimal, and therefore, the significance of the single birth could be justifiably questioned. Since the original report in 1996, we conducted studies on factors affecting the development of mouse oocytes in vitro using oocyte-granulosa cell complexes isolated from secondary preantral follicles of 12-day-old mice without prior organ culture. Various concentrations of FSH, insulin, ascorbic acid, and glucose were found to affect the developmental competence of oocytes grown in vitro [2, 3]. For example, the combination of FSH and insulin and the high concentration of glucose (27.8 mM) used in the original protocol were found to be deleterious to oocyte developmental competence [2, 3]. A revised two-step protocol was devised after consideration of these results and those of other unpublished studies. Here we demonstrate dramatically improved developmental competence of oocytes grown in vitro from primordial follicles using the revised protocol: 59 living offspring were produced from in vitro grown oocytes.

MATERIALS AND METHODS

Animals

Ovaries and oocytes were obtained from (C57BL/6J × SJL/J)F_1 (abbreviated B6SJLF1) females. Sperm for fertilization in studies of preimplantation development were also from B6SJLF1 mice, but for embryo transplantation studies the sperm were obtained from either green fluorescent protein (GFP) homozygous or wild-type (non-GFP carrying) males from the STOCK TgN(GFPU)5Nagy mouse line (stock 3115; JAX Research Systems, Bar Harbor, ME). This enabled the identification of offspring after the transfer of experimental embryos (derived from in vitro-grown oocytes) and control embryos (derived from in vivo-grown oocytes) to oviducts of the same recipients. Males were housed individually for at least 4 wk before use.

Protocols for Oocyte Development In Vitro

The two-step strategy for oocyte development from primordial follicles was essentially the same as described previously; intact ovaries from newborn mice were organ-cultured for 8 days, and oocyte-granulosa cell complexes isolated from them were cultured for 14 days [1]. Briefly, ovaries were isolated from newborn mice (postnatal Day 0), and surrounding tissue, including the ovarian bursa, was removed. The ovaries were placed on Millicell-PC membrane inserts (3.0 μm pore size, 30 mm in diameter; Millipore Corp., Medford, MA) with medium filling only the lower chamber. Each ovary was placed on the membrane with a single drop of medium, and eight ovaries were placed on each membrane. Then medium was removed from the lower chamber until only a thin film covered the ovaries. The medium for organ culture was Waymouth MB752 supplemented with 0.23 mM pyruvic acid, 10 μg/ml streptomycin sulfate, 75 μg/ml penicillin G, and 10% fetal bovine serum (FBS); all were purchased from Sigma Chemical Co. (St. Louis, MO). The organ cultures were maintained for 8 days at 37°C in modular incubation chambers (Billups Rothenberg, Del Mar, CA) thoroughly infused with a gas mixture of 5% CO_2, the balance was air. For the second step of oocyte development, the oocyte-granulosa cell complexes were isolated from the organ-cultured ovaries using collagenase and DNase as described previously [1]. The isolated

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TABLE 1. Comparison of original and revised protocols for oocyte development from primordial follicles.

Objective	Culture period	Original protocol		Revised protocol	
		Medium	Supplements	Medium	Supplements
Ovarian organ culture	8 days	Waymouth	FBS	Waymouth	FBS
Culture/oocyte/Granulosa cell complexes	8 days	Waymouth	BSA ITS FSH (0.5 ng/ml) EGF (1 ng/ml)	Waymouth	BSA Fetuin ITS FSH (0.05 ng/ml) EGF (1ng/ml)
Culture oocyte/Granulosa cell complexes	6 days	Waymouth	BSA Fetuin ITS FSH (0.5 ng/ml) EGF (1 ng/ml)	MEMα	BSA Fetuin ITS
Oocyte maturation	15–17 h	Waymouth	BSA Fetuin FSH (100 ng/ml)	MEMα	BSA Fetuin FSH (100 ng/ml) EGF (1 ng/ml)

complexes were cultured for 14 days on Costar Transwell-Col membranes (3.0 μm pore size, 12 mm in diameter; Corning Inc., Corning, NY) using either the original protocol, as described previously [1], or the revised protocol. The original and revised protocols are summarized and contrasted in Table 1.

For the revised protocol, complexes were cultured for 8 days in Waymouth medium MB752 supplemented as above, except without FBS, but including 3 mg/ml crystallized and lyophilized BSA (A4378; Sigma); 0.05 ng/ml human recombinant FSH (0.005 IU; provided by the National Hormone and Pituitary Program); 1 ng/ml epidermal growth factor (EGF, culture grade; Becton Dickinson Labware, Bedford, MA); 5 μg/ml each of insulin, transferrin, and selenium (ITS, Becton Dickinson Labware); and 1 mg/ml fetuin (prepared by the Microchemistry Service of The Jackson Laboratory as described previously [1]). Medium was then replaced with modified essential medium-α (MEMα without ribonucleosides and deoxyribonucleosides; Invitrogen Corporation, Carlsbad, CA), supplemented with 3 mg/ml BSA, antibiotics as above, ITS, and fetuin, but not EGF or FSH, for 6 days. Complexes were then dislodged from the membrane by sharply jolting the membrane insert with a snap of the finger against the side of the membrane. The complexes were collected and washed three times in fresh MEMα without ITS. The complexes were cultured for 17 h in MEMα supplemented with 3 mg/ml BSA, antibiotics as above, 1 mg/ml fetuin, 1 ng/ml EGF, and 100 ng/ml (0.5 IU) FSH for oocyte maturation. Throughout the 14 days of culture after organ culture, the oocyte-cumulus complexes were maintained at 37°C in modular incubation chambers infused with an atmosphere of 5% CO_2, 5% O_2, and 90% N_2. Approximately half of the culture medium was replaced every 2 days.

After 17 h of incubation for oocyte maturation, the granulosa (cumulus) cells were removed from the 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 and inseminated. 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. These oocytes were not inseminated. 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 recorded as the number of oocytes that had developed competence to undergo GVB in vitro. Mature oocytes were inseminated and preimplantation embryo development was carried out as described previously [1]. After culture for 5 days, embryos reaching the blastocyst stage were stained with Hoechst, flattened with a coverslip, and the total number of cells per blastocyst was determined.

Maturation of Control Oocytes

Oocyte-cumulus cell complexes were isolated from the ovaries of 22-day-old B6SJLF1 mice by puncturing the largest antral follicles with a needle and collecting them using drawn glass micropipettes. The oocytes were matured and fertilized in vitro using the revised protocol as described above for in vitro-grown oocytes.

Presentation of Data and Statistical Analyses

Percentages are presented as the mean percentage of at least three independent experimental replicates; variation between experiments is illustrated using the SEM. For evaluation of the differences between groups, data were subjected to arcsine transformation and ANOVA. When a significant F ratio was defined by ANOVA, groups were compared with the Fisher protected least significant difference post hoc test using Statview software (version 5; SAS Institute Inc., Cary, NC). The number of cells per blastocyst were presented in notched box and whisker plots using Statview software. When notches did not overlap among groups, the groups were considered significantly different ($P < 0.05$) [4].

RESULTS

The competence of oocytes grown in vitro to undergo maturation, fertilization, and complete preimplantation development to the blastocyst stage was determined using both the original and the revised protocols. In addition, these results were compared with those for oocytes isolated from 22-day-old mice and thus, of the same total chronological age grown in vivo but matured and fertilized in vitro.

Germinal Vesicle Breakdown

More oocytes underwent GVB in response to a 17-h stimulation with EGF and FSH after development in vitro using the revised protocol rather than the original protocol (62% versus 47%, respectively; $P < 0.01$) (Fig. 1). However, the percentage of oocytes that were competent to undergo GVB; that is, the number of oocytes undergoing GVB during the initial 17-h ligand-stimulated maturation plus the additional oocytes that underwent GVB during the subsequent 24-h incubation, was not different; 66% in both groups. Thus, almost all of the GVB-competent oocytes matured in response to FSH and EGF when they were grown in vitro using the revised protocol. The higher percentage of ligand-induced maturation obtained using the revised protocol could reflect better development of either the granulosa cells, the oocytes, or both.

Preimplantation Development

Fifty-three percent of the oocytes that underwent GVB cleaved to the two-cell stage after insemination when the oocytes were developed in vitro using the revised protocol compared with 33% using the original protocol ($P < 0.05$).

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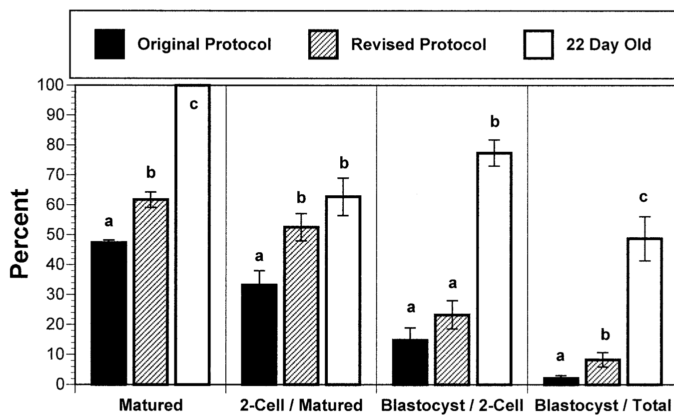

FIG. 1. Developmental competence of mouse oocytes grown in vitro from primordial follicles: a comparison of the original and revised protocols. In vitro-grown oocytes were compared with in vivo-grown oocytes isolated at the germinal vesicle stage from 22-day-old mice and matured and fertilized in vitro. The percent that matured is the percentage that underwent GVB, including both those that had progressed to both metaphase I and metaphase II, in response to FSH and EGF. The bars indicate the mean and SEM of four independent experiments. Each experiment started with approximately 120 oocytes in each group. Means with different letters for each end point are significantly different ($P < 0.05$).

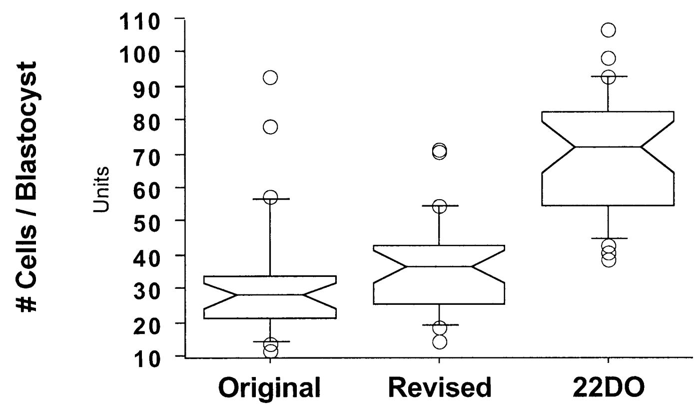

FIG. 2. The number of cells per blastocyst after oocyte growth in vitro using the original and revised protocols as well as after oocyte growth in vivo (22 days old). Results are presented as notched box plots. Lines of the boxes delineate the 25th, 50th, and 75th percentiles and the whiskers depict the 10th and 90th percentiles of a population. The notch defines the 95% confidence interval around the median (50th percentile); thus, groups that display nonoverlapping notches can be considered different ($P < 0.05$) [4]. The number of cells in at least 25 blastocysts was determined for each group.

The percentage of oocytes that cleaved to the two-cell stage was similar for oocytes developed in vitro under the revised protocol and for those developed in vivo and matured in vitro after isolation from 22-day-old mice (63%; $P = 0.18$) (Fig. 1).

The percentage of two-cell stage embryos derived from the original and the revised protocols that developed to the blastocyst stage was not different: 15% versus 23%, respectively ($P = 0.25$). In both cases of in vitro-grown oocytes, the percent completing the two-cell stage to blastocyst transition was less than that of in vivo-grown oocytes isolated from 22-day-old mice and matured in vitro (77%; $P < 0.0001$). However, the proportion of blastocysts to oocytes developed in vitro was greater when the revised protocol was used: 8% versus 2%. The total number of blastocysts produced in all experiments was 20 in the original protocol group and 75 using the revised protocol. Although this was a 4-fold improvement over the original protocol, it was still far lower than the percentage of embryos that developed following oocyte development in vivo (49%). The number of cells per blastocyst was higher in embryos derived from oocytes using the revised versus the original protocol; the median number following the original protocol was 28, versus 37 using the revised protocol ($P < 0.05$). Nevertheless, this is only half the cell number of the blastocysts derived from in vivo-grown oocytes, which contained a median number of 72 cells (Fig. 2). Although not quantified, hatching blastocysts were rarely observed after oocytes developed in vitro using the original protocol, but were common after using the revised protocol.

Completion of Nuclear Maturation

Although complete cytoplasmic maturation enabling development to the blastocyst stage does not require the progression of nuclear maturation to metaphase II [5], the progression of more oocytes to metaphase II would indicate more advanced oocyte development. Therefore, the progression of nuclear maturation of oocytes grown in vitro using the two protocols was determined in more detail. As shown in Figure 3, 44% of the oocytes developed in vitro using the revised protocol matured to metaphase II, compared with only 17% using the original protocol ($P = 0.001$).

Development to Living Offspring

The ability of preimplantation embryos derived from in vitro-grown oocytes produced using the revised protocol to develop to term was determined and compared with that of embryos derived from in vivo-grown oocytes from 22-day-old mice. Two-cell stage embryos from both groups were mixed before transfer. Each oviduct received three embryos

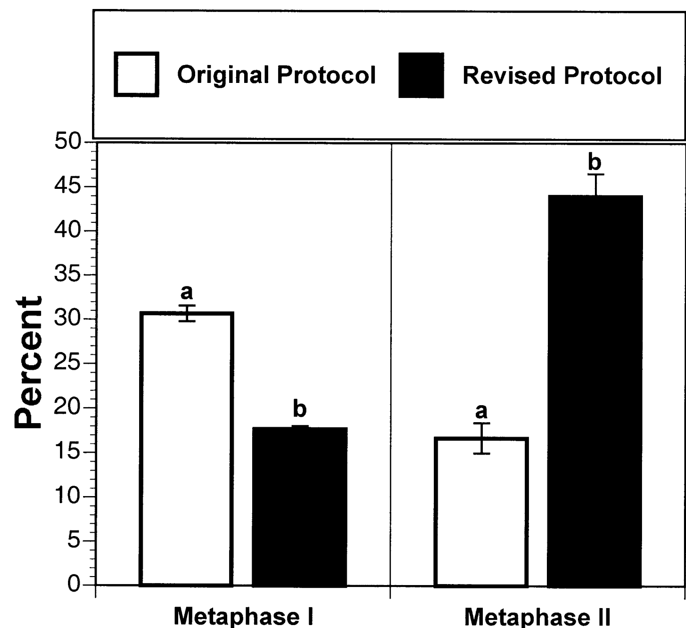

FIG. 3. Comparison of the nuclear maturation of oocytes grown in vitro using the original and revised protocols. The bars indicate the mean and SEM of four independent experiments. Means with different letters for each end point are significantly different ($P < 0.05$). A significantly greater percentage of the oocytes grown in vitro using the revised protocol completed nuclear maturation as indicated by progression to metaphase II and the production of a polar body.

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derived from in vivo-grown oocytes and eight embryos derived from in vitro-grown oocytes. One group was fertilized using sperm from males homozygous for GFP to enable identification of groups after birth. The use of GFP-bearing sperm for fertilization was alternated between the groups for experimental balance. Of 1160 transferred two-cell stage embryos derived from in vitro-grown oocytes, 66 (5.7%) developed to term and 7 pups (10.6%) died at birth. Thus, 59 (27 females, 32 males) of the offspring survived. By comparison, of 437 transferred two-cell stage embryos derived from in vivo-grown oocytes, 76 (17.4%) developed to term and 4 (5.3%) died at birth. Thus, 72 (35 females, 37 males) of the offspring survived. When the groups were compared by chi-square analysis, the percentage of surviving term births was greater ($P < 0.0001$) in the in vivo-grown group. The percentage that died at birth was not different ($P = 0.23$).

DISCUSSION

There are now 59 living offspring derived from oocytes grown in vitro from the primordial follicles of newborn mice. Eggbert is no longer alone. This constitutes strong proof of the principle that oocytes with full developmental potential can be produced in vitro. Nevertheless, oocytes grown in vitro are still not the equivalent of oocytes grown in vivo. The frequency of maturation to metaphase II, completion of the two-cell stage to blastocyst transition, or development to term was still not equivalent to that of in vivo-grown oocytes.

The differences between the original and revised protocols were based on findings using oocyte-granulosa cell complexes cultured after isolation from the secondary preantral follicles of 12-day-old mice, as well as our unpublished experiments. Complexes were cultured for 10 days before maturation and insemination. It was found that the combination of FSH plus insulin was deleterious to oocyte competence to complete preimplantation development [2]. This combination promoted the precocious differentiation of oocyte-associated granulosa cells (OAGCs), suggesting inappropriate differentiation of these cells. This observation was supported by high-resolution two-dimensional polyacrylamide gel analysis showing a large difference in the global pattern of proteins produced by the OAGCs cultured with FSH plus insulin compared with that of either normal cumulus cells or OAGCs cultured under conditions that did not have a deleterious effect on oocyte developmental potential [6]. The original two-step protocol included FSH plus insulin during the culture of oocyte granulosa cell complexes for 14 days (step 2) [1]. However, in experiments not shown here, we found that we could not entirely eliminate FSH plus insulin from the step 2 protocol. When this was attempted, OAGCs did not undergo sufficient proliferation to maintain their necessary association with the oocytes during the early stages of the step 2 culture. It was found by empirical experimentation that FSH, but not insulin, could be omitted during the last 6 days of culture without negatively affecting the association of granulosa cells with oocytes. This avoided the deleterious effects of the FSH plus insulin combination during the key later stages of oocyte development.

The required omission of FSH during these stages of oocyte development in vitro does not suggest that FSH is not important during follicular development in vivo. It is done for practical reasons that do not necessarily attempt to duplicate the hormonal requirements for follicular development in vivo. The microenvironment of the cultured oocyte-granulosa cell complex is dramatically different from that in vivo. In intact follicles, the oocyte-granulosa cell complexes are confined within the follicle wall comprised of mural granulosa cells, a basal lamina, and layers of theca cells. This confinement probably restricts the diffusion of oocyte-derived paracrine factors that are essential for promoting the differentiation of OACGs to the cumulus cell phenotype. When isolated oocyte-granulosa cell complexes are cultured, these factors probably diffuse into the culture medium and do not reach concentrations at the OAGCs sufficient to prevent the differentiation of the mural granulosa cell phenotype and promote the cumulus cell phenotype when stimulated with FSH plus insulin. This ambiguous differentiation of the OAGCs in vitro is probably detrimental to oocyte developmental competence [7–9] and is less severe when FSH is removed during the sensitive stages of oocyte growth.

Waymouth medium MB752, used throughout the original protocol, contains an extraordinarily high glucose concentration, 27.8 mM [10]. However, we subsequently found that a glucose concentration of 5.5 mM for oocyte development in vitro resulted in consistently higher frequencies of cleavage to the two-cell stage and completion of the two-cell stage to blastocyst transition [3]. MEMα has a glucose concentration of 5.5 mM as well as a higher concentration of ascorbic acid (0.25 mM), which was also found to promote more optimal development of the isolated complexes [3] than does Waymouth medium (0.09 mM). Nevertheless, in preliminary experiments (not shown), we found that Waymouth medium supported growth and development of complexes during the early stages of culture better than MEMα, but MEMα was beneficial to the later stages. Consequently, in the revised protocol, after isolation of oocyte-granulosa cell complexes from the organ cultures, they were cultured for 8 days in Waymouth medium, then for 6 days in MEMα (Fig. 1).

In the original 1996 report on oocyte development from primordial follicles in vitro, less than 2% of the two-cell stage embryos developed to the blastocyst stage [1]. Yet, in the experiments described in the present communication, wherein we directly compared the original and revised protocols, almost 15% of the embryos produced using the original protocol completed this transition. Indeed, by almost all criteria, development of oocytes using the original protocol has improved, indicating production of higher-quality oocytes. We have no definitive explanation for this. However, we have continually endeavored to improve the overall environmental conditions in our laboratory to favor improved oocyte and embryo development.

Full oocyte development requires the acquisition of competence to resume and complete the meiotic divisions (nuclear maturation), the accumulation of maternal factor gene products essential for supporting fertilization and early embryogenesis (cytoplasmic maturation), and epigenetic modification of the oocyte genome, including genomic imprinting (epigenetic maturation). Thus, it is truly challenging to achieve full oocyte development in vitro. Our culture system, designed to begin with the primordial follicles of newborn mice, does not include the entire span of oocyte development because the oocytes have completed development to the diplotene stage of meiosis in vivo. A recent study essentially adapted our two-step culture strategy to begin with the primitive gonad, and adjacent mesonephros, isolated from 12.5-day fetuses and achieved remarkable oocyte development [11]. However, the oocytes were unable to resume meiosis. Microsurgical transfer of the germinal

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vesicle from the meiotically incompetent oocytes grown in vitro to the enucleated ooplasm of oocytes grown in vivo resulted in the resumption and completion of nuclear maturation. These oocytes were then fertilized and could develop to yield live offspring [11]. These results dramatically demonstrated that apparently normal epigenetic maturation could occur in vitro—an important achievement. Nevertheless, full oocyte development did not occur in these studies because the oocytes did not acquire competence to resume meiosis, and because the germinal vesicles were removed from the cytoplasm of the in vitro-grown oocytes, it is unknown whether the oocytes were competent to undergo cytoplasmic maturation. In our studies, oocytes grown in vitro completed nuclear, cytoplasmic, and epigenetic maturation, albeit at lower frequencies than oocytes grown in vivo. It will be useful to apply the revised protocols described here to determine whether full oocyte development, beginning before the initiation of meiosis in the fetal gonad, can be achieved.

In our original study, the single living offspring produced, Eggbert, encountered late onset health problems that included obesity and neurological abnormalities [12]. Because only one mouse was produced, it could not be assumed that the health problems were related to oocyte or embryo culture. The 59 offspring produced in this study from oocytes grown in vitro, along with littermate controls, are undergoing extensive prospective analysis of long-term health.

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