***In vitro* development of mouse fetal germ cells into mature oocytes**

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Abstract

Little is known about the mechanisms underlying primordial follicular formation and the acquisition of competence to resume meiosis by growing oocytes. It is therefore important to establish an *in vitro* experimental model that allows one to study such mechanisms. Mouse follicular development has been studied *in vitro* over the past several years; however, no evidence has been presented showing that mature oocytes can be obtained from mouse fetal germ cells prior to the formation of primordial follicles. In this study, a method has been established to obtain mature oocytes from the mouse fetal germ cells at 16.5 days postcoitum (dpc). From the initiation of primordial follicular formation to the growth of early secondary follicles, ovarian tissues from 16.5 dpc fetal mice were cultured *in vitro* for 14 days. Subsequently, 678 intact secondary follicles were isolated from 182 mouse fetal ovaries and cultured for 12 days. A total of 141 oocytes inside antral follicles were matured *in vitro*, and 102 oocytes underwent germinal vesicle breakdown. We found that 97 oocytes were fertilized and 15 embryos were able to form morula–blastocysts. We also analyzed various genomic imprinting markers and showed that the erasure of genomic imprinting markers in the parental generation was also imposed on the oocytes that developed from fetal germ cells. Our results demonstrate that mouse fetal germ cells are able to form primordial follicles with ovarian cells, and that oocytes within the growing follicles are able to mature normally *in vitro*.

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Introduction

The assembly and subsequent development of primordial follicles are crucial for mouse oogenesis. Primordial follicles are formed from granulosa cells encasing individual oocytes in the mouse fetal ovary after 17 days of gestation (Pepling & Spradling 1998, 2001, Epifano & Dean 2002). Female germ cells within the mouse fetal ovary are arrested in the prophase of the first meiotic division, and become surrounded by a single layer of granulosa cells contained within an outer basal lamina (Byskov *et al.* 1997, Kezele *et al.* 2005). Interactions between oocytes and pregranulosa cells are crucial for the initial formation of primordial follicles, and the initial stages of folliculogenesis are independent of gonadotropins and involve cell-autonomous and non-cell-autonomous factors (Epifano & Dean 2002). With periodicity, cohorts of these primordial follicles, representing the lifetime complement of germ cells available to females, are recruited into a growth phase that culminates in meiotic maturation and ovulation of the mature oocytes

into the oviduct in preparation for fertilization (Epifano & Dean 2002). Regulative mechanisms of primordial follicle formation and development are only partially understood, and more information underlying these processes could be obtained by culturing follicles *in vitro* at the earliest stages of formation and growth (Smitz & Cortvriendt 2002).

During the last few years, the formation and development of mouse primordial follicles have been investigated *in vitro* (Eppig & O'Brien 1996, Eppig *et al.* 1996, Klinger & de Felici 2002, Obata *et al.* 2002a, O'Brien *et al.* 2003, Lenie *et al.* 2004, Niwa *et al.* 2004, Shen *et al.* 2006a, 2006b). The primordial follicles within the newborn mouse ovaries were able to achieve complete development and maturation *in vitro*, although the frequency of preimplantation development was low (Eppig & O'Brien 1996, Eppig *et al.* 1996, O'Brien *et al.* 2003). The mature oocytes could not be directly obtained from the mouse fetal germ cells using *in vitro* culture (McLaren & Southey 1997, McLaren 2000, Chuma & Nakatsuji 2001, Klinger & de Felici 2002,

Obata *et al.* 2002a; Niwa *et al.* 2004). Mouse oocytes could develop to the size of 50 μm from 16.5 days postcoitum (dpc) fetal germ cells and show signs of chromatin differentiation, but the oocytes were unable to go on to acquire meiotic/developmental competence *in vitro* (Klinger & de Felici 2002). However, several studies have demonstrated that the developmental potential of mouse primordial follicles could be restored by the transplantation of ovaries under the kidney capsule (Cox *et al.* 2000, Liu *et al.* 2000, 2001). Recently, using the ectopic transplantation technology, our group demonstrated that oocytes generated from the 16.5 dpc fetal germ cells had the potential to be fertilized *in vitro* and to support early embryonic development (Shen *et al.* 2006a). However, regulation of the *in vitro* formation of mouse primordial follicle derived from fetal germ cells, the selection and initiation of growth of the primordial follicles out of the dormant pool, and the oocytes generated *in vitro* that had full developmental potential to support the embryonic development remain enigmatic. As the store of female gametes is maintained within the primordial follicles, the real challenge is to establish an *in vitro* culture system for studying primordial follicles. The formation and development of primordial follicles, as well as the transition from primordial to mature follicle, can be studied using this system. Finally, this system could potentially provide an almost unlimited source of oocytes for biomedical use (Smitz & Cortvrindt 2002).

The purpose of this study is to determine the developmental competence of the germ cells from the 16.5 dpc fetal ovaries to differentiate into mature oocytes by *in vitro* culture. The results demonstrate that 16.5 dpc mouse fetal germ cells are able to aggregate into primordial follicles with the ovarian pregranulosa cells *in vitro*, and the oocytes from the growing follicles are able to mature and support early embryonic development *in vitro*.

Material and Methods

Ovarian tissue isolation and culture in vitro

Upon the approval of study plan by the Institutional Animal Care and Use Committees of Qingdao Agricultural University, 16.5 dpc mouse fetal ovaries were obtained from pregnant CD-1 mice (Vital River, Beijing, China), and the copulation plug was designated at 0.5 dpc. The 16.5 dpc mouse fetal ovaries without attached mesonephroses were cultured *in vitro*. Briefly, the ovaries were placed on Millicell-PC membrane inserts (3.0 μm pore size, 10 mm diameter; Millipore Corp., Medford, MA, USA) with medium filling only the lower chamber, and each ovary was placed on each membrane. Then the medium was removed from the lower chamber until only a thin film covered the ovaries. The medium for organ culture was DMEM/F12 + α -MEM

(1:1; Gibco-BRL) supplemented with 0.23 mM pyruvic acid, 3% (w/v) BSA (Sigma), insulin–transferrin–selenium mix (ITS-mix; 5 mg/ml, 5 mg/ml, and 5 ng/ml respectively; Sigma), 100 U/ml penicillin G, and 100 mg/ml streptomycin sulfate. The organ cultures were maintained for 14 days at 37 °C in modular incubation chambers (Billups Rothenberg, Del Mar, CA, USA) thoroughly infused with a gas mixture of 5% CO₂ and 95% air (O'Brien *et al.* 2003). The medium was changed every 48 h with replacement of half of the complete medium.

Follicles isolation and culture in vitro

Fourteen days after mouse ovarian tissue culture, the intact early secondary follicles (110–130 μm) were mechanically isolated from the cultured ovaries using needles, and then cultured *in vitro* for 12 days (Liu *et al.* 2001, Shen *et al.* 2006b). Selected follicles were individually washed and placed in the culture droplets. Medium A consisted of α -MEM with 5% heat-inactivated fetal calf serum (Gibco-BRL), 100 mIU/ml follicle-stimulating hormone (FSH), 10 mIU/ml luteinizing hormone (LH; Sigma), and ITS-mix. Culture dishes (10 cm Petri dish; BD Bioscience, Bedford, MA, USA) containing sixty 10 μl culture droplets covered with 10 ml mineral oil were preincubated overnight at 37 °C, 100% humidity, and 5% CO₂ in the air. On day 2 after culture, 10 μl fresh medium A were added to each droplet. From day 4 onward, the depleted medium was refreshed by exchanging 10 μl fresh medium every other day. The diameters of mouse follicles and oocytes cultured *in vitro* were measured daily. On day 12, 10 μl medium were replaced with 10 μl mature medium B to induce final oocyte maturation. Mature medium B consisted of α -MEM supplemented with 1.5 IU/ml rhCG (Sigma) and 5 ng/ml recombinant epidermal growth factor (rEGF; Sigma). After 16–18 h, cumulus expansion of the cumulus–oocyte complexes (COCs) was assessed. Oocyte maturation status was adjudged by germinal vesicle breakdown (GVBD) and extrusion of the first polar body (Shen *et al.* 2006a, 2006b).

The early secondary follicles were mechanically isolated from the 12-day-old CD-1 ovaries in the control group, and were cultured *in vitro* for 12 days using the protocol as described above for *in vitro* growth.

Assessment of steroid production in vitro

From follicle culture of day 6, medium A was refreshed every 2 days by retrieving and adding 10 μl . From the surviving follicles, the medium was pooled per culture dish and stored at –20 °C for hormone analysis. Estradiol (E₂) and progesterone were measured using RIAs; E₂ was measured using a very sensitive (4 pg/l) and reproducible RIA (total coefficient of variation (CV), 10%) from clinical

assays with a measurement range for E₂ from 4 to 500 ng/l. Progesterone secretion was measured using RIA from CIS Biointernational with an analytical sensitivity of 40 ng/l and a precision of 8% or less (CV %). The assays of E₂ were validated for use by sampling the culture medium every other day, and the assays of progesterone were validated at days 12 and 13 respectively (Lenie *et al.* 2004, Shen *et al.* 2006a).

Expression of specific genes in the different follicular stages

RNA samples were isolated from the different stages of follicles ($n=10$), and oligo-dT-primed cDNA was synthesized using superscript reverse transcriptase (RT) according to the manufacturer's protocol (TaKaRa, Kyoto, Japan). One microliter of each RT reaction product was used in each 25 μ l PCR mix primed with gene-specific oligonucleotides. The following primers were used to detect mouse-specific genes in the different follicular stages: *Ddx4* is a marker for postmigratory germ cells until postmeiotic stages, 5'-GGT CCA AAA GTG ACA TAT ATA CCC-3' and 5'-TTG GTT GAT CAG TTC TCG AGT-3' (420 bp long); growth differentiation factor 9 (*Gdf9*), 5'-CCA GCA GAA GTC ACC TCT ACA A-3' and 5'-ACA TGG CCT CCT TTA CCA CA-3' (240 bp long); the mouse homolog of the yeast meiosis-specific homologous recombination gene, disrupted meiotic cDNA 1 homolog (*Dmc1h*), 5'-GGA CAT TGC TGA CCG CTT CAA CGT-3' and 5'-GGC GAT CCT CAG TTC TCC TCT TCC-3' (427 bp long); *Og2x* is an oocyte-specific homeobox gene expressed in germ cell cysts and in primordial and growing oocytes, 5'-CCT TCA GTC ACA GTT TCC GTA T-3' and 5'-GGG AGG TTC TGG CAA GCA AT-3' (226 bp long); the synaptonemal complex protein 3 (*Sycp3*), 5'-ATG ATG GAA ACT CAG CAG CAA GAG A-3' and 5'-TTG ACA CAA TCG TGG AGA GAA CAA C-3' (325 bp long); and LH/choriogonadotropin receptor (*Lhcgr*), 5'-AAT CTC TCC TTT GCA GAC TTT TG-3' and 5'-AGC ATA GGT GAT GGT GTG CCA-3' (216 bp long). *Gapdh* primers were used as a control supplied by the RT-PCR kit. The cycling conditions were as follows: 30 s at 94 °C, 30 s at 60 °C, and 1 min at 72 °C (25 cycles; Shen *et al.* 2006b).

DNA methylation analysis

Genomic DNA was prepared from the oocytes ($n=15$) that developed *in vitro* from mouse fetal germ cells in experimental group, control group, and normal mice. DNA was treated with a sodium bisulfite solution as described previously (Shen *et al.* 2006b). Region 2 of the *Igf2r* gene and the 5' upstream region of *Peg3* were amplified using Ex-Taq DNA polymerase (TaKaRa). The PCR primers were as follows: *Igf2r*, 5'-GAG GTT AAG GGT GAA AAG TTG TAT-3' and 5'-CAC TTT TAA ACT

TAC CTC TCT TAC-3' (450 bp long); *Peg3*, 5'-TTT TGT AGA GGA TTT TGA TAA GGA GGT G-3' and 5'-CCC CAA ACA CCA TCT AAA CTC TAC AAA C-3' (288 bp long). The cycling conditions were as follows: 30 s at 94 °C, 30 s at 60 °C, and 1 min at 72 °C (35 cycles). Amplified fragments were cloned into the plasmid vector pGEM-T Easy (Promega), and 20 samples in each experiment sequenced using an ABI PRISM 3100 Genetic Analyzer (Applied Biosystems, Foster City, CA, USA).

In vitro fertilization and embryonic culture in vitro

To test the developmental competence of the oocytes derived from fetal germ cells *in vitro*, the oocytes were fertilized *in vitro*. The caudal epididymis was removed from 10- to 12-week-old CD-1 male mice and placed in 1 ml drops of a mutant potassium simplex optimized medium (mKSOM) supplemented with 0.4% (w/v) BSA in sperm dispersion dishes. The dispersion dishes were placed in a 5% CO₂ incubator for 20 min to allow the sperm to disperse. Sperm suspension of 10 μ l was added to 90 μ l drops of mKSOM+BSA in the fertilization dishes. Capacitation was allowed to proceed for 45–60 min at 37 °C in the incubator. After capacitation, COCs were transferred to the 100 μ l fertilization droplets. Incubation was allowed to proceed for 4 h at 37 °C in a 5% CO₂ atmosphere. Finally, about ten inseminated oocytes were incubated in 20 μ l drops of KSOM+BSA in culture dishes for 48 h. Then the medium was replaced with 20 μ l mKSOM+BSA, continuing culture for 48 h. On day 4, the embryonic development was determined at the late morula–early blastocyst stages (Shen *et al.* 2006a, 2006b).

Statistical methods

Follicle formation and survival data, oocyte and follicle diameter, and nuclear maturity were analyzed using χ^2 -analysis for likely trends. The oocyte proportions that underwent GVBD, fertilization, and late morula–early blastocyst formation were compared by contingency table analysis, followed by χ^2 -testing. Probability <0.05 was considered statistically significant.

Results

Mouse fetal ovarian culture in vitro

Mouse 16.5 dpc fetal ovaries were isolated from pregnant mice, and histological examination of the fetal ovaries revealed that no typical primordial follicles were present (data not shown). Subsequently, each fetal ovary without attachment of mesonephros was cultured *in vitro* (Fig. 1A). A total of 231 fetal ovaries were cultured *in vitro* and 197 ovaries (85.3%) survived after 14 days of culture. Subsequently, the intact early secondary follicles were mechanically isolated from the cultured ovaries using

Figure 1 Growth and differentiation characteristics of secondary follicles from fetal ovaries cultured *in vitro*. (A) shows 16.5 dpc mouse fetal ovary, (B and C) show the fetal ovaries cultured *in vitro* for 14 days and the secondary follicles isolated from the cultured fetal ovaries respectively. (D–H) show the follicles isolated from ovaries cultured *in vitro* for 2, 4, 6, 8, and 10 days respectively. (I) shows the oocyte–granulosa cells complexes ovulated from the mature follicle on day 13. Scale bars represent: (A) 500 μ m; (B) 150 μ m; (C, D, and G) 75 μ m; (E and F) 50 μ m; (H and I) 100 μ m.

needles (Fig. 1B and C). Morphological examination of the follicles recovered after 14 days revealed that the follicles had proceeded to the secondary follicle stage. Moreover, the follicles grown in the ovary tissue *in vitro* were similar to the follicles derived from 12-day-old normal mice. In our experiments, we were able to obtain three to eight intact secondary follicles from a single cultured fetal ovary. We also found that the membrane of secondary follicles derived from mouse ovaries cultured *in vitro* was able to be breached by needles, and that the granular cells within the follicles were able to be dispersed from the primary oocytes.

Follicular growth characteristics *in vitro*

In the experimental group, 678 intact secondary follicles were isolated from 182 mouse ovaries cultured *in vitro*. These were subsequently cultured *in vitro* for 12 days. During this period, the follicles progressively grew from a spherical to a diffuse structure and then displayed an antral-like cavity (Fig. 1D–H). A total of 441 follicles (65.0%) survived during the 12-day culture period. From day 8, an antral-like cavity was observed. A total of 153

follicles that survived (34.7%) became antral follicles by day 10, with the COCs located in the antral-like cavity (Fig. 1H). In the control group, 314 follicles derived from 12-day-old mouse ovaries were cultured *in vitro*. Seventy-seven percent of the follicles survived after 12-day culture, and 74% of the follicles that survived became antral follicles by day 10 *in vitro* (Shen *et al.* 2006a, 2006b). These results indicate that mouse follicular development showed significant differences between the experimental and control groups ($P < 0.01$).

After culturing the cells, we measured the diameters of the oocytes and follicles for a comparison with the experimental and control groups (Fig. 2). In the experimental group, the mean diameter of the oocytes ($n = 183$) was $72.1 \pm 5.4 \mu$ m, while in the control group, it ($n = 104$) was $76.9 \pm 4.1 \mu$ m. The mean diameters of the follicles from the ovaries cultured for 12 days *in vitro* ($n = 158$) and the control group ($n = 114$) were 347 ± 53 and $413 \pm 44 \mu$ m respectively. These data regarding oocytes and follicle showed no significant differences between the experimental and control groups. Compared with published results (Liu *et al.* 2000, 2001, Lenie *et al.* 2004, Shen *et al.* 2006a, 2006b), the sizes of the oocytes and follicles

Figure 2 Characteristics of mouse follicles and oocytes derived from a 16.5 dpc fetal ovary *in vitro*. Diameters of the oocytes and the follicles in the experimental and control groups grown *in vitro* respectively.

generated from the fetal germ cells in this study were similar to that from the secondary follicles cultured *in vitro*.

In the experimental group, the concentration of E_2 progressively increased as the time of incubation also increased (Fig. 3A). However, it remained lower than that from the control cultures of early preantral follicles. The basal progesterone production before hCG stimulus remained higher in the cultures of experimental group than in the cultures of control group. Subsequently, the production of progesterone increased dramatically at 18 h after stimulation with hCG/EGF inducing ovulation, and the progesterone production stayed lower in the cultures of experimental group than in the cultures of control group (Fig. 3B). There was no significant difference between the experimental and control groups in the production of E_2 and progesterone.

Figure 3 (A) Estradiol concentration (ng/l) in media of the experimental and control groups. (B) Progesterone concentration (μg/l) in conditioned media of the experimental and control groups.

Molecular characterization of oocytes derived from fetal germ cells *in vitro*

We further examined the expression of follicle-specific genes (*Ddx4*, *Gdf9*, *Dmc1h*, *Og2x*, *Sycp3*, and *Lhcgr*) in different stages of *in vitro*-cultured follicles by RT-PCR. As shown in Fig. 4, expression of these follicle-specific genes was detected as early as day 0 in the follicle culture *in vitro*, which is equal to mouse ovary *in vivo* around postnatal day 12. In general, their expression patterns were similar to that of the follicles from various stages of postnatal mouse ovaries (Hübner *et al.* 2003, Lenie *et al.* 2004, Rajkovic *et al.* 2004, Shen *et al.* 2006b).

To determine whether methylation of imprinted genes was completed during the oogenesis observed during our *in vitro* culture method, we examined the differentially methylated regions (DMRs) of the maternal imprinted gene *Igf2r* in the oocytes of the experimental group. In mammals, parental imprinted memories persist in somatic cells after fertilization, and it is necessary for them to be erased and re-established during germ cell development to reflect the gender of the individual. The erasure process of genomic imprinting memory proceeds in mouse primordial germ cells (PGCs) from 10.5 to 11.5 dpc, and the maternal-specific imprints are established during oogenesis (Kaneko-Ishino *et al.* 1995, Kono *et al.* 1996, Miyoshi *et al.* 1998, Obata *et al.* 1998, 2002b, Bao *et al.* 2000, Surani 2001, Lee *et al.* 2002, Zwaka & Thomson 2005). The *Igf2r* gene, a maternally expressed imprinted gene, has been shown with a fully methylated pattern in the maternal allele and an unmethylated pattern in the paternal allele in somatic cells, and begins to be methylated in mouse oocytes after 13.5 dpc (Brandeis *et al.* 1993). As shown in Fig. 5, we analyzed the DNA methylation status in DMRs of *Igf2r* and in the control gene *peg3*, a paternally expressed imprinted gene. In experimental group, the *Igf2r* gene was methylated at DMR CpG sites in the oocytes developed

Figure 4 Expression of follicle stage-specific genes in mouse follicles derived from 16.5 dpc fetal ovary after *in vitro* tissue culturing for 14 days. Detection of follicle-specific gene mRNA in different stages including 0, 4, 8, and 12 days *in vitro* grown follicles from the fetal ovaries in the experimental and control groups.

in vitro, and the DNA clones with methylated sites accounted for more than 65.5% of all the CpG sites. The results of the methylated patterns of imprinted gene *Igf2r* show the significant difference among the experimental group, the control group (92.2%), and the normal oocytes (96.3%; $P<0.01$).

Oocyte maturation and fertilization in vitro

To test the developmental capacity of the oocytes derived from fetal germ cells *in vitro*, COCs from the inside of the antral follicles were matured in a medium supplemented with hCG and EGF for 16–18 h. The results are summarized in Tables 1 and 2. The oocytes which extruded the first polar body were judged to be metaphase II.

In the experimental group, 141 oocytes inside antral follicles were matured *in vitro*, 102 (72.3%) oocytes underwent GVBD and progressed to MII stage achieving 53.9% of GVBD oocytes (Fig. 6A; Table 1). In the control group, 139 oocytes were matured *in vitro*, 79.9% oocytes underwent GVBD and progressed to MII stage achieving 57.7% of GVBD oocytes. There were no differences in the GVBD formation rates and MII oocyte rates, when comparing the experimental and control groups.

To assess the capacity of the matured oocytes derived from the fetal germ cells cultured *in vitro*, GVBD oocytes from the experimental and control groups were fertilized *in vitro*, and zygotes were examined for cleavage to the

two-cell stage 24 h after fertilization *in vitro* and for late morula and early blastocyst developmental competence *in vitro*. In the experimental group, 97 oocytes were fertilized, 44 embryos (45.4%) were able to cleave to the two-cell stage and 15 two-cell stage embryos (34.1%) could form late morula–early blastocysts (Fig. 6B and C; Table 2). In the control group, 109 oocytes were fertilized, 76 embryos (69.7%) could cleave to two-cell stage, and 53 two-cell stage embryos (69.7%) could form the late morula or early blastocysts. There was a significant difference in the two-cell embryonic ($P<0.05$) and blastocyst development rates ($P<0.01$) between the experimental and control groups.

Discussion

In this study, we obtained mature oocytes from 16.5 dpc mouse fetal germ cells *in vitro*. Our results demonstrate that 16.5 dpc mouse fetal germ cells are able to form primordial follicles with pregranulosa cells *in vitro*, and that the oocytes within the growing follicles are able to mature and support the embryonic preimplantation development *in vitro*. Furthermore, we confirmed that the erasure of genomic imprinting markers that had been imposed in the parental generation was imposed in the oocytes that developed from fetal germ cells.

Before the formation of the primordial follicles, germ cell clusters undergo programmed breakdown and massive apoptosis of oocytes during the later stages of

Figure 5 Methylation status of the differentially methylated regions (DMRs) of the imprinted genes, *Igf2r* and *peg3*, in the oocytes from experimental group, control group, and normal mice. Each line represents an individual clone. In total, 30 CpG sites within the DMR of *Igf2r* gene, and 17 CpG sites within the DMR of *peg3* gene were analyzed. Squares indicate CpG sites within the regions analyzed; filled and open squares indicate methylated and unmethylated cytosines respectively. The number of DNA samples sequenced (left) and the percentage of the methylated CpG sites out of all the CpG sites (right) are represented to the left and right of each line respectively.

Table 1 *In vitro* maturation of mouse oocytes derived from fetal germ cells.

Groups	No. of oocytes	No. of GVBD oocytes	No. of MII oocytes
Experimental group	141	102 (72.3%)	55 (39.0%)
Control group	139	111 (79.9%)	64 (46.0%)*

A total of 141 oocytes were matured *in vitro* and the ratios of germinal vesicle breakdown (GVBD) and MII oocytes are in terms of the GVBD and MII oocytes from the GV oocytes respectively. * $P < 0.05$.

gestation. Only 33% of oocytes were able to survive and were surrounded by a single layer of granulosa cells to form the primordial follicles (Pepling & Spradling 1998, 2001). These problems caused a decrease in the rate of formation and development of the primordial follicles. Researchers found that mouse oocytes derived from fetal germ cells were unable to resume meiosis and progress to the last stage of growth *in vitro* (Klinger & de Felici 2002, Obata *et al.* 2002a, Niwa *et al.* 2004, Farini *et al.* 2005). The efforts to solve these problems with an *in vitro* culture system were limited because very little is known about the mechanisms underlying the initiation of oocyte growth, the molecular interactions between the oocytes and somatic cells during the follicle formation, the formation of gap junctions in the follicle, and the growth initiation and selective mechanisms of the follicle (Klinger & de Felici 2002, Lee *et al.* 2002).

Several possible factors might account for the incompetence of oogenesis *in vitro*. One is the abnormal gap junction between the follicular granulosa cells and oocytes that might influence the development of oocytes and block the meiosis (Carabatsos *et al.* 2000, Eppig 2001, Klinger & de Felici 2002, Hahn *et al.* 2005). Several studies have demonstrated that the gap junctions during the development of follicles were required for the progression of the follicular development, and the intercellular connections were thought to be important for transporting small molecules facilitating the growth of mouse oocyte (Carabatsos *et al.* 2000, Eppig 2001, Epifano & Dean 2002, Matzuk *et al.* 2002). Klinger *et al.* demonstrated that mouse oocytes cultured *in vitro* for 18–20 days derived from 15.5 to 16.5 dpc fetal germ cells were unable to complete the final growth stage

Table 2 *In vitro* fertilization of mouse oocytes derived from fetal germ cells.

Groups	No. of fertilized oocytes	No. of two-cell embryos	No. of morula/blastocysts
Experimental group	97	44 (45.4%)	15 (15.5%)
Control group	109	76 (69.7%)*	53 (48.6%) [†]

Ninety-seven GVBD oocytes were fertilized *in vitro* and the ratio of developmental embryo is in terms of the embryos from the GVBD oocytes. * $P < 0.05$, [†] $P < 0.01$.

corresponding to the *in vivo* antral follicle stage, and insufficient maintenance of gap junction communication between follicular oocyte and granulosa cells *in vitro* reduced paracrine signaling transduction and blocked the oocyte growth and development (Klinger & de Felici 2002). This in turn could depend on accompanying granulosa cell differentiation regulated by gonadotropins and by signals from the oocyte itself (Eppig & O'Brien 1996, Eppig *et al.* 1996). In this regard, selective deletion of connexins expressed in the follicle has resulted that defects both in signaling between oocyte and granulosa cells and intercellular coupling between granulosa cells severely affected oocyte growth and the acquisition of meiotic competence. In fact, oocytes lacking connexin-37 are able to grow up to a maximum diameter of about 50 μm (Simon *et al.* 1997), similar to that achieved by the oocytes in Klinger's culture system (Klinger & de Felici 2002). Similarly, oocytes from connexin-43-deficient mice can grow at decreased rate (final mean diameter 53 μm) and fail to undergo GVBD (Ackert *et al.* 2001). Another cause is absence of synchrony between nuclear and cytoplasm maturation, which is necessary for the acquisition of the competence to resume meiosis (McLaren & Buehr 1990, McLaren & Southee 1997, Chuma & Nakatsuji 2001, Pepling & Spradling 2001, Obata *et al.* 2002a). In order to resume meiosis, oocyte volume needs to reach 80% of its maximal volume; mouse oocytes generated *in vitro* are unable to reach this size (Eppig & O'Brien 1996, Eppig *et al.* 1996). The premeiotic germ cells within mouse fetal ovary are able to be induced into GV stage oocytes *in vitro*. However, these GV stage oocytes could not mature *in vitro* due to their incompetent cytoplasm (Bao *et al.* 2000, Obata *et al.* 2002a, Niwa *et al.* 2004). Recently, incompetence of folliculogenesis *in vitro* was solved using a two-step culture method utilizing a combination of *in vivo* transplantation under the kidney capsule and *in vitro* culture (Shen *et al.* 2006a, 2006b). Our early research demonstrated that the fetal germ cells were able to complete two meiotic divisions and generate a large number of mature oocytes, which were highly competent in supporting the embryonic development *in vitro* (Shen *et al.* 2006a,b). Mouse kidney capsule, as an ectopic site, provides a suitable environment for the development of mouse follicles (Cox *et al.* 1996, 2000, Liu *et al.* 2000, 2001). However, whether mouse oocytes derived from the fetal germ cells without the environment of kidney capsule were able to resume meiosis and progress to the last stage of growth *in vitro* is still unknown, and advanced research should be done for the critical factors required for the induction of meiosis *in vitro*.

In previous studies, it was reported by Eppig *et al.* that the oocyte–granulosa cell complexes from mice younger than 6 days of age are unable to sustain development *in vitro* (Eppig & O'Brien 1996, Eppig *et al.* 1996). However, early secondary mouse follicles from 8- or 12-day-old mouse ovaries could be cultured and

Figure 6 *In vitro* maturation and fertilization of oocytes from cultured fetal ovaries *in vitro*. (A) The oocytes matured *in vitro* derived from 16.5 dpc mouse fetal ovaries. (B and C) Development of oocytes derived from *in vitro* culturing after fertilization (A). The bars of A, B, and C represent 100, 50, and 100 μm respectively.

matured *in vitro* (Liu *et al.* 2001, Lenie *et al.* 2004). When the ovaries of newborn mice were cultured *in vitro* with whole organ for 8 days, and then the growing oocyte–granulosa cell complexes were isolated from the organ-cultured ovaries and cultured for an additional 14 days, these oocytes were able to be fertilized *in vitro*, and supported the development to term (Eppig & O'Brien 1996, Eppig *et al.* 1996, O'Brien *et al.* 2003). The culture conditions for purified mouse PGCs in the absence of somatic cell support had been established to study the molecular dissection of the processes governing the development of such cells crucial for early gametogenesis (Farini *et al.* 2005). Under these culture conditions, PGCs were able to proliferate at high rate, undergo meiosis, and even to progress to a completed meiotic prophase I; however, they did not go on to grow and enter the meiotic prophase II. However, PGCs, when cultured within intact mouse fetal ovary, are able to be induced into GV stage oocytes *in vitro* and to mature in culture by transferring their nuclei into enucleated oocytes fully grown *in vivo* (Obata *et al.* 2002a, Niwa *et al.* 2004).

It seems that the ovarian environment is crucial for the early development of mouse follicles before postnatal day 8. Eppig (2001) and Epifano & Dean (2002) further demonstrated that the communication between oocytes and granulosa cells is bidirectional, and a complex interaction of regulatory factors regulates the development of both types of cells, which is essential not only for oocyte development but also for follicular development – beginning with the initial assembly of the primordial follicle and continuing throughout ovulation. In our initial experiments, germ cells were isolated from 16.5 dpc mouse fetal ovaries and aggregated into ovary-like cell clusters with the 16.5 dpc fetal ovarian somatic cells. Furthermore, by *in vitro* culturing, only a few primordial follicles can be formed in the aggregated cell clusters (data not shown). Therefore, we changed our strategy and used fetal germ cells preserved in the natural fetal ovary as the starting materials, rather than isolated primary oocytes, and then performed tissue culture *in vitro* for 14 days (including 2 days fetal ovarian development and 12 days postnatal ovarian development). Subsequently, the early secondary follicles of the fetal ovary were isolated as soon as possible and cultured *in vitro* for 12 days, following the method of previous studies (Eppig & O'Brien 1996, Eppig *et al.* 1996, O'Brien *et al.* 2003, Lenie *et al.* 2004, Liu *et al.* 2000, 2001,

Shen *et al.* 2006a, 2006b). Our results demonstrated that incompetent folliculogenesis *in vitro* can be overcome by this two-step method utilizing a combination of 14-day ovarian tissue culture *in vitro* and 12-day single follicle culture *in vitro*, and further suggested that the non-serum medium is able to promote the formation of primordial follicles in our experiment other than the serum medium described in O'Brien experiment, and somatic cell support in the context of the intact 16.5 dpc mouse fetal ovary is most favorable for the development of germ cells *in vitro*. Furthermore, in our experiments, mouse fetal ovaries were cultured *in vitro* before follicular formation as described by Eppig's group (Eppig & O'Brien 1996, Eppig *et al.* 1996, O'Brien *et al.* 2003). Together, the detailed mechanism of interaction between mouse somatic and germ cells to form the follicles and their initiation into a growth phase *in vitro* would enable us to exploit the primordial follicle as a starting point for regulating mammalian fertility (Eppig 2001, Epifano & Dean 2002). This study may be valuable for mouse folliculogenesis. In future, we will further optimize the method and attempt to accomplish the whole process of mouse oogenesis *in vitro* from mouse PGC.

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