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Animal Experimentation

Comparison of Maturation, Fertilization, Development, and Gene Expression of Mouse Oocytes Grown In Vitro and In Vivo

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***Purpose*:** To investigate the difference of in vitro and in vivo grown oocytes, we compared maturation, fertilization, development, and maternal gene expression from both in vitro and in vivo grown mouse oocytes.

***Methods*:** The preantral follicles isolated from 12-day-old mice were cultured on Transwell-COL membrane inserts. After culture, maturation, fertilization, and developmental rates were assessed. RT-PCR (reverse transcription—polymerase chain reaction) was performed to examine the expression of β-actin, GDF-9, and IGF-II in matured oocytes.

***Results*:** No difference in the nuclear maturation was detected between in vitro and in vivo grown oocytes, but the mean oocyte diameter of the in vitro group was smaller than that of the in vivo group. The fertilization rate was significantly lower in the in vitro group than in the in vivo group ($p < 0.05$). The capacities of in vitro grown oocyte to cleave and develop to blastocysts were significantly lower than those of the in vivo grown oocytes ($p < 0.001$). Moreover, blastocyst of in vitro group had fewer total cells than those of in vivo group ($p < 0.05$). In regards to the expression of genes in mature oocytes, growth differentiation factor-9 (GDF-9) expression was similar between the two groups, but β-actin was significantly reduced in the in vitro group compared to the in vivo group. Particularly, the expression of insulin-like growth factor II (IGF-II) was not found in the in vitro grown oocytes.

***Conclusions*:** These results showed that in vitro grown oocytes did not have the same developmental capacity as in vivo grown oocytes. We assume that the aberrant expression of maternal-derived genes in the in vitro grown oocytes may cause the poor embryo viability.

KEY WORDS: Gene expression; in vitro; maturation; mouse; preantral follicle.

INTRODUCTION

There are thousands of oocytes present in the mammalian ovaries. However, most of them undergo degeneration during the growth and maturation, only a very few oocytes become available for ovulation (1). The mammalian oocytes isolated from antral follicles can be matured, fertilized and developed into live young in mice (2), rats (3), sheep (4), cattle (5), pigs (6), and humans (7). However, the mammalian ovaries have only limited number of antral follicles available for the in vitro maturation.

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The ovarian preantral follicles are an enormous and potentially valuable source of oocytes that could be used for clinical, agricultural, and zoological purposes. However, developing an optimal in vitro culture technique to bring the immature oocytes in preantral follicles to mature oocytes is an important prerequisite. To date, many researchers have published their efforts to develop culture techniques and their insights into the metabolism of follicles, production of steroids during the growth, and effects of gonadotrophins and growth factors on the follicular development in mice (8–10), cattle (11), pigs (12), and even humans (13).

Murine species have been widely used to develop the culture system for preantral follicles and to study follicular growth, since they are small, readily available, and grow to Graafian follicle within a short time-span. Particularly, the studies for the culture of mouse preantral follicles have demonstrated that not only the growth and maturation of the oocytes but also fertilization and embryos development were possible (10,14,15). Furthermore, live young were obtained by transferring embryos derived from in vitro grown oocytes (14,15). Earlier studies show that mouse oocytes grown, matured, and fertilized in vitro have limited developmental capacity. However, information on the cause of lower developmental capacity of in vitro grown oocytes is lacking.

Thus, examination of the difference of mouse oocytes grown in vitro and in vivo to verify the cause of lower developmental capacity is necessary. The aim of this study was to examine maturation, fertilization, and developmental rates and expression of maternal genes in the mouse oocytes derived from the in vitro grown preantral follicles, and to compare these findings with those obtained from oocytes grown and matured in vivo.

MATERIALS AND METHODS

Animals

The mice used in this study (ICR) were housed in a temperature- and light-controlled room, under a photoperiod of 12-h light:12-h dark. All experimental protocols and animal handling procedures were reviewed and approved by the Animal Ethics Committee of Eulji General Hospital and Eulji University School of Medicine.

Isolation of Preantral Follicles

The ovaries were aseptically removed from 12-day-old ICR female mice ($n = 30$). They were immersed into Leibovitz L-15 medium containing 1 mg/mL collagenase (Type 1A; C-2674, Sigma, St. Louis, MO) and 0.2 mg/mL DNase I (DN-25, Sigma, St. Louis, MO) for 20 min at 37°C and repeatedly drawn in and out of the pipette until the ovaries were dissociated into individual follicles. A total of 1588 preantral follicles to be cultured were selected by the following criteria: i) intact round follicular structure with two to three layers of granulosa cells; ii) the oocyte had to be visible, round and centrally located within the follicle.

In Vitro Growth and Maturation of Preantral Follicles

The culture medium was αMEM medium supplemented with 5% fetal bovine serum, 100 mIU/mL FSH (Metrodin-HP, Sereno, Aubonne, Switzerland), and 10 mIU/mL LH (L-5259, Sigma, St. Louis, MO). The follicles were cultured on Transwell-COL membrane inserts (3.0-μm pore size, 24.5-mm diameter; Costar, Cambridge, MA) in six-well cluster dishes to prevent the loss of structural integrity between the oocyte and granulosa cells. The follicles were cultured for 10 days at 37°C and 5% CO_2 in air. Half of the medium was changed every 2 days. After 10 days of growth in vitro, follicles were allowed to mature for 16–18 h in medium supplemented with 1.5 IU/mL hCG (Profasi, Sereno, Aubonne, Switzerland).

As a control, in vivo grown oocytes were collected from the ovaries of 22-day-old ICR mice and matured in the same medium in which in vitro grown oocytes were matured.

A part of matured follicles were selected randomly and adherent cumulus cells were removed. The maturation status and the diameter of the oocytes, excluding the zona pellucida, were examined with an inverted microscope and the ocular micrometer.

In Vitro Fertilization

In vitro matured oocyte-granulosa cell complexes were placed into 50-μL drops of Whittingham's T_6 medium supplemented with 30 mg/mL bovine serum albumin (A-3311, Sigma, St. Louis, MO) under mineral oil.

As a control, in vivo matured oocytes were obtained from 23-day-old ICR mice. The mice were superovulated with 5 IU pregnant mare serum gonadotrophin (PMSG) followed 48 h later by 5 IU of human chorionic gonadotrophin (HCG). Fifteen to sixteen hours

after the HCG injection, oocytes were recovered from the oviducts then placed into the drops of fertilization medium.

The sperms collected from the cauda epididymis of mature ICR male mice were incubated for 2 h in fertilization medium before insemination. The oocytes were inseminated with spermatozoa with concentration of 1–2 $\times$ 10^6 per mL. Four to six hours after the insemination, oocytes were washed and cultured for in vitro development.

In Vitro Development

The fertilized oocytes were cultured in 20-μL drops of KSOM medium supplemented with 10% (v/v) serum substitute supplement (SSS, Irvine, Santa Ana, CA), 1% (v/v) essential amino acids (M-7145, Sigma, St. Louis, MO), and 0.5% (v/v) nonessential amino acids (B-6766, Sigma, St. Louis, MO) under mineral oil. The oocytes were examined the following day, and the number of 2-cell embryos was noted. The 2-cell embryos were further cultured for 4 days until the blastocyst stage.

Flourescence Staining

The chromatin configuration of GV (germinal vesicle) oocytes (10 days after culture), the detailed status of fertilized oocytes (6 h after insemination) and the cell number of blastocysts (5 days after insemination) were evaluated by fluoresence staining. The oocytes and blastocysts were fixed in 2% formaldehyde in PBS for 10 min at room temperature. The oocytes and blastocysts were then placed on slides with a drop of mounting medium consisting of the 3:1 ratio of glycerol versus PBS containing 2.5 mg/mL sodium azide and 2.5 μg/mL Hoechst 33342 (B-2261, Sigma, St. Louis, MO). A cover slip was placed on top of the oocytes and blastocysts, and the edge was sealed with fingernail polish. The stained oocytes and blastocysts were examined under a fluorescence microscope.

Assessment of Zona Pellucida Hardening

In vitro and in vivo grown and matured oocytes were treated with 1% hyalruonidase (H-3506, Sigma, St. Louis, MO) and denuded of adherent granulosa cells by drawing them in and out a pasteur pipette and collected in PBS. The time required for the digestion of the zona pellucida by 1 mg/mL α-chymotrypsin (C-4129, Sigma, St. Louis, MO) was used as an indicator of zona hardening as described previously (16). The zona pellucida digestion time is defined as the time required for 50% of the oocytes to become completely zona-pellucida-free as observed under the inverted microscope.

Gene Expression

Mature oocytes were washed in three changes of PBS containing 0.1% PVP and then transferred in a minimal volume of PBS into 300 μl of TRIzol^{TM} Reagent (Gibco BRL, USA.). Total RNA was extracted according to the manufacturer's instructions. Reverse transcription (RT) was performed with total RNA isolated from 30 oocytes by using 1st strand cDNA Synthesis Kit (Boehringer Mannheim, Germany). Polymerase chain reaction (PCR) was conducted on three oocyte equivalents for β-actin, growth differentiation factor-9 (GDF-9), and Insulin-like growth factor-II (IGF-II). The sequences of the primer for PCR (listed in Table I) were designed according to published cDNA sequences. The PCR products were resolved on 2% agarose gels containing 0.5 μg/mL ethidium bromide and visualized with UV transilluminator. The amounts of specific mRNAs were analyzed using an image-analyzing system with a computer software (Vilber Lourmat, France).

Embryo Transfer

Morphologically normal 2-cell embryos that was derived from in vitro growth, maturation, and

Table I. Primers Used for RT-PCR and Product Size

Gene	Primer sequence	Product (bp)
β-actin	5′-GTGGGCCGCTCTAGGCACCAA-3′ 5′-CTCTTTGATGTCACGCACGATTTC-3′	539
GDF-9	5′-GGTTCTATCTGATAGGCGAGG-3′ 5′-GGGGCTGAAGGAGGGAGG-3′	446
IGF-II	5′-GGCCCCGGAGAGACTCTGTGC-3′ 5′-GCCCACGGGGTATCTGGGGAA-3′	255

Table II. Maturation Rates of Oocytes Derived from In Vitro and In Vivo Grown Mouse Preantral Follicles

Source	No. of follicles cultured	No. (%) of oocytes survived	GV (%)	GVBD (%)	Meta II (%)	Oocyte diameter (μm)
In vitro	147	109 (74.1)*	20 (18.3)	10 (9.2)	79 (72.5)	69.6 ± 2.1*
In vivo[a]	105	105 (100)*	18 (17.1)	13 (12.4)	74 (70.5)	73.3 ± 3.0*

Note. GV: Germinal vesicle, GVBD: germinal vesicle breakdown, Meta II: metaphase II.
[a] Oocytes collected from the ovaries of 22-day-old mice and matured in medium containing 1.5 IU/mL hCG for 16–18 h.
* $p < 0.05$.

fertilization were transferred to the oviduct of pseudopregnant female ICR recipients.

Statistical Analysis

The statistical significance of the data was analyzed using a Student's t-test and chi-square (χ^2) test. A statistical significance was established at the $p < 0.05$ level.

RESULTS

Comparison of Maturation Rates

The percentage of the oocytes that had undergone germinal vesicle breakdown (GVBD) and had extruded the first polar body (metaphase II) was determined. As shown in Table II, the percentage of metaphase II oocytes of the in vitro group (72.5%) was not different from that of the in vivo group (70.5%). The oocytes isolated from 12-day-old mice were 56.7 ± 2.5 μm (mean $\pm$ SEM) in diameter, and the mean size had increased during the culture period. However, the mature oocytes of the in vitro group (mean: 69.6 ± 2.1 μm) were smaller than the mature oocytes of equivalent chronological age of the in vivo group (23-days old; mean: 73.3 ± 3.0 μm).

Comparison of Fertilization Rates

As shown in Table III, the fertilization rates of in vitro group (76.5%) was significantly lower ($p < 0.05$) than that of in vivo group (90.2%). The percentage of normal monospermic oocytes in the fertilized oocytes was not different between the two groups (84.6 vs. 85.5%). The percentage of polyspermic oocytes was also similar between the two groups (15.4 vs. 14.5%).

Table III. Fertilization Rates of Mouse Oocytes Grown and Matured In Vitro and In Vivo

Source	No. of oocytes examined	No.(%) of oocytes fertilized		
		Total	Monospermic	Polyspermic
In vitro	85	65 (76.5)*	55 (84.6)	10 (15.4)
In vivo	92	83 (90.2)*	71 (85.5)	12 (14.5)

* $p < 0.05$.

Comparison of Developmental Rates

The embryos cleaved to the 2-cell stage after insemination, as shown in Table IV, in vitro group (57.8%) were significantly lower ($p < 0.001$) than those in vivo group (84.4%). When the embryos were examined 5 days after insemination, 14.4% of the in vitro grown oocytes developed to the blastocyst stage. It was significantly less ($p < 0.001$) than that of the in vivo grown oocytes (56.6%). As shown in Table V, the total cell number of the blastocysts derived from the in vitro grown oocytes (39.0 ± 10.8) were significantly lower ($p < 0.05$) than those of blastocysts derived from the in vivo grown oocytes (60.5 ± 12.5).

Comparison of Chromatin Configuration

On day 10 of the in vitro culture, most of the in vitro grown oocytes were progressed from NSN (meiotic incompetent GV oocyte; nucleolus is not surrounded by chromatin) to SN (meiotic competent GV oocyte; nucleolus is surrounded by chromatin) pattern (Fig. 1). The percentage of SN pattern in the in vivo grown oocytes was somewhat less than that of the in vitro grown oocytes, but there was no statistical difference between these two groups (Fig. 2)

Comparison of Zona Pellucida Hardening

As shown in Fig. 3, the time required for digestion of the zona pellucida of in vitro grown and matured oocytes (11.7 ± 0.8 min) was longer than that of in vitro matured oocytes (8.0 ± 1.3 min) and in vivo-matured oocytes (6.0 ± 1.2 min). Particularly, the zona pellucida digestion time of oocytes grown in vitro were twice longer compared to that of freshly ovulated mature oocytes grown in vivo.

Table IV. Developmental Rates of Mouse Oocytes Grown and Matured In Vitro and In Vivo After In Vitro Fertilization

Source	No. of oocytes examined	No. (%) of embryos developed to			
		2-Cell	4-Cell	Morular	Blastocyst
In vitro	232	134 (57.8)**	96 (41.4)**	62 (26.7)**	33 (14.4)**
In vivo	205	173 (84.4)**	162 (79.0)**	133 (64.9)**	116 (56.6)**

** $p < 0.001$.

Comparison of the Expressions of Maternal Genes

To compare the difference of the expressions of specific genes in mature oocytes the expressions of growth differentiation factor-9 (GDF-9), β-actin and insulin-like growth factor-II (IGF-II) mRNA in the in vitro and in vivo grown oocytes were studies with RT-PCR. The expression level of GDF-9 mRNA was not different between the two groups. The expression level of β-actin mRNA was significantly lower in the in vitro grown oocyte when compared with the in vivo counterpart. IGF-II mRNA was expressed in the in vivo grown oocyte, but not in the in vitro grown oocytes (Figs. 4 and 5).

Embryo Transfers

The result of embryo transfer is shown in Table VI. After fertilization of oocytes derived from the in vitro growth and maturation, One-hundred eighty one 2-cell embryos were transferred to the oviducts of two pseudopregnant recipients (15–38 embryos per recipient), and two recipients became pregnant. Six live pups (3.3%) were born: three males and three females. These pups appeared to be normal and proved to be fertile after puberty.

DISCUSSION

It has been reported that oocytes grown and matured in vitro have reduced capacity to be fertilized (15) and higher rate of abnormal fertilization (17) as compare with their in vivo counterparts. In this experiment, we confirmed that oocytes grown and matured in vitro has a lower rate of fertilization than oocytes grown and matured in vivo. This phenomenon is probably due to incomplete cytoplasmic maturation of oocytes and the failure of the activation mechanism development triggered by sperm penetration (17). It is assumed that long-term in vitro culture results hardening of the zona pellucida, thereby prevents the penetration of the sperm into the oocyte. This assumption is supported by the observation that the time required for digestion of zona pellucida of oocytes grown and matured in vitro, using α-chymotrypsin, was longer than that of in vivo grown oocytes.

Table V. Number of Cells Per Blastocyst Derived from In Vitro and In Vivo Grown Oocytes

Source	No. of blastocysts examined	No. of cells (Mean ± SEM)	Range
In vitro	35	39.0 ± 10.8*	21–61
In vivo	32	60.5 ± 12.5*	42–85

* $p < 0.05$.

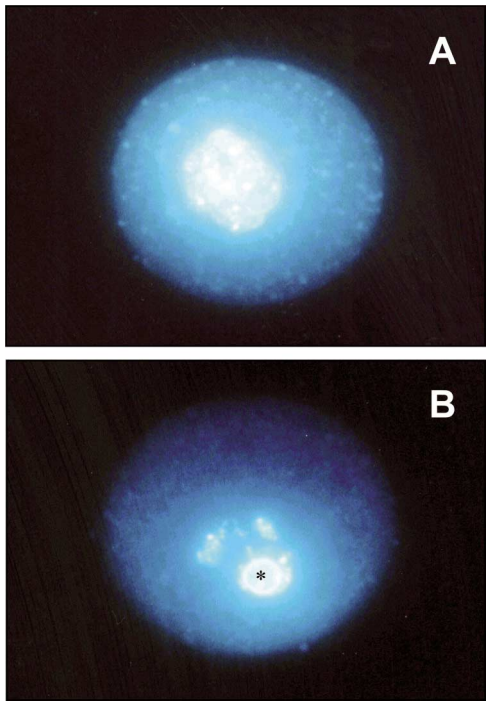

Fig. 1. Fluorescent microscopy of chromatin configuration in mouse GV oocyte. A: Meiotic incompetent oocyte (NSN type; nucleolus is not surrounded by chromatin). B: Meiotic competent oocyte (SN type; nucleolus is surrounded by chromatin). Asterisk indicates Hoechst-positive ring of chromatin surrounding the nucleolus.

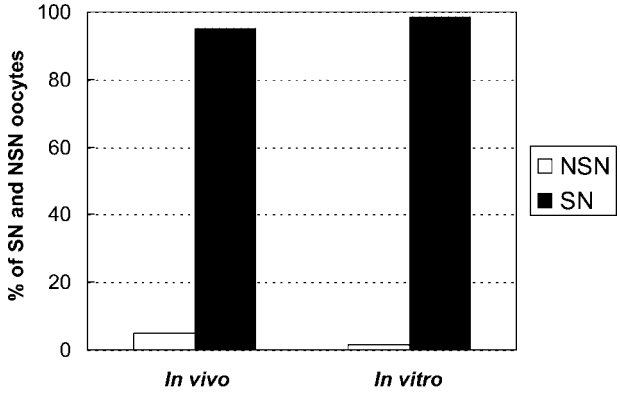

Fig. 2. Comparison of chromatin configuration in the in vitro and in vivo grown mouse oocytes. SN, surrounded nucleolus (meiotic competent GV oocyte; NSN, nonsurrounded nucleolus (meiotic incompetent GV oocyte).

According to the previous studies about the developmental capacity of oocytes grown and matured in vitro, less than 50% of fertilized oocytes underwent development to the blastocyst stage (14,15). In this experiment, the rate of development to blastocysts is only 12%. The poorer rate of blastocyst development could be explained by three factors. The first factor is the difference in the mouse strain used. We used the ICR strain for experiment while many other researchers had used F_1 hybrid strain. Generally, ICR strain is sensitive to a cell block in which the development stops at the 2-cell stage, while F_1 hybrid strain is not susceptible to this phenomenon (18). The second explanation is the difference of O_2 concentration in culture condition. The concentration of O_2 is an important factor to culture preantral follicles. It is known that concentration of 20% O_2 is an optimal concentration for the culture of intact preantral follicles

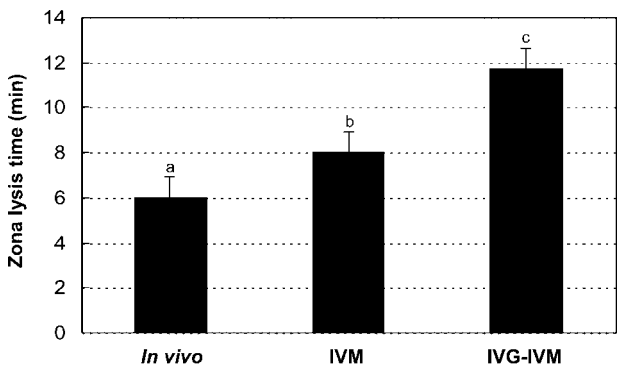

Fig. 3. Comparison of the zona hardening of the in vivo matured (in vivo), in vitro matured (IVM) and in vitro grown and matured (IVG-IVM) mouse oocytes. [a,b,c]Different superscripts above columns donate significant differences ($p < 0.05$).

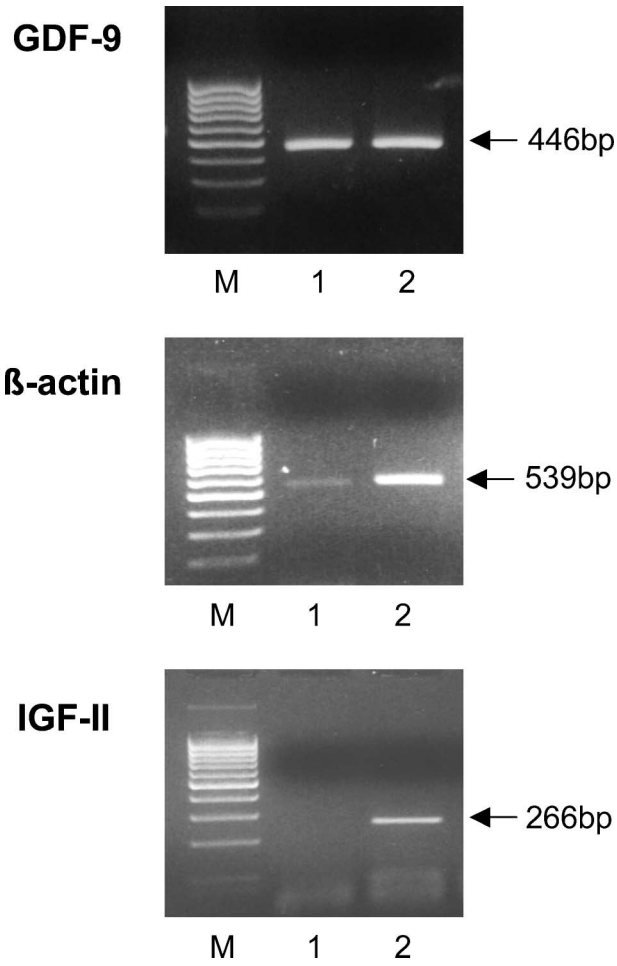

Fig. 4. Representative gels of RT-PCR analysis of GDF-9, ß-actin, and IGF-II gene transcripts. M: molecular size marker; lane 1: in vitro grown and matured oocyte; lane 2: in vivo grown and matured oocyte.

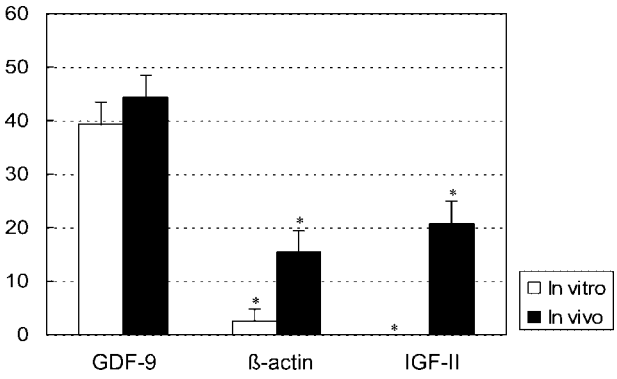

Fig. 5. Comparison of the expression patterns of GDF-9, ß-actin, and IGF-II gene transcripts in oocyte grown and matured in vitro and in vivo. Asterisks above columns denote significant differences ($p < 0.05$).

Table VI. Result of Transfer of Embryos Derived from the In Vitro Grown, Matured, and Fertilized Oocytes

No. of Recipient	No. of embryos transferred	No. of Pregnant	No.(%) of offsprings	
8	181	2	6 (3.3)	M: 3, F: 3

Note. M: Male, F: female.

isolated by manual dissection (19). On the contrary, for preantral follicles isolated by enzyme treatment, 5% O_2 is believed as an optimal concentration because partial damage of theca cells and basal lamina cause more oxidative stress. Eppig and Wigglesworth (20) reported that 20% O_2 concentration resulted lower survival and these oocytes could not develop to blastocysts after fertilization. Finally, we did not add EGF in maturation medium. It is known that EGF promotes nuclear and cytoplasmic maturation of oocytes. Previous studies for the preantral follicle culture showed impressive beneficial effects of EGF, combined with FSH or LH, on maturation, fertilization, and development to blastocysts (9,15).

The diameter of oocytes is an important index for the cytoplasmic maturation and developmental competency of oocytes. In our experiment, none of the in vitro grown oocytes grew as large as the oocytes isolated from the antral follicles of 23-day-old mice, which were at the same chronological age as the in vitro grown oocytes isolated 12 days of age and cultured for 11 days. Thus, in vitro grown oocytes, under these conditions, were not equivalent in size to the in vivo grown oocytes. This result is very similar to other studies (14,17).

Initially, all oocytes in preantral follicles are in the NSN state, but while growing they either continue to develop to the NSN configuration or are shifted into the SN configuration (21). The transition of GV oocytes from the NSN to SN configuration is important to acquire the meiotic competency to resume the first meiosis (22). Hartshorne *et al*. (23) reported that in cultured mouse ovarian follicles, the chromatin organization in the oocyte normally changed from the NSN to SN configuration. This study demonstrated that the transition rate of oocyte chromatin organization from NSN to SN in the in vitro grown oocyte was similar to that of the in vivo grown oocytes at the same chronological age (23-day old). Thus, this study confirmed that in vitro grown oocytes may normally acquire the meiotic competence to resume the first meiotic division during the culture period.

During the oocyte growth, gene expression is highly active in order to accumulate the maternal products that are required, after fertilization, to sustain the first step of the embryonic development (24). Several experiments have shown that maternal factors are able to modify developmental ability (25) and gene expression in the zygote (26).

β-actin is a housekeeping gene, which is a key component of the cytoskeleton. In this study, the expression of β-actin mRNA in the in vitro grown oocyte group was significantly lower compared to the in vivo group. GDF-9 is an oocyte-specific factor and a member of the transforming growth factor-β (TGFβ) superfamily that is expressed at the high level in mammalian oocytes beginning from the type 3a primary follicles (one-layer growing primary follicles) but not in oocytes of quiescent primordial follicles (27). The GDF-9 knockout mice showed an arrest of follicle development beyond the type 3b stage (one-layer primary follicle stage) (28). In addition, this mice showed the defect in oocyte meiotic competence, including abnormal GVBD, and spontaneous parthenogenetic activation of the oocytes (29). Moreover, it reported that GDF-9 is an important factor for the growth and differentiation of cultured early ovarian follicles (30). In our study, there was no difference in the expression of GDF-9 mRNA between in vitro and in vivo grown oocyte.

IGF-II has been known to stimulate the growth and metabolism of early embryos (31). It is also an important survival factor that protect a range of cell types from apoptosis (32). The report of Stojanov *et al.* (33) showed that IGF-II was present in the oocyte and early zygote as maternal transcripts, and was rapidly lost after fertilization. This transcript was not detected by the early 2-cell stage, but detected again in the late 2-cell stage. Our study demonstrated that IGF-II mRNA was not present in the in vitro grown oocyte, whereas there was IGF-II mRNA expression in the in vivo grown oocyte. The specific gene expression patterns in this study suggest that the aberrations in the expression of specific genes in the in vitro grown oocyte may cause the poor embryo viability.

There were several reports of production of live young after transfer of embryos derived from the in vitro grown, matured, and fertilized oocytes. However, only about 5% of the transferred embryos developed to the live young (14,15). We also demonstrated the production of live young from oocytes grown in vitro from preantral follicles which were fertilized and transferred at the 2-cell embryo stage. The rate of live young produced in our laboratory was 3.3%. The low birth rate may be a consequence of the compromised developmental capacity of the in vitro grown oocytes.

Of note, the total cell number is regarded as an important index for developmental capacity of the

blastocyst. We could also speculate that insufficient cytoplasmic maturation and aberrant gene expressions of the in vitro grown oocyte caused the smaller cell number of the blastocyst. Further studies are needed to improve the culture system for preantral follicles and to verify the cause of the lower developmental capacity of in vitro grown oocytes.

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