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Premeiotic fetal murine germ cells cultured in vitro form typical oocyte-like cells but do not progress through meiosis

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Abstract

A convenient method for fetal murine premeiotic germ cells to develop into oocytes in vitro has been established. Fetal ovaries from mice, collected 12.5 d postcoitus (dpc), were organ-cultured in vitro using a medium for organ growth, and the developmental potential regarding oocyte formation was determined. After 28 d of culture, premeiotic female germ cells developed into oocytes with a mean (±SD) diameter of 73.3 ± 7.7 μm. However, follicles developed in vitro versus in vivo had fewer granulosa cells (32 ± 2.6 vs. 142 ± 9.5, respectively; $P < 0.01$), and the ovaries had less mRNA for *Cx37* and *Cx43* ($P < 0.01$). Oocytes in the first meiotic division phase were isolated from cultured ovaries or after hormone treatments. After exposure to okadaic acid at a final concentration of 1 μM, oocytes derived from premeiotic fetal female germ cells were able to undergo germinal vesicle breakdown but failed to complete the first meiotic division. Furthermore, the intracellular content of GSH in oocytes cultured in vitro was lower than that of oocytes matured in vivo ($P < 0.01$). In conclusion, premeiotic germ cells derived from murine fetuses as early as 12.5 dpc were able to differentiate into germinal vesicle–stage oocytes but were unable to complete meiosis I in vitro.

Keywords: Meiosis; Mouse; Oocyte; Oogenesis; Premeiotic germ cells

1. Introduction

Mammalian oogenesis, a critical process during mammalian development, has been studied extensively over the past two decades, through in vitro culturing of single follicles, ovarian tissues, or granulosa cell–free oocytes [1–14]. In vitro development of murine oocytes from premeiotic fetal germ cells has been attempted. Earlier studies by Chuma and Nakatsuji have shown that in vitro–cultured oocytes derived from 10.5 to 11.5 d postcoitus (dpc) fetal germ cells only underwent the first phase of meiotic transition, up to the leptotene stage [8]. Klinger and de Felici developed a multistep in vitro culture system to generate fetal murine oocytes [9]. In their studies, 50-μm oocytes were formed through direct stimulation of fetal germ cells with Kit ligand (KITL) or by coculturing them with granulosa cells [9]. Recently, Obata et al. and Niwa et al. reported that 12.5 dpc fetal murine ovaries could be cultured for 28 d in vitro, with oocytes reaching 63.9 μm in diameter [10,15]. However,

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it has been difficult to develop meiotically mature oocytes from fetal murine germ cells in vitro, due to poor understanding of the regulation of oogenesis [4,8–10,16]. In our previous studies, we established a new method of inducing premeiotic germ cells to differentiate and develop into mature oocytes using a sequential in vitro culture and in vivo transplantation procedure [14]. More recently, we demonstrated that the 16.5 dpc fetal murine germ cells were able to form primordial follicles by coaggregating with ovarian pre–granulosa cells in vitro, and the oocytes from the growing follicles were able to mature in vitro and develop into embryos [12]. However, the mechanisms regulating premeiotic fetal germ cell differentiation during in vitro oogenesis, and the contributions of intercommunications between oocytes and granulosa cells to such a process, remain enigmatic and warrant further investigations [16]. In this study, we investigated the developmental competence of murine germ cells from 12.5 dpc fetal ovaries to differentiate into mature oocytes under in vitro culture conditions.

2. Materials and methods

2.1. Isolation and culture of fetal ovaries

The Institutional Animal Care and Use Committees of Qingdao Agricultural University approved all animal procedures. Fetal ovaries were obtained 12.5 dpc from pregnant CD-1 mice (Vital River, Beijing, China), based on the presence of a copulation plug (0.5 dpc). The fetal ovaries, without attached mesonephroses, were selected and cultured in 300 μL tissue-specific medium in a 24-well plate (BD Biosciences, Bedford, MA, USA). Fetal ovaries on 24-well plates were cultured at 37 °C in modular incubation chambers (Billups Rothenberg, Del Mar, CA, USA), thoroughly infused with a gas mixture of 5% CO_2 and air. The next day, the medium was increased to 600 μL, and the depleted medium was refreshed by exchanging with 300 μL of fresh medium every other day [14].

The day at which isolated ovaries were cultured in vitro was defined as Day 0. Fetal ovaries were harvested and subsequently cultured in Medium A for 14 d, Medium B for 8 d, and Medium C for 6 d. Medium A consisted of a 1:1 combination of DMEM/F12 and α-minimal essential medium (α-MEM; Gibco-BRL, Carlsbad, CA, USA), supplemented with 10% heat-inactivated fetal calf serum (FCS; Gibco-BRL), 0.23 mM pyruvic acid, 100 U/mL penicillin G, and 100 mg/mL streptomycin sulfate. Medium B consisted of DMEM/F12 and α-MEM (1:1), supplemented with 3 mg/mL BSA, 1 mg/mL fetuin, 1% insulin-transferrin-selenium mix (ITS-mix; 1 mg/mL, 0.55 mg/mL, and 5 ng/mL, respectively; Gibco-BRL), 5 mIU/mL follicle-stimulating hormone (FSH), 1 ng/mL recombinant epidermal growth factor (rEGF; Sigma, St. Louis, MO, USA), 0.23 mM pyruvic acid, 100 U/mL penicillin G, and 100 mg/mL streptomycin sulfate. Medium C consisted of α-MEM, 3 mg/mL BSA, 1 mg/mL fetuin, 1% ITS mix, 0.23 mM pyruvic acid, 100 U/mL penicillin G, and 100 mg/mL streptomycin sulfate [12–14,17–19].

2.2. Evaluation of germ cells undergoing meiotic prophase I

Female germ cells were isolated from 12.5, 13.5, and 14.5 dpc CD-1 fetal genital ridges after the ethylenediamine tetraacetic acid (EDTA)-puncturing method [14]. The cells were placed directly in phosphate-buffered saline (PBS) medium and dispersed on 4-well plates (BD Biosciences) coated with poly-L-lysine (Sigma). To check whether the isolated germ cells entered into meiosis, germ cells were stained with anti-SCP3 antibody (Santa Cruz Biotechnology Inc., Santa Cruz, CA, USA). Cells were fixed and treated as described [8]. Briefly, after fixation, cells were incubated overnight at 4 °C with a 1:200 dilution of the anti-SCP3 antibodies, and TRITC-conjugated anti-rabbit IgG was used as the secondary antibody. The meiotic prophase stages were determined by characteristic patterns of SCP3 immunostaining within germ cells [14].

2.3. Gene expression in follicles

Samples of RNA (300 ng) isolated from fetal ovaries (n = 5) at various in vitro developmental stages were used for cDNA synthesis through reverse transcription, according to the manufacturer's protocol (TaKaRa, Dalian, China). An aliquot (1 μL) of the RT reaction products was used in a 25-μL PCR reaction with gene-specific primers. The following genes were amplified (Table 1): the synaptonemal complex protein 3 (*Scp3*); the murine homologue of the yeast meiosis-specific homologous recombination gene (*Mvh*); disrupted meiotic cDNA 1 homologue (*Dmc1*); growth differentiation factor 9 (*Gdf9*); zona pellucida proteins, *Zp1-3*; and gap junction genes *Cx37* and *Cx43*. For these analyses, *Gapdh* was used as a positive control for amplification. The PCR conditions were as follows: 30 s at 94 °C, 30 s at 60 °C, and 1 min at 72 °C (25 cycles) [12–14].

Quantification of *Cx43* and *Cx37* mRNA was conducted using an ABI 7500 real-time PCR instrument (Applied Biosystems, Foster City, CA, USA). The PCR primer sequences (Table 1) were designed using Primer

Table 1
Primers used for RT-PCR amplification of cDNA fragments of ovary-specific genes derived from murine ovaries.

Gene	Primers	Annealing temperature (°C)	Fragment size (bp)
Scp3	Upper: 5′-ATG ATG GAA ACT CAG CAG CAA GAG A-3′ Lower: 5′-TTG ACA CAA TCG TGG AGA GAA CAA C-3′	65	325
Dmc1	Upper: 5′-GGA CAT TGC TGA CCG CTT CAA CGT-3′ Lower: 5′-GGC GAT CCT CAG TTC TCC TCT TCC-3′	63	427
Gdf9	Upper: 5′-CCA GCA GAA GTC ACC TCT ACA A-3′ Lower: 5′-ACA TGG CCT CCT TTA CCA CA-3′	61	240
Zp1	Upper: 5′-CTG AGG ATT GCC ACG GAT AA-3′ Lower: 5′-GGA GTC AAG GAG CAT GAA GGT-3′	60	324
Zp2	Upper: 5′-GCT ACA CAC ATG ACT CTC AC-3′ Lower: 5′-GGT GAC TCA CAG TGG CAC TC-3′	60	380
Zp3	Upper: 5′-TTG AGC AGA AGC AGT CCA GC-3′ Lower: 5′-CGG TTG CCT TGT GGA TGG TC-3′	60	441
Mvh	Upper: 5′-GGT CCA AAA GTG ACA TAT ATA CCC-3′ Lower: 5′-TTG GTT GAT CAG TTC TCG AGT-3′	63	420
Cx37	Upper: 5′-AAG AGC GGT TGC GGC AGA AAG AGG G-3′ Lower: 5′-GCA GGT TGA GCA CCA GGG AGA TGA C-3′	65	324
Cx43	Upper: 5′-TGT GGG CA AGA CAC GAA TAT G-3′ Lower: 5′-GAC AAG GTC CAA GCC TAC TCC C-3′	63	221
LHR	Upper: 5′-AAT CTC TCC TTT GCA GAC TTT TG-3′ Lower: 5′-AGC ATA GGT GAT GGT GTG CCA-3′	60	216
Actin	Upper: 5′-TCG TGG GCC GCT CTA GGC AC-3′ Lower: 5′-TGG CCT TAG GGT TCA GGG GG-3′	60	243
Gapdh	Upper: 5′-GTC ATT GAG AGC AAT GCC AG-3′ Lower: 5′-GTG TTC CTA CCC CCA ATG TG-3′	60	215

Express software (Applied Biosystems). Amplification reactions were performed in 25-μL reaction volume containing 1 μL cDNA, 12.5 μL of 2 × SYBR green master mix (TaKaRa, Dalian, China), 9.5 μL RNase-free water, and 1 μL each of forward and reverse gene specific primers (5 μM), according to the manufacturer's protocol. Cycle amplification conditions were as follows: 95 °C for 10 min followed by 40 cycles of 95 °C for 15 sec and 60 °C for 1 min. All data were normalized to the median. The average and standard errors were calculated from triplicate measurements. All experiments were repeated 3 times, and values shown are mean ± SEM.

2.4. Immunohistochemistry of LHR

Portions of fetal ovary were blocked in PBS with 10% goat serum and 0.1% Triton X-100 for 30 min at room temperature before incubating with primary antibodies. Ovarian tissues were incubated overnight at 4 °C with antibodies directed against LHR (1:500; Boster, Wuhan, China). After 3 washes in PBS, these tissues were incubated for 30 min at room temperature with biotinylated anti-rabbit secondary antibody at a dilution of 1: 500 (Beyotime, Haimen, China), washed 3 times in PBS for 5 min each, and then incubated with DAPI (Beyotime) for 5 min at room temperature. Negative controls, omitting the primary antisera, were included in each experiment.

2.5. Conditioned medium collected from follicles cultured in vitro

Primary or early secondary follicles from 8-d-old CD-1 mice were cultured in vitro for 14 d in 10-cm culture dishes (BD Biosciences) containing 10 μL culture droplets ($n = 60$) that were covered with 10 mL mineral oil (Sigma). Medium for murine follicle culture consisted of α-MEM supplemented with 5% heat-inactivated FCS, 100 mIU/mL FSH, 10 mIU/mL luteinizing hormone (LH; Sigma), and ITS mix. The plate was preincubated overnight at 37 °C, 100% humidity, and 5% CO_2 in air. Selected follicles were washed and placed individually in the culture droplets. After culture for 4 d, 10 μL fresh medium was added to each droplet. From Day 6 onward, half of the medium was collected and replaced with fresh medium. The collected medium was filtered and used immediately or stored at −20 °C [13,14].

2.6. Maturation of oocytes in vitro

Following 28 d of culturing in vitro, oocytes derived from 12.5 dpc fetal murine ovaries were transferred to maturation medium and incubated for 16 to 18 h.

Maturation medium consisted of α-MEM with 3 mg/mL BSA, 1 mg/mL fetuin, 100 mIU/mL FSH, 10 mIU/mL LH, 1 ng/mL EGF, 0.23 mM pyruvic acid, 100 U/mL penicillin G, and 100 mg/mL streptomycin sulfate. Oocytes were collected at germinal vesicle (GV), germinal vesicle breakdown (GVBD), and MII stages, respectively.

In Experimental Group I, murine oocytes derived from premeiotic germ cells in vitro were induced to mature by adding okadaic acid (OA; Sigma) to a final concentration of 1 μM [9,20]. After OA treatment for 3 h, oocytes were washed and further cultured for 16 to 18 h in mature medium (under the conditions described earlier).

In Experimental Group II, from Day 22 of ovary organ culture onward, murine ovaries were cultured for 6 d in conditioned medium that consisted of 80% maturation medium and 20% collected follicle medium, as described earlier.

In the Control group, oocytes denuded of cumulus cells (DOs) were isolated from large antral follicles within ovaries of 21-d-old CD-1 mice and matured in maturation medium or the conditioned medium as described earlier for Experimental Groups I and II.

2.7. Assessment of steroid production in vitro

During in vitro culturing of ovaries, 3 mL medium was collected from 10 wells of 24-well plates and stored at −20 °C pending hormone analysis. Concentrations of estradiol (E2) in the media collected from ovary cultures were measured using a sensitive (<4 pg/L) and reproducible RIA assay system (from Clinical Assays; total coefficient of variation [CV], <10%; Sorin Fueter, Brussels, Belgium) with an E2 measurement range from 4 to 500 ng/L. The assays were performed every 4 d to validate the E2 content in the culture medium [13,21]. The measurements were repeated on three occasions. Primary or early secondary follicles from 8-d-old CD-1 mice were cultured in vitro for 14 d, as described earlier, and the medium was collected as a control.

2.8. Assay for intracellular GSH

The intracellular content of GSH, the level of glutathione, was measured as described by Ge et al. [22]. Cumulus-free oocytes were washed 3 times in FHM. Distilled water (5 μL) containing 50 oocytes was transferred to a 1.5-mL microfuge tube, and then 5 μL of 1.25 M phosphoric acid was added to the tube. Samples were frozen at −80 °C and thawed at room temperature. This procedure was repeated 5 times. The samples were stored at −80 °C until analyzed. Concentrations of GSH in the oocyte were determined by the 5,5′-dithiobis(2-nitrobenzoic acid) (DTNB)-oxidized GSH reductase-recycling assay, according to the manufacturer's protocol (Beyotime). Absorbance was monitored continuously at 405 nm with a spectrophotometer for 25 min, with readings recorded every 5 min. Standards (0.5, 1.0, 2.0, 5.0, 10.0, and 15.0 μM) of GSH and a sample without GSH were also assayed. The amount of GSH in each sample was divided by the number of oocytes to determine the intracellular GSH concentration per oocyte. All experiments were repeated 6 times, and values shown are the mean ± SEM.

2.9. Western blot analysis

Lysate from 300 oocytes were collected in SDS sample buffer and heated to 100 °C for 4 min. After cooling on ice and centrifuging at 10,000 × *g* for 4 min, samples were frozen at −20 °C until used. Total proteins were separated by sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS-PAGE) with a 4% stacking gel and a 10% separating gel for 20 min at 60 V and 3.5 h at 120 V, respectively, and then electrophoretically transferred onto nitrocellulose membranes for 2 h at 200 mA at 4 °C. The membrane was then blocked overnight at 4 °C in TBST buffer (20 mM Tris, 137 mM NaCl, 0.1% Tween-20, pH 7.4) containing 5% low-fat milk. To detect pERK1/2, blots were cut according to the 68-kDa molecular weight marker and were incubated for 2 h in TBST with 1:500 murine anti-p-ERK1/2 antibodies (Santa Cruz). After 3 washes for 10 min each in TBST, the parts of the membrane were incubated for 1 h at 37 °C with horseradish peroxidase (HRP)-conjugated rabbit anti-mouse IgG diluted 1:1000 in TBST. Membranes were washed 3 times in TBST and then processed using the enhanced chemiluminescence (ECL) detection system (Beyotime). To re-probe total ERK2, the part of the membrane was washed in stripping buffer (100 mM b-mercaptoethanol, 20% SDS, and 62.5 mM Tris, pH 6.7) to strip off bound antibody after ECL detection at 50 °C for 30 min. The membrane was re-probed with polyclonal rabbit anti-ERK2 antibody (Santa Cruz) diluted 1:300, incubated with HRP-labeled goat anti-rabbit IgG diluted 1:1000, and finally processed as described above. All experiments were repeated at least 3 times [23,24].

2.10. Statistical methods

After arcsine transformation, the rates of fetal murine ovary survival were analyzed by ANOVA and

differences located with Tukey's test. The diameter of oocyte and follicle, number of granulosa cells, relative mRNA level of genes, GSH and estradiol concentrations according to each developmental stage of murine oocyte in vitro or in vivo were analyzed by ANOVA and differences located with Tukey's test. All analyses were done with the Statistical Analysis System [25].

3. Results

3.1. Oocytes generated from 12.5 dpc premeiotic murine fetal germ cells

We analyzed the expression of SCP3 in germ cells from 12.5, 13.5, and 14.5 dpc murine ovaries by immunostaining. The SCP3 protein was detected in female germ cells of 13.5 and 14.5 dpc embryos but not 12.5 dpc embryos, indicating that the female germ cells of 12.5 dpc fetal ovaries were in the premeiotic stage (Fig. 1).

Murine 12.5 dpc fetal ovaries were cultured in vitro. After 2 d of culture, the ovary grew into a thin layer (Fig. 2A). On Day 6 (start of culture = Day 0), round germ cells and primary oocytes started to appear within the ovary (Fig. 2B). On Day 10, a typical primordial follicle structure, which consisted of an oocyte surrounded by flattened pre–granulosa cells, was observed within the cultured murine ovaries (Fig. 2C). On Day 14, the murine oocytes grew rapidly and started to clearly display follicle morphology. The follicles consisted of a 35- to 40-μm oocyte, surrounded by one or two layers of granulosa cells (Fig. 2D). On Day 18, the ovarian oocytes were 60 μm and the germinal vesicle structure was formed (Fig. 2E). On Day 22, the oocytes reached 60 to 70 μm and were surrounded by multiple layers of granulosa cells. A follicular structure was evident. These granulosa cells slowly proliferated into two or three layers, without the formation of an antral-like cavity (Fig. 2F). On Day 26, the oocytes grew to more than 70 μm. The follicular structures became diffused (Fig. 2G). On Day 28, the mean diameter of oocytes was 73.3 ± 7.7 μm, with the largest oocyte measured 85 μm (Fig. 2H). Oocytes derived from cultured fetal murine ovaries of 12.5 dpc were able to dissociate from the ovarian tissue spontaneously or after stimulation with FSH and LH, and they were round and had a typical zona pellucida and germinal vesicle structure (Fig. 2I).

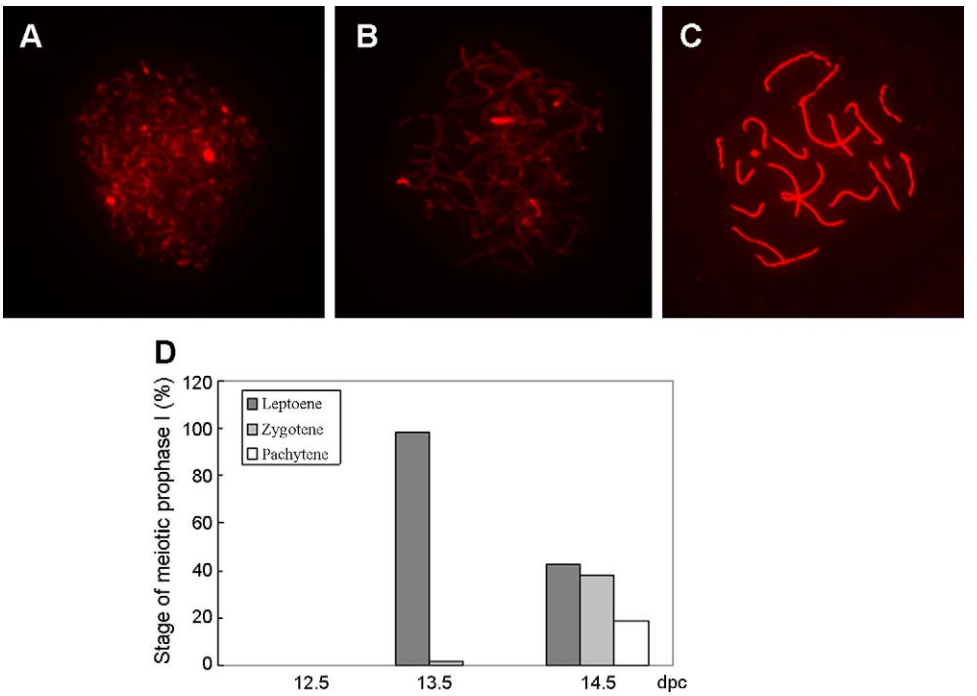

Fig. 1. Evaluation of murine germ cells entering into progression through meiotic prophase I. (A) The preleptotene/leptotene stage of meiotic prophase in cultured 13.5 dpc female germ cells was identified by immunostaining patterns (fluorescent dots) with anti-SCP3 antibody. (B, C) Zygotene and pachytene stages of meiotic prophase in 14.5 dpc female germ cells were identified by diffuse immunofluorescence staining with anti-SCP3 antibody. (D) Percentages of germ cells in various meiotic stages in 12.5, 13.5, and 14.5 dpc fetal murine ovaries.

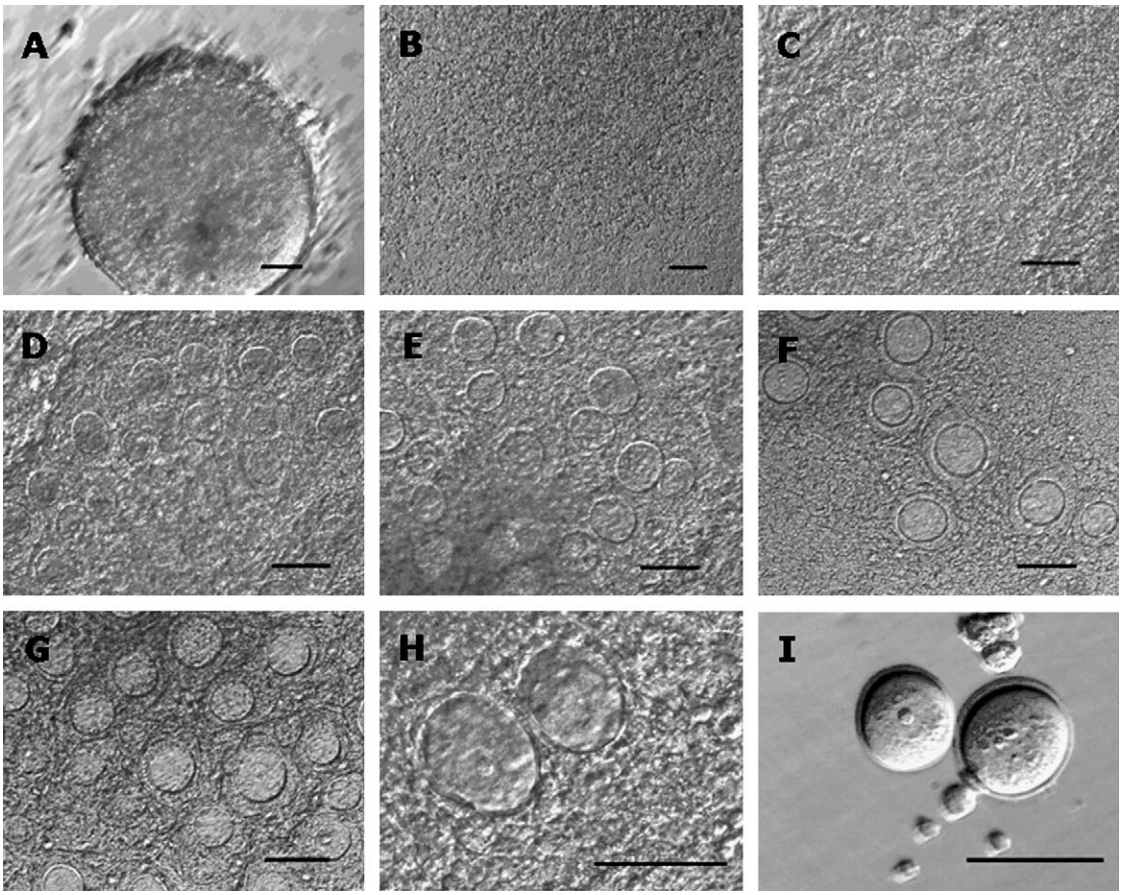

Fig. 2. Generating developed oocytes derived from premeiotic female fetal germ cells of 12.5 dpc in vitro. (A–H) Developed ovarian oocytes derived from 12.5 dpc fetal murine ovaries cultured in vitro for 2, 6, 10, 14, 18, 22, 26, and 28 d. (I) Representative oocytes, with typical germinal vesicle and zona pellucida from cultured fetal murine ovaries. Scale bars: (A–G) 50 μm; (H, I) 100 μm.

3.2. Developmental characterization of oocytes derived from premeiotic germ cells

The experimental group contained a total of 211 cultured fetal ovaries; of these, 165 survived after 28 d of culture. The mean diameter of the growing oocytes within the cultured ovaries is shown (Fig. 3A). Overall, 50 to 100 oocytes with a diameter >65 μm could be obtained from an ovary. The mean diameter of oocytes (n = 319) in the experimental group at Day 28 was 73.3 ± 7.7 μm; oocytes from 21-d-old control mice were 84.6 ± 4.4 μm (n = 170). The diameter of the follicle differed ($P < 0.01$) between the experimental group and the control group at Day 14 of fetal murine ovary culture (Fig. 3B). The mean diameter of the follicles in the experimental group (n = 157, not including primordial follicles) at Day 22 was 91.4 ± 5.9 μm, whereas the mean diameter of the follicles in the control group (n = 112, not including primordial follicles) at Day 15 (after birth) was 144.2 ± 13.1 μm.

We counted granulosa cells, as well as the layers of granulosa cells surrounding the oocyte (Fig. 3C, D). The average number of the follicular granulosa cells (not including primordial follicles) in the experimental group (n = 134) at Day 22 was 32 ± 2.6, with an average of 1.6 ± 0.2 layers. The control group (n = 87) from 15-d-old mice had an average of 142 ± 9.5 granulosa cells and 3.4 ± 0.8 layers, respectively. The numbers of follicular granulosa cells differed between the two groups ($P < 0.01$).

3.3. Gene expression in oocytes derived from premeiotic germ cells

As shown in Fig. 4A, the expression of ovarian-specific genes was detectable in the cultured ovaries at Day 4 (time equivalent to 16.5 dpc murine ovary in vivo). The gene expression pattern in the cultured ovaries was similar to those of in vivo ovaries at corresponding stages. We further examined (RT-PCR)

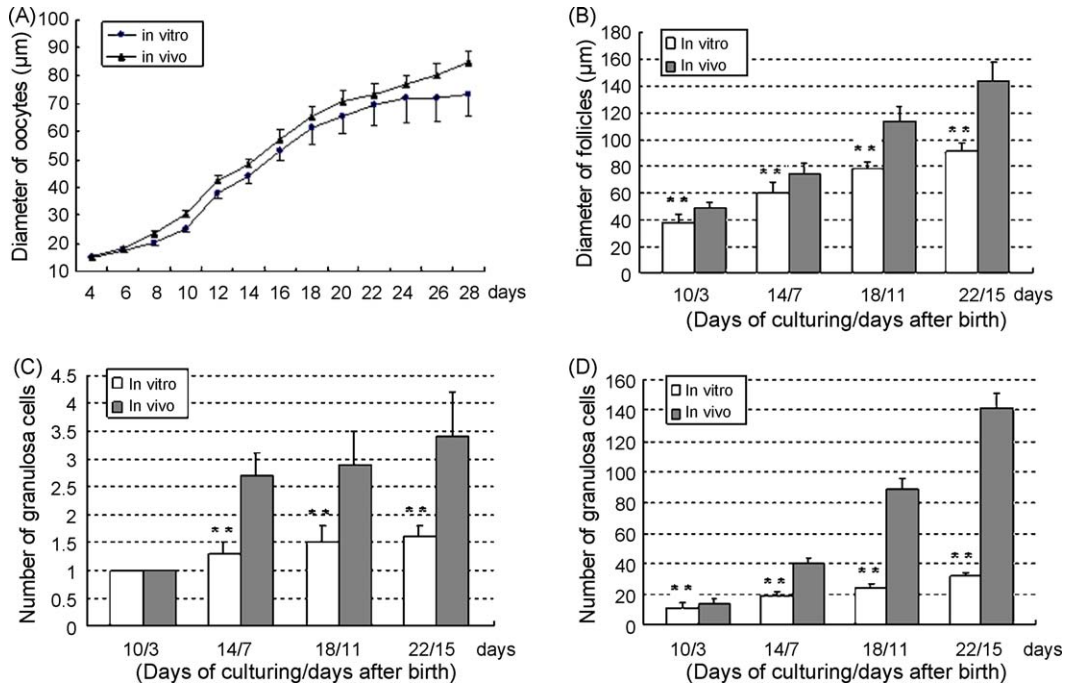

Fig. 3. Developmental characteristics of murine ovarian follicles derived from 12.5 dpc fetal ovaries in vitro. (A, B) Diameter of murine oocytes and follicles, respectively, derived from 12.5 dpc fetal murine ovaries cultured in vitro. (C) The number of granulosa cell layers in the cross section of ovarian follicles derived from 12.5 dpc fetal ovaries in vitro. (D) Mean numbers of granulosa cells in the cross section of ovarian follicles derived from 12.5 dpc fetal ovaries in vitro. Normal follicular oocytes developed in vivo were analyzed as the control groups, respectively. **Value differed from control ($P < 0.05$).

the expression of *Cx37* and *Cx43* genes in ovaries cultured for various periods; both *Cx37* and *Cx43* were detected. To quantify the differences in expression of gap junction genes between ovaries cultured in vitro and those developed in vivo, we further examined the expression of *Cx37* and *Cx43* using real-time PCR technology. As shown in Fig. 4B, C, mRNA levels of *Cx43* and *Cx37* in ovaries cultured in vitro were lower than those in ovaries developed in vivo ($P < 0.01$).

As shown in Fig. 5, there was no apparent difference in LHR protein expression (immunohistochemistry) between ovaries derived from 12.5 dpc fetal mice cultured in vitro for 14 and 21 d. Furthermore, follicular theca cells in the fetal ovaries cultured in vitro for 14 and 21 d were not around the follicular structure but dispersed within the ovaries.

The concentration of E2 in the culturing media progressively increased with incubation (Fig. 6A). The mean concentration of E2 for the experimental group was 174 ± 16 ng/L at Day 28, whereas in the controls it was 296 ± 25 ng/L ($P < 0.05$).

As shown in Fig. 6B, a total of six samples (50 oocytes per sample) from three replicates were assayed. The intracellular concentration of GSH in oocytes cultured in vitro (7.53 ± 2.15 pmol/oocyte) was lower than that in oocytes matured in vivo (23.17 ± 5.7 pmol/oocyte; $P < 0.01$).

3.4. In vitro maturation of oocytes from 12.5 dpc premeiotic murine fetal germ cells

The developmental potential of the oocytes derived from fetal murine premeiotic germ cells was evaluated by calculating the proportions of oocytes that had undergone GVBD and/or reached MII stage after culturing for 16 to 18 h in a medium supplemented with FSH and EGF. None of the oocytes in the in vitro developed group were undergoing GVBD. In the control group, out of 109 oocytes denuded of cumulus cell–oocyte complexes (COCs) derived from 22-d CD-1 murine antral follicles cultured in vitro, 88.1% underwent GVBD, of which 63.5% progressed to the MII stage. We further checked whether oocytes developed from premeiotic fetal murine germ cells could complete meiosis after treatment with OA or by culture in the conditioned medium collected from cultured follicles in vitro. In the group treated with OA, 61 of 74 (82.4%) oocytes derived from 12.5 dpc premeiotic murine fetal germ cells and cultured in vitro for 28 d underwent GVBD but did not proceed to MII (Fig. 7A, B). Similar

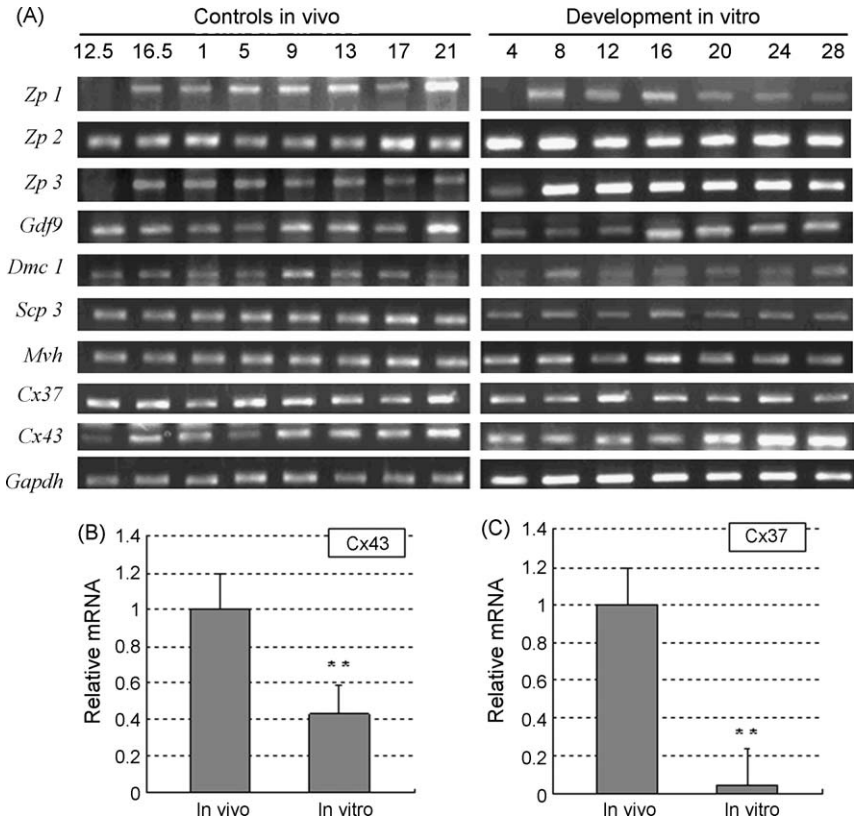

Fig. 4. Detection of gene expression of murine oocytes derived from premeiotic fetal germ cells in vitro. (A) Detection of follicle-specific gene expression in various stages, including the 12.5 dpc fetal murine ovaries, and 12.5 dpc, 16.5 dpc, and 1-, 5-, 9-, 13-, 17-, and 21-d murine ovaries in the control groups, and 4-, 8-, 12-, 16-, 20-, 24-, and 28-d in vitro–grown ovarian oocytes in experimental groups, respectively. (B, C) Quantification of *Cx43* and *Cx37* mRNAs in 12.5 dpc fetal ovaries cultured in vitro for 14 d, respectively, with murine ovaries developed in vivo as control. **Value differed from control ($P < 0.01$).

results were obtained in the control group, with 33 of 39 (84.62%) oocytes derived from 21-d-old murine COCs undergoing GVBD. Furthermore, GVBD was not observed for the oocytes (52 oocytes) cultured in the conditioned medium. In addition, ERK1/2 was not activated in the oocytes derived from premeiotic fetal germ cells cultured in vitro for 28 d followed by maturation in vitro for 16 to 18 h (Fig. 7C, D).

4. Discussion

In the current study, premeiotic female germ cells derived from fetal murine ovaries as early as 12.5 dpc were able to develop to oocytes in vitro. Furthermore, premeiotic female fetal murine germ cells were able to differentiate into GV oocytes >65 μm but were not able to progress to the meiotic division phase.

In the past few years, research on mammalian oogenesis has been focused on the inducement of primordial germ cells (PGCs) to differentiate into oocytes in vitro [9,11,26–30]. There has been increasing attention on molecular regulation of primordial follicle development to mature follicles in vitro [3,15,17,31–33]. Mature murine oocytes can currently be generated in vitro from primary oocytes of primordial follicles but not from premeiotic fetal germ cells. In earlier studies, de Felici's group reported that the isolated PGCs recovered from 11.5 and 12.5 dpc gonads underwent rapid apoptotic degeneration without somatic cell support, supporting the notion that the interplay between the germ cells and the somatic cells in fetal ovaries is essential for oocyte development [4,12–14,27]. When explanted into a cultured aggregate of embryonic lung tissue, germ cells were able to enter into meiosis at the same chronological time as those in ovaries in vivo [4]. If very few germ cells enter the female genital ridge, the supporting cell lineage fails to differentiate and the oocytes subsequently die or are eliminated, even though granulosa cell differentiation is initiated [4]. Eppig et al. and Epifano et al. further demonstrated that the communication between oocytes and granulosa cells is bidirectional and that a complex

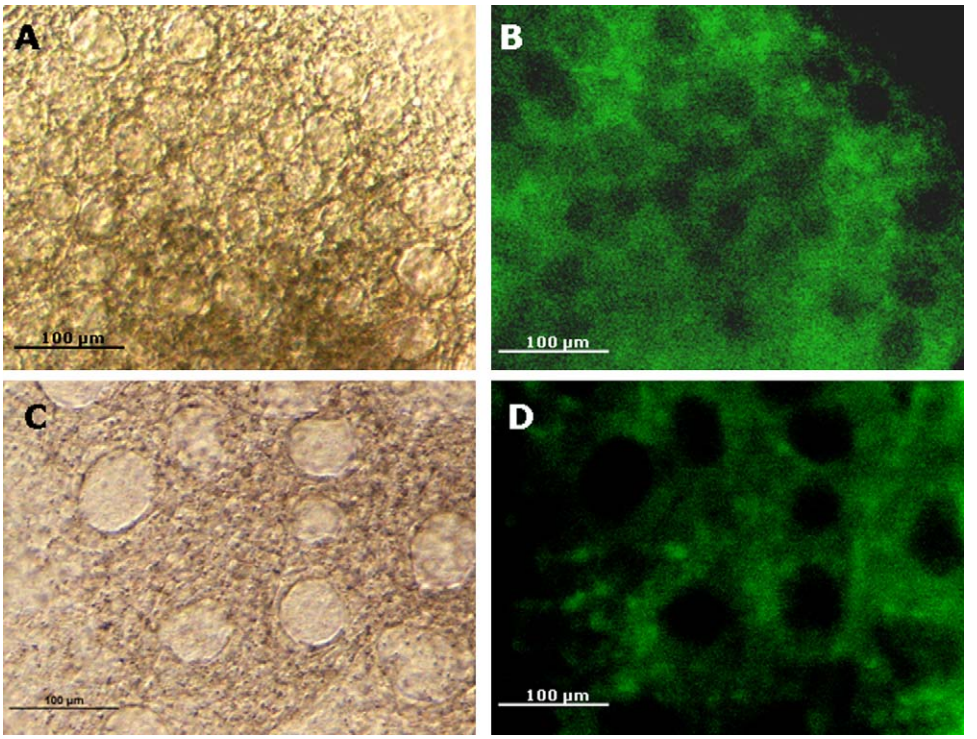

Fig. 5. Immunolocalization of LHR of murine ovaries derived from 12.5 dpc fetus. (A, C) Morphology of ovaries derived from 12.5 dpc fetuses, cultured in vitro for 14 and 21 d, respectively. (B, D) Immunolocalization of LHR expressed in the fetal ovaries cultured in vitro for 14 and 21 d, respectively. Scale bars = 100 μm.

interplay of regulatory factors governs the development of both types of cells. This is essential not only for oocyte development but also for follicular development, starting with the assembly of primordial follicle and continuing throughout follicular development [34–36]. Most recently, Farini et al. established a coculture method on somatic cell monolayer with Kit ligand (KITL), fibroblast growth factor 2 (FGF2), and leukemia inhibitory factor (LIF), which allowed purified murine PGCs to develop to the diplotene stage, but no mature oocytes were obtained [15]. Obata et al. and Niwa et al. cultured intact murine 12.5 dpc fetal ovaries for 28 d in vitro; they reported that the resulting oocytes could not complete secondary meiosis [10,15,30]. In our initial experiments, premeiotic germ cells were isolated from 12.5 dpc fetal murine ovaries and aggregated to ovary-like cell clusters with the 12.5 dpc fetal ovarian somatic cells (data not shown). In another study, we demonstrated that only a few primordial follicles were formed in the aggregated cell clusters by in vitro culture or transplantation under the kidney capsule of a mouse [37]. To overcome these problems, we adopted a new method by initiating differentiation using premeiotic germ cells from natural fetal ovaries, rather than isolated PGCs. Using this new method, a large number of follicles were formed, and the oocytes in the follicles were able to progress to the GV stage with a diameter >75 μm (Fig. 2 I). Based on these results, we inferred that somatic cell support in the context of fetal murine ovary is an optimal condition for germ cell development in vitro [13].

Several factors may contribute to the incompetence of oogenesis in vitro. Synchrony between nuclear and cytoplasm maturation is necessary for the acquisition of the competence to resume meiosis [4,8,38–41]. In addition, abnormal gap junctions between the follicular granulosa cells and oocytes could suppress development of oocytes and meiosis [9,42–44]. Another challenge is that the oocytes generated in vitro were unable to grow to 80% of their maximal volume, an adequate size that is required for normal meiosis [3]. Efforts to overcome these hurdles have been limited by the lack of adequate understanding of the mechanisms underlying the initiation of oocyte growth, the formation of gap junctions in the follicle, and the entry into meiosis of growing oocytes [45–47]. In the current study, 12.5 dpc fetal ovaries were organ-cultured in vitro in organ growth medium, and developmental potential of murine oocytes was analyzed. We demonstrated that oocytes with a mean diameter of 73.3 μm

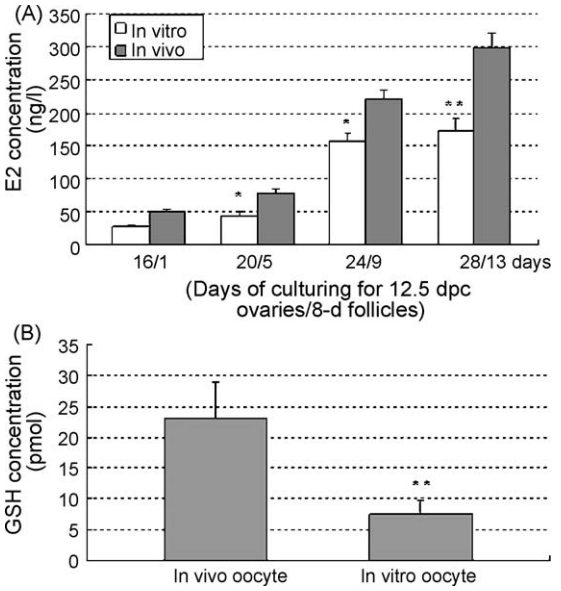

Fig. 6. (A) Estradiol concentration (ng/L) in media of fetal murine ovaries (isolated 12.5 dpc) and ovaries cultured in vitro for 16, 20, 24, and 28 d, and from media of follicles isolated from ovaries of 8-d-old mice and cultured in vitro for 1, 5, 9, and 13 d (control groups). The columns represent the mean ± SEM for estradiol production from fetal ovaries cultured in three repeated experiments. (B) Intracellular concentrations of GSH in the oocytes of 12.5 dpc fetal ovaries cultured in vitro for 28 d, with oocytes at the GV stage as controls. *Value differed from control ($P < 0.05$).

could be obtained from the premeiotic female germ cells after 28 d of culture. The diameter of these oocytes exceeded 80% of the maximal volume, a threshold for meiosis progression. These oocytes, isolated from ovaries cultured either spontaneously or with inducement, were able to enter the first meiotic division. Mammalian oocytes secrete regulatory factors to support the proliferation and differentiation of the surrounding granulosa cells in the ovarian follicles, and these regulatory factors were transferred through the gap junctions between the oocyte and the surrounding granulosa cells [3,10]. In the current study, the average number of follicular granulosa cells (excluding primordial follicles) in fetal murine ovaries cultured in vitro was significantly lower than that developed in vivo. Furthermore, differentiation of granulosa cells was not detected in cultured fetal murine ovaries, due to the absence of apparent formation of an antral-like cavity. To analyze the formation of gap junctions, and to compare the expressive difference of gap junction genes between the in vivo developed ovaries and in vitro–cultured ovaries, we further examined the mRNA level of *Cx37* and *Cx43* using real-time PCR technology. The mRNA levels of *Cx37* and *Cx43* genes of ovaries cultured in vitro were significantly lower than those in ovaries developed in vivo.

In this study, we confirmed premeiotic female fetal murine germ cells were able to differentiate into GV oocytes but were unable to progress to the meiotic division phase. In several studies, OA, a potent inhibitor of protein phosphatases 1 and 2A, induced noncompetent oocytes to resume meiosis [9,20]. Therefore, we used OA to study the regulatory effects of protein phosphatases on mitogen-activated protein kinase (MAPK) phosphorylation and meiotic resumption during murine oocyte maturation in vitro. In the group treated with OA, 61 of 74 (82.4%) oocytes derived from 12.5 dpc premeiotic murine fetal germ cells cultured in vitro for 28 d underwent GVBD but did not reach the MII stage (Fig. 7A, B). In contrast with the oocytes of preantral follicles and COCs, in vitro–grown oocytes have a low ability to respond to OA and to resume meiosis in vitro [9]. Thus, in vitro–grown oocytes had two main meiotic defects. First, they were unable to undergo spontaneous or FSH/EGF-induced GVBD, and second, after OA-induced GVBD, they were unable to progress into meiosis II. In the past several years, Klinger et al. also demonstrated that 48.7% in vitro–grown oocytes were able to respond to OA treatment and underwent GVBD but were not able to extrude the first polar body [9]. If added to the culture medium, OA quickly reversed the inhibitory effects of butyrolactone I, a specic inhibitor of maturation-promoting factor (MPF), on MAPK phosphorylation and meiosis resumption [20]. Several studies showed that the OA can mediate meiotic resumption in the absence of MPF activation [9,48]. Perhaps an OA-sensitive protein phosphatase mediates the inhibition of MAPK activation [39]. Furthermore, we found that MAPK was not activated in the oocytes derived from premeiotic fetal murine germ cells cultured in vitro for 28 d followed by maturation in vitro for 16 to 18 h. Meanwhile, several studies reported that MAPK activity was not necessary for GVBD, so the lack of such activity does not explain their inability to complete this process [20,39]. When GV-stage oocytes were exposed to OA, chromosome condensation occurred prematurely and GVBD were accelerated, whereas meiotic spindle assembly and meiotic progression to metaphase I stage were inhibited [9]. The deleterious action of OA on cytoskeleton assembly was necessary for progression into meiosis, explaining the inability of OA-induced GVBD oocytes to progress into meiosis II. The relatively large fraction of oocytes induced to undergo GVBD by OA, which were unable to progress beyond the early phase of GVBD (∼20%) or with compacted mass of chromatin

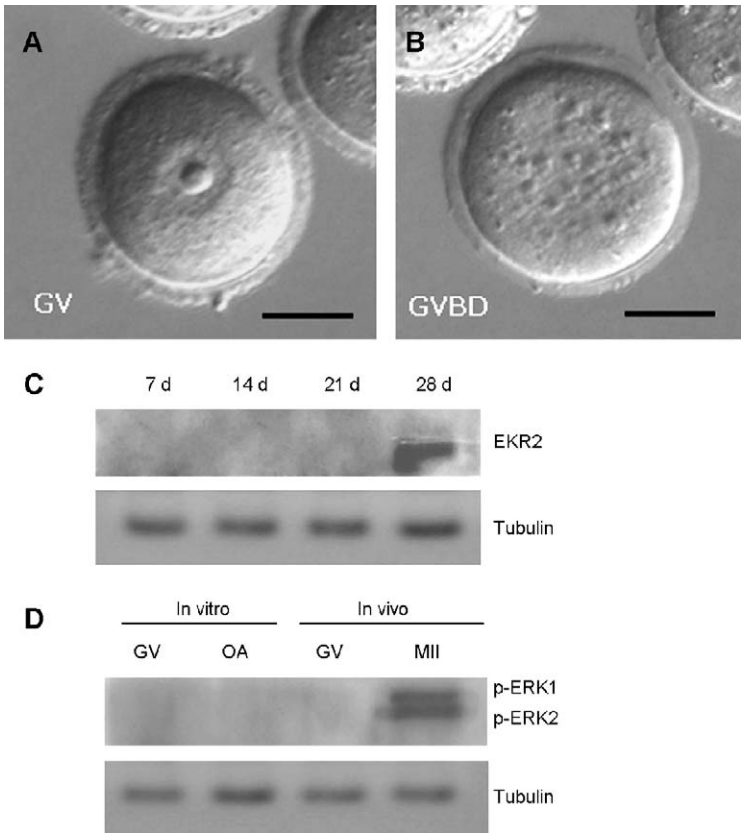

Fig. 7. In vitro maturation of oocytes from fetal murine ovaries cultured in vitro for 28 d; phosphorylation of ERK1/2 after in vitro maturation was detected by Western blotting. (A) Oocytes derived from fetal (12.5 dpc) ovaries in vitro. (B) GVBD oocytes induced by OA (A). The scale bars of (A) and (B) represent 25 μm. (C) Expression of ERK2 in oocytes derived from 12.5 dpc premeiotic fetal murine germ cells cultured in vitro for 28 d. (D) Phosphorylation of ERK1/2 in the oocytes that derived from the premeiotic fetal murine germ cells in vitro, cultured in vitro for 28 d and induced to mature with OA, and the oocytes derived from 21-d-old murine antral follicles were matured in vitro as the control groups.

(∼50%) by 24 h after OA treatment, revealed the high frequency of cytoplasmic immaturity in promoting and sustaining meiotic M-phase [9]. Furthermore, in the current study, cytoplasmic maturity of oocytes grown in vitro from 12.5 dpc fetal murine gonads was significantly lower than that of oocytes grown in vivo, according to the level of GSH. Thus, it was very difficult for these immature oocytes produced in vitro to complete meiosis I after treatment with OA. Moreover, the conclusion that there is no detectable ERK activity in the in vitro–produced oocytes after 3-h culture in OA does not exclude the possibility that OA induces a rapid and transit ERK phosphorylation sufficient for GVBD. These questions should be answered in future studies.

In summary, we developed a method by which female germ cells within 12.5 dpc fetal murine ovaries can be induced to differentiate and develop into GV oocytes in vitro. We demonstrated that premeiotic 12.5 dpc female fetal germ cells were unable to enter the second meiosis and mature in vitro. Our method offers a convenient tool for studying oogenesis and may facilitate further investigations into the regulative mechanism under PGC and oocyte differentiation.

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