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# Generation of Functional Oocytes and Spermatids from Fetal Primordial Germ Cells after Ectopic Transplantation in Adult Mice[1]

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## ABSTRACT

**Primordial germ cells (PGCs) are undifferentiated germ cells in developing fetuses. As these cells give rise to definitive oocytes and spermatozoa that contribute to new life in the next generation, their development must be under strict control, regarding genetic and epigenetic aspects. However, we do not know to what extent their development depends on the specific milieu. In this study, we transplanted mouse PGCs collected from male and female gonads at 12.5 days postcoitum, together with gonadal somatic cells, under kidney capsules of adult mice. The transplanted PGC and gonadal somatic cells constructed testis-like and ovary-like tissues, respectively, under the kidney capsules within 4 wk. Normal-appearing round spermatids and fully grown germinal vesicle (GV) oocytes developed within these tissues. Ectopic spermatogenesis continued thereafter, while oogenesis consisted of only a single wave. The injection of these round spermatids directly into mature in vivo-derived oocytes led to the birth at term of normal pups. PGC-derived GV oocytes were isolated, induced to mature in vitro, and injected with normal spermatozoa. The injected oocytes were successfully fertilized and developed into normal pups. Our findings demonstrate the remarkable flexibility of PGC development, which can proceed up to the functional gamete stage under spatially and temporally noninnate conditions. This transplantation system may provide a unique technical basis for induction of the development of early germ cells of exogenous origins, such as those from embryonic stem cells.**

*intracytoplasmic sperm injection, oocyte development, ovary, spermatid, testis*

## INTRODUCTION

Mammalian germ cells first emerge from epiblast cells in early postimplantation embryos as primordial germ cells (PGCs) [1–3]. After specification into germ cells, PGCs undergo dynamic genetic and epigenetic changes that are quite different from those of somatic cells [4]. The most profound change occurs at meiosis, which generates haploid sets of chromosomes and the genomic imprinting that ensures the parent allele-specific functional differences of a subset of genes. All such changes at the genomic and cellular structural levels are requisites for the formation of functional gametes from PGCs and the generation of new life in the next generation. These changes are under the strict control of autonomous systems and from external milieu, including the surrounding somatic cells and the extracellular matrix. Generally, the development of PGCs can be roughly divided into three stages: an early specification stage at the base of the allantois, a middle migratory stage in the hindgut and mesentery, and a late differentiation stage within the genital ridge [5]. Therefore, the complex series of developmental events in PGCs should proceed precisely in a spatially and temporally dependent manner.

Based on the recent developments of cell/tissue culture systems and accumulating knowledge of germ cell biology, many attempts have been made to culture germ cells in vitro by mimicking their development in vivo. However, the complexity of the process of germ cell development has hampered the induction of immature germ cells into functional gametes under culture conditions. Although obstacles may differ between male and female germ cells, meiotic divisions that are predisposed to prophase arrest and erroneous chromosomal segregation are common problems that are difficult to overcome. Therefore, the number of reports of germ cell cultures leading to the successful birth of normal offspring, thus providing definitive evidence of the development of functional gametes, remains very limited. Male germline cells, for example, can only be cultured from pachytene spermatocytes to round spermatids to produce normal pups in mice [6]. Production of female gametes, metaphase II oocytes, was undertaken practically only when culture was started by using early prophase I germinal vesicle (GV) oocytes [7].

Orthotopic or ectopic transplantation of isolated germ cells into host animal bodies represents another strategy for manipulating PGCs or immature germ cells into producing functional gametes. This strategy is most successful with male germ cells in mice, where tubules or the efferent ducts of neonatal testes provide a highly efficient route to introduce immature male germ cells. Using this transplantation system, isolated spermatogonia, germline stem cells, and even early PGCs harvested from 8.5-day-old fetuses can be used to produce spermatids or spermatozoa, which were proven to have fertilizing ability through natural mating or intracytoplasmic sperm injection (ICSI) [8–10]. In contrast, in the case of females, it is generally difficult to perform intraovarian transplantation because the ovaries are filled with follicles and connective tissues and bleed easily after puncture. Recently, we succeeded in transplanting cultured thecal stem cells into ovarian stroma to assess their ability to differentiate in vivo [11]. However, to avoid excessive bleeding during transplantation, it was necessary to remove the host ovary from

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the bursa first and then restore it after cell transplantation. To date, in situ direct intraovarian transplantation of female germ cells has been reported only for isolated female germline stem cells [12].

The present study was undertaken to examine the feasibility of ectopic transplantation of PGCs into adult animals as a means of obtaining functional gametes. We chose the mouse as a model because we consider the production of offspring to be critically important to prove the ultimate normality of the gametes generated. We collected PGCs from gonads at Day 12.5 because this is the earliest stage at which a large number of female and male PGCs can be collected separately based on the appearance of the gonads. After complete dissociation was achieved using enzymatic treatment, PGCs together with gonadal somatic cells were transplanted under the kidney capsules of ovariectomized females.

## MATERIALS AND METHODS

### Animal Care and Mating

All mice were maintained under specific-pathogen-free conditions at the Bioresource Center (RIKEN, Japan). They were housed under controlled lighting conditions (daily light period, 0700 h to 2100 h). Female mice were mated with males overnight and were checked for vaginal plugs the next morning (0.5 day postcoitum [dpc] = noon of the day when a vaginal plug was detected). All animal experiments were conducted in accordance with the guidelines of the RIKEN Tsukuba Institute.

### Transplantation of PGCs Reaggregated with Gonadal Somatic Cells Under the Kidney Capsule

Gonadal cell reaggregation and transplantation were performed according to the protocol described by Qing et al. [13], with minor modifications. Briefly, C57BL/6NCrSlc (B6) females (Japan SLC, Shizuoka, Japan) were mated with green fluorescent protein (GFP)-expressing transgenic C57BL/6-Tg(CAG-EGFP)C14-Y01-FM131Osb males [official symbol, Tg(CAG-EGFP)131Osb] or DBA/2CrSlc males (Japan SLC) to obtain B6-GFP or B6D2F1 (BDF1) embryos, respectively.

Genital ridges (gonads with adjacent mesonephros) were collected from 12.5-dpc embryos, and each gonad was separated from the mesonephros by using a dissecting microscope and sharp needles. The sex of the isolated gonad was determined by its appearance. Four gonads of the same sex were mixed in a 1.5-ml centrifuge tube and dispersed in a 100-μl solution of 0.25% trypsin plus 0.2% collagenase IV at 37°C for 15 min, followed by pipetting 5–10 times. After the addition of 900 μl of culture medium 199 (Sigma, Tokyo, Japan) containing 10% fetal bovine serum (Biowest, France) and 1% antibiotics (penicillin-streptomycin; Invitrogen, Tokyo, Japan), cells were resuspended in 100 μl of culture medium. After the addition of phytohemagglutinin-P (final concentration, 35 μg/ml; Sigma), cell suspensions were incubated at 37°C for 10 min. Subsequently, the suspensions were centrifuged twice at 300 × *g* for 4 min to make reaggregated pellets as grafts. The reaggregated grafts were gently picked up with a truncated 200-μl micropipette, placed on isopore membrane filters (pore size, 3.0 μm; Millipore, Bedford, MA) in culture medium, and cultured for 12–18 h at 37°C. Finally, grafts were transplanted under the kidney capsules of bilaterally ovariectomized recipient female mice of matched strains. The number of transplants per single kidney was one or two.

### Microinjection of Round Spermatids Derived from Transplanted PGCs

Four weeks after transplantation, the grafts that had been reconstructed by using male gonads were removed from the kidney capsules of recipient mice and dissociated mechanically, as described for intact mouse testes [14]. Briefly, the grafts were placed in erythrocyte lysing buffer (155 mM $NH_4Cl$, 10 mM $KHCO_3$, 2 mM ethylenediamine tetraacetic acid, pH 7.2) at 4°C, the presumed tunica albuginea was removed, and the seminiferous tubule masses were transferred into cold (4°C) phosphate-buffered saline (PBS) supplemented with 5.6 mM glucose, 5.4 mM sodium lactate, and 0.1 mg/ml polyvinyl alcohol (GL-PBS). The tubules were cut into small pieces and pipetted gently to disperse cells into the GL-PBS. Subsequently, the cell suspension was filtered through a 38-μm nylon mesh, washed twice by centrifugation (300 × *g* for 4 min), and resuspended in 50 μl of GL-PBS. We found presumptive round spermatids in these suspensions but not elongated spermatids or spermatozoa. Therefore, in the next series of experiments, we performed round spermatid injection (ROSI), as described below.

To collect oocytes for ROSI, 7- to 10-wk-old female BDF1 mice (Japan SLC) were superovulated via injections of equine chorionic gonadotropin (7.5 IU and 2.5 IU), followed by injections of human chorionic gonadotropin (hCG; 7.5 IU and 2.5 IU) 48 h later. Mature metaphase II (MII) oocytes were collected from the oviducts 15–17 h after hCG injection. Cumulus cells were released by treating cultures with 0.1% hyaluronidase in potassium-modified simplex optimization medium (KSOM) [15]. The oocytes were transferred to fresh KSOM and incubated at 37°C in an atmosphere of 5% $CO_2$ in air for up to 90 min before ROSI.

ROSI was performed with a piezo-driven micromanipulator (Prime Tech Ltd., Ibaraki, Japan) [16]. The cover of a plastic dish (Falcon product no. 1006; Becton Dickinson, Franklin Lakes, NJ) was used as a microinjection chamber. Several small drops (~4 μl) of bovine serum albumin (BSA)-free HEPES-buffered KSOM (Hepes-KSOM), with or without 10% polyvinylpyrrolidone (PVP), were placed on the bottom of the plate and covered with mineral oil. Graft-derived cells were placed in one of the PVP droplets. Before oocytes were injected with the nuclei of round spermatids, they were activated by treatment with $Ca^{2+}$-free KSOM containing 2.5 mM $SrCl_2$ for 20 min at 37°C. Oocytes that had reached telophase II at 40–90 min after the onset of activation treatment were each injected with a round spermatid. They were kept in Hepes-KSOM at room temperature (24°C) for 10 min before culture in KSOM at 37°C under 5% $CO_2$ in air.

### In Vitro Maturation of GV Oocytes Derived from Transplanted PGCs and ICSI

Grafts reconstructed with female gonads were picked up from recipient kidneys 4 wk after transplantation. Grafts containing several follicles were mechanically processed to isolate GV oocytes and then subjected to in vitro maturation (IVM), as described previously [17]. Briefly, after antral follicles in the grafts were punctured with sharp needles in a Hepes-buffered 1:1 mixture of TYH (119.3 mM NaCl, 4.78 mM KCl, 1.26 mM $CaCl_2H_2O$, 1.19 mM $KH_2PO_4$, 25.07 mM $NaHCO_3$, 5.56 mM glucose, 1 mM pyruvic acid, 4 mg/ml BSA, 5 mg/100 ml streptomycin, 7.5 mg/100 ml penicillin G) and minimal essential medium alpha (TaM) (Invitrogen), the GV oocytes were collected using a filtered mouth pipette and subjected to IVM for 15–18 h in TaM at 37°C under 5% $CO_2$ in air. Oocytes that reached the MII stage via IVM were subjected to ICSI, as the zona pellucida of graft-derived oocytes was hardened during IVM and fertilization was not possible using conventional in vitro fertilization (data not shown).

ICSI was performed using freeze-thawed sperm from a B6 or Japanese Fancy 1 (JF1/Msf) strain. Frozen sperm (kept for several months in a deep freezer at −80°C) suspended in human tubal fluid medium were thawed by a 3-min incubation in a water bath at 37°C and were stored at 4°C for up to 120 min. The thawed sperm were placed in one of the PVP droplets on the microinjection chamber. The head of a single spermatozoon was separated from its tail by applying a few piezo pulses to the head–tail junction. The isolated sperm head was injected into an oocyte derived from the grafts stored in Hepes-buffered KSOM. Manipulated oocytes were kept at room temperature for 10 min before culturing in KSOM at 37°C under 5% $CO_2$ in air.

### Embryo Transfer and Delivery of Offspring

Micromanipulated embryos that reached the two-cell stage after 24 h of culture in KSOM were transferred into the oviducts of Day-1 pseudopregnant ICR mice (CLEA Japan, Tokyo, Japan). On Day 20, the recipient female mice were examined for the presence of fetuses, and live pups were nursed by lactating ICR foster mothers.

### Histological Analyses

Some of the transplanted grafts removed on Day 28 or 56 after transplantation were fixed overnight in 4% paraformaldehyde in PBS or Bouin solution and were routinely embedded in paraffin. Embedded samples were sectioned (4 μm) and subjected to hematoxylin-eosin (HE) staining and immunohistochemistry. For immunohistochemistry, rehydrated sections were pretreated in a microwave for 10 min in the presence of 10 mM sodium citrate buffer (pH 6.0) for antigen retrieval. Sections were then blocked for 1 h at room temperature in 0.5% blocking reagent (Perkin-Elmer Japan, Yokohama, Japan). Primary antibody incubations were carried out overnight at 4°C in blocking solution, using rabbit anti-mouse Vasa homologue (MVH) protein antibody (1:5000; a gift from Dr. Toshiaki Noce) [18], rabbit anti-heat-shock protein 1-like (HSPA1L)/Hsc70t antibody (1:2000; a gift from Dr. Hirokazu Fujimoto)

[19], and rabbit anti-synaptonemal complex protein 3 (SYCP3) antibody (1:1000; Ab code 15092; Abcam, Japan). The immunoreactions of each primary antibody were visualized using biotin-labeled secondary antibodies and a Vectastain ABC elite kit (Vector Laboratories, Burlingame, CA).

### *Measurement of Serum Hormone Levels*

Blood samples were collected from anesthetized host mice at 4 wk after transplantation, using cardiac puncture, and then centrifuged to separate the serum. Serum testosterone levels were determined using an enzyme immunoassay kit (Cayman Chemical Co., Ann Arbor, MI) according to the manufacturer's instructions. For quantification of serum estradiol levels, samples were applied to a liquid chromatography-tandem mass spectrometry apparatus (ASKA Pharma Medical Co., Ltd., Kawasaki, Japan). Age- and sex-matched nontransplanted animals with or without ovariectomy were used as controls. Data were analyzed statistically using a Student *t* test, and significance was set at a $P$ value of $<0.05$.

## RESULTS

### *Gonadal Cells Formed Testis- or Ovary-Like Tissue Structures Under the Kidney Capsule*

We transplanted 12.5-dpc PGCs, which had just initiated sex-dependent differentiation, ectopically, together with age-matched somatic cells, to examine the extent to which they could undergo normal development. Before transplantation, four 12.5-dpc gonads were completely dissociated to single cells by enzymatic treatment (Fig. 1C). The mean number of cells collected from a single male and female gonad was $2.90 \times 10^5 \pm 0.07 \times 10^5$ SEM (including $1.00 \times 10^5 \pm 0.09 \times 10^5$ PGCs) and $1.84 \times 10^5 \pm 0.08 \times 10^5$ (including $6.24 \times 10^4 \pm 0.07 \times 10^4$ PGCs), respectively ($n = 4$ gonads). Cells were reaggregated overnight as a single graft and transplanted under the kidney capsules of bilaterally ovariectomized female mice (Fig. 1A). In the first series of experiments, we used gonads from B6-GFP fetuses to distinguish transplanted reaggregated tissues from recipient host cells. Male and female reaggregated grafts developed testis- and ovary-like tissues, respectively, within 4 wk after transplantation (Fig. 1D and E). Similar tissue regeneration and development activities were observed with BDF1-derived donor cells for both sexes (Fig. 1F). After this time point, however, the male testis-like tissues became swollen and vacuolar, whereas the female ovary-like structures were atrophic in appearance (Fig. 2). Teratoma formation was not observed in host animals at any time point analyzed. These results indicate that embryonic gonadal cells of both sexes reconstructed the gonadal structure ectopically under the kidney capsule and that their maximum development was reached at around 4 wk after transplantation. In some experiments, we reaggregated gonadal cells from different donor strains (e.g., GFP-expressing and non-GFP-expressing animals) and confirmed chimeric distribution in the reconstructed tissues (Fig. 1G).

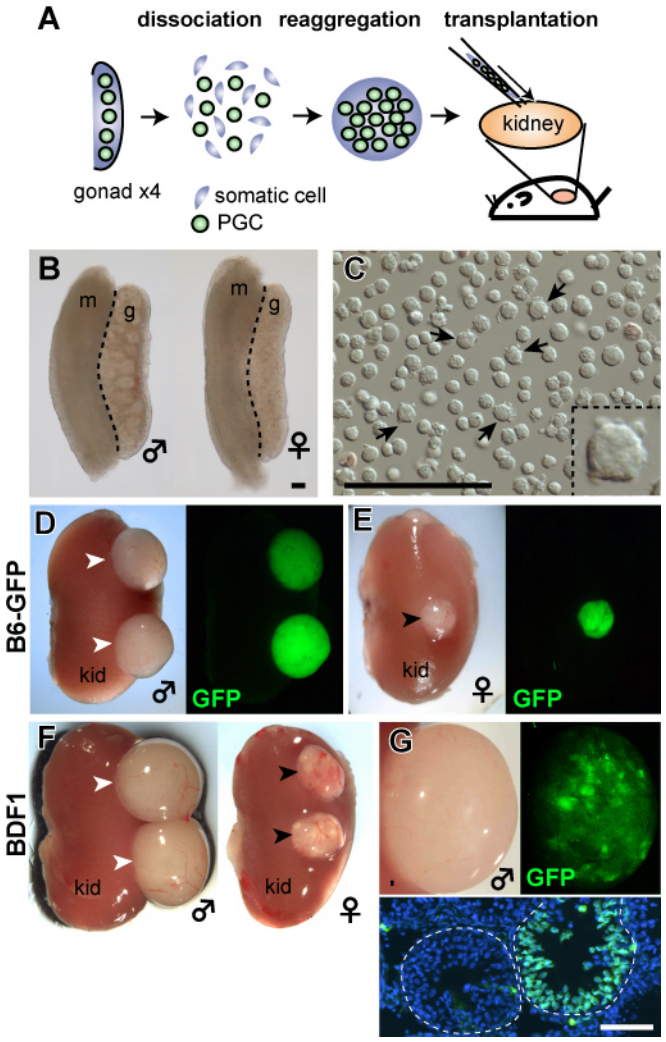


FIG. 1. Transplantation of PGCs with gonadal somatic cells under the kidney capsule. **A**) Schematic shows the transplantation procedure used in this study. Two pairs of sex-matched gonads at 12.5 dpc were dissociated, reaggregated, and transplanted under the kidney capsules of ovariectomized female recipient mice. **B**) Appearance of male and female gonads (g) with adjacent mesonephros (m) of 12.5-dpc embryos is shown. **C**) Microscopic appearance of gonadal dissociates of 12.5-dpc female gonads. Arrows and inset indicate PGCs with their characteristic lamellipodia. **D**–**F**) Gross appearance of grafts at 4 wk after transplantation under the kidney (kid) capsule is shown. Male and female grafts formed testis-like (white arrowheads) and ovary-like (black arrowheads) tissues, respectively, in both B6-GFP (**D** and **E**) and BDF1 (**F**) strains. **G**) A testis-like tissue generated by mixing gonadal cells from different strains (GFP and non-GFP mice). The chimeric composition of the entire tissue was confirmed by GFP fluorescence in the gross appearance (**upper right panel**) and in a histological section (**lower panel**). Bar = 100 μm.

### *Male PGCs from BDF1 Mice, but Not from B6-GFP Mice, Completed Meiosis Under the Kidney Capsule*

Next, we histologically examined the testis-like tissues reconstructed in males. The HE-stained sections largely resembled those of normal adult testes, containing many seminiferous tubules and mesenchymal cells that filled the interstitial spaces (Fig. 3A–H). However, we found that the development of germ cells within the seminiferous tubules was greatly affected by the genotype of the donor cells (Fig. 3A–H). The seminiferous tubules derived from B6 donor cells contained only a few cell layers (Fig. 3A), whereas those from BDF1 donor cells formed thick epithelia with several cell layers (Fig. 3E). For precise identification of germ cells, we stained the sections by using an antibody to MVH/DDX4, a marker of spermatogonia-to-round spermatid stages [18]. As expected, the thick epithelia of BDF1-derived tissues were strongly stained for DDX4; in contrast, those of B6-GFP-derived tissues showed no positively stained cells, except for the occasional presence of presumptive spermatogonia (Fig. 3B). Furthermore, the seminiferous tubules from BDF1 tissues were positive for the SYCP3 protein (a marker of meiotic cells) and for the HSPA1L/Hsc70t protein (a marker of elongated spermatids), suggesting the completion of meiosis in these testis-like tissues. We found presumptive round spermatids with characteristically small nuclei and nucleolus-like struc-

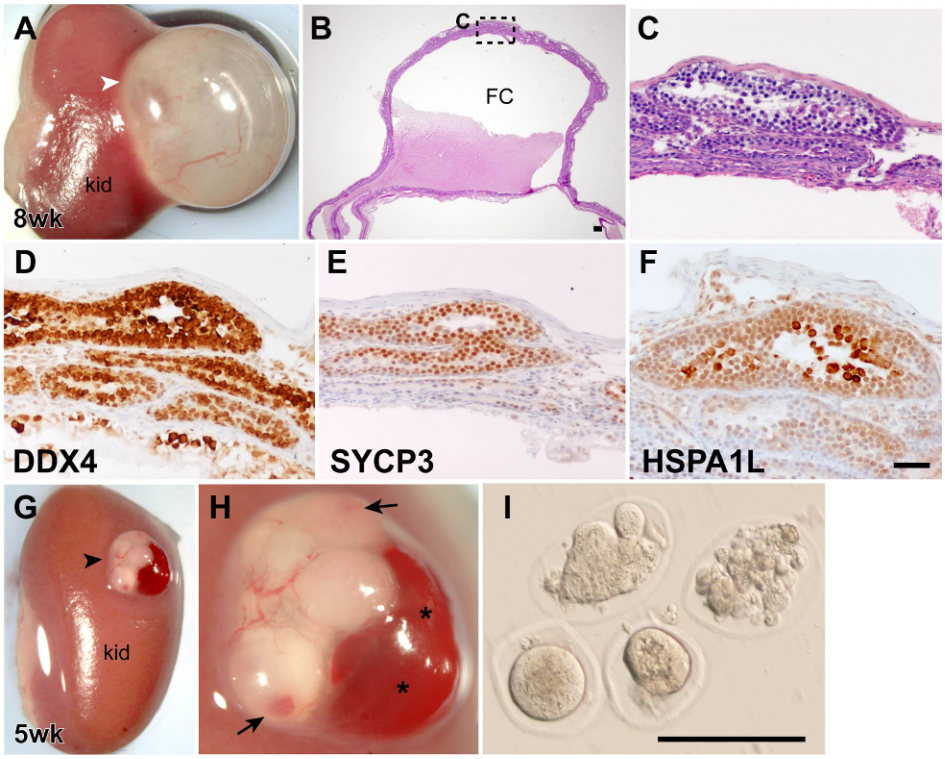


FIG. 2. Male (**A**–**F**) and female (**G**–**I**) grafts collected at different time points (>4 wk after transplantation) are shown. **A**) Gross appearance of a testis-like tissue (white arrowhead) from BDF1 mice at 8 wk posttransplantation. The size of this structure was much larger than that observed at 4 wk (see Fig. 1F). As shown in an HE-stained section (**B**), the enlargement was caused by extraordinarily swollen seminiferous tubules that contained fluid (FC). In the periphery, some seminiferous tubules maintained their normal epithelial structure and underwent spermatogenesis, even at 8 wk after transplantation (**C**), as shown by positive staining for DDX4 (**D**), SYCP3 (**E**), and HSPAL1 (**F**). (**G** and **H**) An ovary-like tissue from BDF1 mice shows a hemorrhagic appearance at 5 wk after transplantation. In addition to a few large blood-containing follicles (or corpuses; asterisks), many follicles exhibited a red spot (arrows), indicating previous ovulation. Although a few oocytes were collected from the remaining follicles, they were completely degenerated (**I**). Bar = 100 μm.

tures in HE-stained sections of BDF1 tissues (Fig. 3I). In the seminiferous tubules containing germ cells, the mean frequency of round spermatids was 0.33% ± 0.09% SEM (n = 4) of the germ cell population. At 8 wk after transplantation, the testis-like tissues were occupied mostly by large vacuoles originating from extraordinarily swollen seminiferous tubules. However, there were several seminiferous tubules containing round spermatids in the peripheral areas of these testis-like

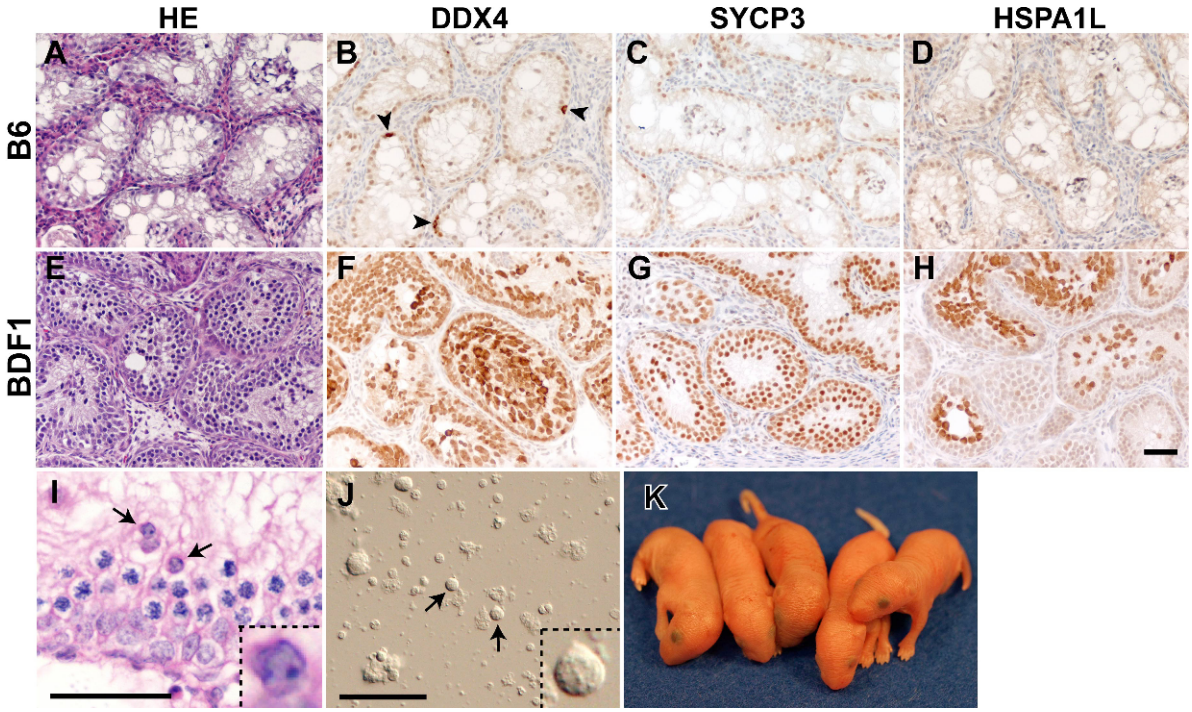


FIG. 3. Histological views of testis-like tissues at 4 wk after transplantation and collection of round spermatids are shown. B6-GFP-derived (**A**–**D**) and BDF1-derived (**E**–**H**) tissues were very different regarding their spermatogenic ability. As shown by HE staining (**A** and **E**) and immunostaining for DDX4 (**B** and **F**; wide-range germ cell marker from spermatogonia to spermatids), SYCP3 (**C** and **G**; meiotic cell marker), and HSPA1L (**D** and **H**; elongated spermatid marker), only a few presumptive spermatogonia survived in B6-GFP-derived tissues (**B**, arrowheads), whereas BDF1-derived tissues contained thick spermatogenic layers with postmeiotic spermatogenic cells. Presumptive spermatids were observed in an HE-stained section (**I**, arrowheads and inset) and dissociated suspension (**J**, arrowheads and inset) of BDF1-derived tissues. **K**) Newborn offspring produced by ROSI using these presumptive round spermatids from BDF1 grafts. Bars = 100 μm.

TABLE 1. In vitro maturation of oocytes and development of embryos after microinsemination with PGC-derived male and female gametes.

| | | In vitro maturation of oocytes | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|
| Sex of PGCs | Strain of mice | Total GV | Matured to MII (%) | Total injected | Survived (%) | Two-cell (% per survived) | Embryos transferred | Implanted (% per ET)[a] | Term fetuses (% per ET)[a] |
| XY | BDF1 | | | 214 | 181 (84.6) | 135 (74.6) | 135 | 42 (31.1) | 8 (5.9) |
| XX | B6 | 165 | 109 (66.1) | 109 | 64 (58.7) | 45 (70.3) | 42 | 2 (4.8) | 0 (0) |
| | BDF1 | 312 | 263 (84.3) | 263 | 171 (65.0) | 118 (69.0) | 104 | 26 (25.0) | 6 (5.8) |

[a] ET, embryos transferred.

tissues, similar to those observed at 4 wk, indicating that spermatogenic waves continued in the reconstructed tissues from under the kidney capsule for at least 8 wk (Fig. 2).

### *Production of Normal Pups from Round Spermatids Derived from Transplanted PGCs*

To examine the developmental potential of presumptive spermatids in BDF1 testis-like tissues, we performed ROSI into in vivo-derived BDF1 oocytes. We mechanically dissociated the testis-like tissues at 4 wk after transplantation and observed the isolated cells with an inverted microscope. Using differential interference contrast microscopy, we identified normal appearing round spermatids with characteristically round and small nuclei and nucleolus-like structures but not elongated spermatids in the cell suspension (Fig. 3J). In four replicated ROSI experiments, the majority (84.6%) of oocytes survived injection, and 74% of oocytes developed to the two-cell stage on the following day (Table 1). After the transfer of 135 two-cell embryos into pseudopregnant female mice, eight pups (three males and five females [5.9% per transfer]) were born at term (Fig. 3K and Table 1). One pup died within a day, but the remaining seven pups grew to adulthood and showed a variety of coat colors, as expected from the BDF1 × BDF1 mating (black, brown, diluted black, and diluted brown), and normal fertility when mated with mice of the opposite sex.

### *Female PGCs Developed into GV Oocytes Under the Kidney Capsule and Matured In Vitro*

We also undertook histological examination of the ovary-like tissues derived from female 12.5-dpc gonadal cells. At 4 wk after transplantation, an ovarian mass from each of the B6 and the BDF1 strains contained many antral follicles (Fig. 4A and E). Histologically, there seemed to be no distinct boundary between the cortex and the medulla, and large antral follicles were also observed in the central area of the tissue. Interestingly, we found several polyovular follicles (containing more than one oocyte in a single follicle) in both strains (Fig. 4A and E, asterisks). Mechanical dissociation of the ovary-like tissues enabled the collection of a mean of 20 ± 14.2 SEM ($n = 8$) and of 28.4 ± 9.7 ($n = 11$) GV oocytes from B6-GFP- and BDF1-derived tissues, respectively (total, 165 and 312, respectively [Fig. 4B and F]). These oocytes were fully grown, with diameters of 65–70 μm. After undergoing IVM for 15–18 h, >50% of oocytes were extruded from the first polar body and reached MII, regardless of genotype (Fig. 4C and G and Table 1). At 5 wk after transplantation, ovary-like tissues showed a hemorrhagic appearance, with many follicles exhibiting a red spot, indicative of previous ovulation (Fig. 2G and H). Although a few oocytes were collected from the remaining follicles, these cells were completely degenerated (Fig. 2I). This finding suggests that only a single wave of oogenesis occurred in the reconstructed ovary-like tissues.

### *Production of Pups from BDF1 Graft-Derived Oocytes*

To examine the developmental potential of the MII oocytes derived from ovary-like grafts, these oocytes were subjected to ICSI. As shown in Table 1, more than half of the sperm-injected oocytes survived in both the B6-GFP and the BDF1 strains (58.7% and 65.0%, respectively), and most of them developed to the two-cell stage (70.3% and 69.0%, respectively) (Fig. 4D). After these two-cell embryos were transferred into recipient female mice, only 4.8% of B6-GFP/ICSI embryos implanted in the uteri of recipients and no live embryos reached term (Table 1). In contrast, 25.0% (26/104) of the BDF1/ICSI embryos transferred underwent implantation, and six pups (two males and four females) were obtained at term (5.8% per transferred embryo [Fig. 4H and Table 1]). One female pup died after 1 wk, but the remaining five pups grew to adulthood, and both sexes showed normal fertility.

### *Serum Hormone Levels in Host Animals*

To determine whether the testis- and ovary-like tissues generated under the kidney capsules were endocrinologically functional, we measured the serum levels of testosterone and estradiol in the host animals. As shown in Figure 5, the serum concentrations of these hormones were significantly higher in host animals than in the nontransplanted, ovariectomized animals, although they were still lower than that of intact controls. This finding clearly indicates that the testis- and ovary-like tissues generated under the kidney capsules were also endocrinologically functional.

## DISCUSSION

The present study provides the first demonstration that male and female PGCs transplanted together with somatic cells under the kidney capsule of adult animals undergo normal development to form functional gametes, which subsequently supported full-term embryonic development. This finding unequivocally indicates that PGCs may exhibit remarkable spatial and temporal flexibility in their development, as long as they remain under in vivo conditions. The key to the successful development and survival of PGCs was most likely the coexistence of a set of appropriate somatic cells. As the preparation of somatic cell suspensions from fetal gonads of defined ages is a relatively easy procedure with most laboratory or domestic species, the transplantation procedure presented in this study would be applicable to a broad range of experiments in the field of germ cell biology. This preparation would be of particular significance for the germ cells of females, as orthotopic transplantation of female germ cells into the ovaries is generally unattainable because they are rapidly eliminated from the host tissues after physical damage or other unknown causes. Recently, we developed a two-step intraovarian method for transplantation of thecal stem cells [11]; however, the same technique did not work well for primordial germ cells (Matoba

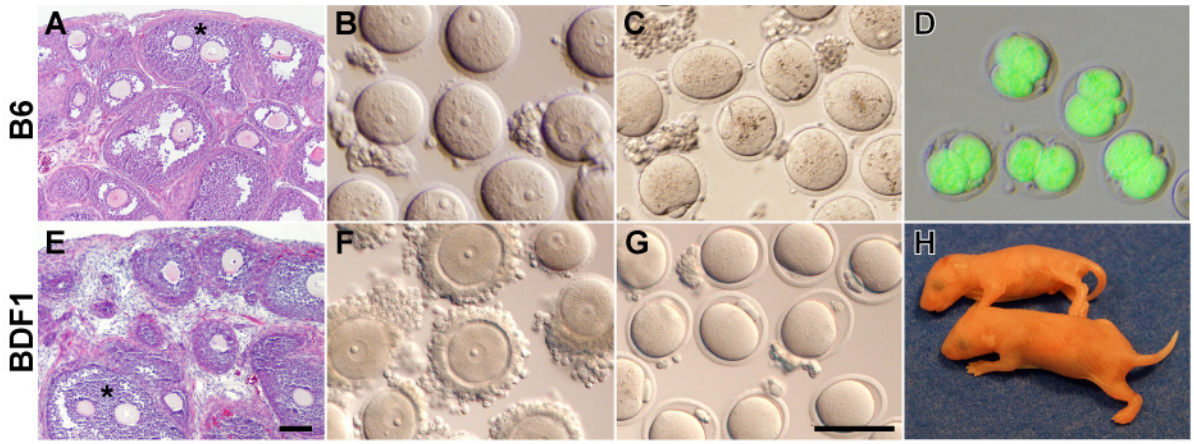


FIG. 4. Histological observation of ovary-like tissues at 4 wk after transplantation and collection of oocytes. The histological appearance of B6-GFP-derived (**A**) and BDF1-derived (**E**) tissues was indistinguishable from each other. HE staining of sections. Asterisks indicate polyovular follicles. **B** and **F**) GV oocytes isolated from B6-GFP and BDF1 grafts via mechanical dissociation. **C** and **G**) MII oocytes obtained via in vitro maturation of these GV oocytes. MII oocytes in both groups developed into the 2-cell stage at a similar rate after ICSI (**D**: 2-cell stage from GFP-bearing B6-GFP oocytes). However, embryos from BDF1, but not B6-GFP mice, developed into normal offspring after embryo transfer (**H**; see Table 1). Bar = 100 μm.

and Ogura, unpublished data). In contrast to transplantation of female germ cells, male germ cells can be readily transplanted into the seminiferous tubules and will develop to a certain extent, depending on experimental conditions such as the species and age of the animals and the immunological factors involved [20].

Although PGCs transplanted under the kidney capsule developed into functional gametes that could be used for normal fertilization, the efficiency of gametic production was apparently different between males and females. In males, the testis-like tissues continued spermatogenesis for at least 8 wk after transplantation, despite the occasional enlargement of the tubular lumens. In contrast, germ cell development in the ovary-like tissues apparently showed age-related changes and reached maximal development at around 4 wk after transplantation, when most oocytes developed into the fully grown GV stage within antral follicles. Thereafter, the follicles started to degenerate (by Week 5) and were replaced with interstitial tissues. Histological examination of the ovary-like tissues suggests there was no hierarchical follicular development, which normally governs continuous oogenesis waves in ovaries. Therefore, in these ovary-like tissues, most follicles developed synchronously, and only a single oogenesis wave occurred. This was probably due to the absence of medulla cortex components in these follicles, thus leading to the indiscriminate exposure of developing follicles to a high level of follicle-stimulating hormone in the ovariectomized recipient females [21]. Therefore, the timing of retrieval of transplanted female germ cells from the reconstructed tissues may be critically important when they are transplanted under the kidney capsule. Regarding this transplantation method, Qing et al. [13] should be credited for the first successful generation of PGC-derived GV oocytes under the kidney capsule, although they did not report subsequent embryonic development, unlike that described in the present study. The unavailability of ICSI or the genetic background of the oocytes (see below) may have hampered the functional examination of the oocytes generated. Interestingly, according to Hashimoto et al. [22], the fetal ovaries or their reaggregates seemed to ovulate periodically for several months when transplanted into the ovarian bursa.

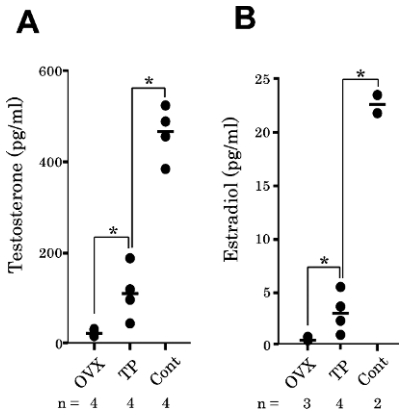


FIG. 5. Serum hormone levels of host animals that received male (**A**) and female (**B**) gonadal cells under their kidney capsule. The testosterone and estradiol levels in the host animals (TP) were significantly higher compared with those detected in nontransplanted ovariectomized animals (OVX) in both groups, whereas they did not reach the levels detected in intact males and females, respectively (Cont). Asterisks indicate the presence of significant differences between the groups ($P < 0.05$).

We also found apparent genotype-dependent differences in the outcome of the transplantation experiments, in terms of the efficiency of germ cell production and the subsequent development of reconstructed embryos. In males, the development of germ cells in the testis-like tissues was critically affected by the donor genotype. The BDF1-derived PGCs developed into round spermatids, which are haploid germ cells that complete meiosis; however, B6-derived PGCs arrested their development at very early premeiotic stages. First, we did not expect that the transplanted PGCs would undergo spermatogenesis beyond meiosis, as their surrounding environment was the same as that of the cryptorchidism-induced experiments. In mice, a cryptorchid testis can be easily created by suturing the testis onto the inside wall of the abdomen, where the temperature is >37°C; this may induce heat stress and apoptosis in male germ cells. According to the literature, male germ cells from the majority of strains, including B6-GFP, are more sensitive to heat-shock stress than strains such as AKR and MRL/MpJ, which are less

sensitive [23]. We recently confirmed that BDF1 testes survived the cryptorchid operation better than B6-GFP testes, at least for 4 wk (Matoba and Ogura, unpublished data). Therefore, it is likely that the high developmental potential of PGCs from BDF1 is attributable to their high tolerance to heat stress. Presently, however, we do not know whether this characteristic was due to F1 hybrid vigor or to the paternal DBA/2 genome.

In females, the GV oocytes derived from B6-GFP and BDF1 PGCs were equally able to develop into MII oocytes, zygotes, and even two-cell embryos. After embryos were transferred, however, very few of the two-cell embryos from B6-GFP donors underwent implantation, and no embryos survived to term. Although it is well known that B6-GFP oocytes can be practically used for the production of offspring via ICSI [24], they are usually in vivo-ovulated oocytes. Generally, oocytes from inbred strains are more vulnerable to in vitro manipulation than F1 hybrid strains; therefore, experiments involving IVM, ICSI, and nuclear transfer in mice usually use F1 oocytes [17, 25, 26]. Such genotype-related differences in developmental competence may appear most frequently at the two-cell stage, the so-called typical developmental block in mice [27], which was probably the case in our study.

The fact that PGCs can develop into functional gametes ectopically in adult mice has important practical implications. Several studies have reported the in vitro generation of germ cells from embryonic stem cells (ESCs) in mice, monkeys, and humans via spontaneous differentiation or culturing as embryoid bodies [28]. However, it is generally very hard to induce the meiotic division of these germ cells in vitro, primarily because of the complexity of the pairing and segregation of the homologous chromosomes during the first meiosis. In female germ cells derived from mouse ESCs, for example, the nuclear distribution of the SCP3 protein was not associated with the chromosomes, indicating that normal chromosomal pairing did not occur [29]. To date, therefore, successful in vitro oogenesis studies are limited to the use of oocytes that have already entered meiosis I [7, 30–32]. Induction of spermatogenesis in vitro is also very difficult because of the inability of testicular cells to reconstruct the three-dimensional structure of seminiferous epithelium in culture. As far as we know, two meiotic divisions of spermatogenic cells can proceed reproducibly only when their culture is started using late spermatocyte stages [6]. As our transplantation system provides a platform for the development of male and female PGCs into functional gametes, ESC-derived PGCs may also develop over the meiotic phase when transplanted together with appropriate somatic cells. We presently believe this is the most practical and reliable way to examine the normality of germ cells derived from ESCs.

Conversely, we ponder the differences between the in vivo and in vitro conditions that support germ cell development. Our study clearly demonstrates that PGCs can develop even under a spatially and temporally noninnate condition. In other words, the presence of somatic cell support and use of ''inside the body'' conditions may be the only requisites for the development of germ cells. The critical in vivo-specific properties may include blood vessel formation [33, 34] and some other physiological factors that are lacking under in vitro conditions. More detailed investigation of the in vivo microenvironment, such as that under the kidney capsule, may allow the future generation of a new culture system that supports long-term germ cell development.

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## REFERENCES

1. Extavour CG, Akam M. Mechanisms of germ cell specification across the metazoans: epigenesis and preformation. Development 2003; 130:5869–5884.
2. Hayashi K, de Sousa Lopes SM, Surani MA. Germ cell specification in mice. Science 2007; 316:394–396.
3. Saitou M. Specification of the germ cell lineage in mice. Front Biosci 2009; 14:1068–1087.
4. Sasaki H, Matsui Y. Epigenetic events in mammalian germ-cell development: reprogramming and beyond. Nat Rev Genet 2008; 9:129–140.
5. Molyneaux K, Wylie C. Primordial germ cell migration. Int J Dev Biol 2004; 48:537–544.
6. Marh J, Tres LL, Yamazaki Y, Yanagimachi R, Kierszenbaum AL. Mouse round spermatids developed in vitro from preexisting spermatocytes can produce normal offspring by nuclear injection into in vivo-developed mature oocytes. Biol Reprod 2003; 69:169–176.
7. Picton HM, Harris SE, Muruvi W, Chambers EL. The in vitro growth and maturation of follicles. Reproduction 2008; 136:703–715.
8. Brinster RL, Zimmermann JW. Spermatogenesis following male germ-cell transplantation. Proc Natl Acad Sci U S A 1994; 91:11298–11302.
9. Ogawa T, Dobrinski I, Avarbock MR, Brinster RL. Transplantation of male germ line stem cells restores fertility in infertile mice. Nat Med 2000; 6:29–34.
10. Chuma S, Kanatsu-Shinohara M, Inoue K, Ogonuki N, Miki H, Toyokuni S, Hosokawa M, Nakatsuji N, Ogura A, Shinohara T. Spermatogenesis from epiblast and primordial germ cells following transplantation into postnatal mouse testis. Development 2005; 132:117–122.
11. Honda A, Hirose M, Hara K, Matoba S, Inoue K, Miki H, Hiura H, Kanatsu-Shinohara M, Kanai Y, Kono T, Shinohara T, Ogura A. Isolation, characterization, and in vitro and in vivo differentiation of putative thecal stem cells. Proc Natl Acad Sci U S A 2007; 104:12389–12394.
12. Zou K, Yuan Z, Yang Z, Luo H, Sun K, Zhou L, Xiang J, Shi L, Yu Q, Zhang Y, Hou R, Wu J. Production of offspring from a germline stem cell line derived from neonatal ovaries. Nat Cell Biol 2009; 11:631–636.
13. Qing T, Liu H, Wei W, Ye X, Shen W, Zhang D, Song Z, Yang W, Ding M, Deng H. Mature oocytes derived from purified mouse fetal germ cells. Hum Reprod 2008; 23:54–61.
14. Ogura A, Yanagimachi R. Round spermatid nuclei injected into hamster oocytes from pronuclei and participate in syngamy. Biol Reprod 1993; 48: 219–225.
15. Lawitts JA, Biggers JD. Culture of preimplantation embryos. Methods Enzymol 1993; 225:153–164.
16. Kimura Y, Yanagimachi R. Intracytoplasmic sperm injection in the mouse. Biol Reprod 1995; 52:709–720.
17. Miki H, Ogonuki N, Inoue K, Baba T, Ogura A. Improvement of cumulus-free oocyte maturation in vitro and its application to microinsemination with primary spermatocytes in mice. J Reprod Dev 2006; 52:239–248.
18. Toyooka Y, Tsunekawa N, Takahashi Y, Matsui Y, Satoh M, Noce T. Expression and intracellular localization of mouse Vasa-homologue protein during germ cell development. Mech Dev 2000; 93:139–149.
19. Tsunekawa N, Matsumoto M, Tone S, Nishida T, Fujimoto H. The Hsp70 homolog gene, Hsc70t, is expressed under translational control during mouse spermiogenesis. Mol Reprod Dev 1999; 52:383–391.
20. Rodriguez-Sosa JR, Dobrinski I. Recent developments in testis tissue xenografting. Reproduction 2009; 138:187–194.
21. Bronson FH, Channing CP. Suppression of serum follicle-stimulating hormone by follicular fluid in the maximally estrogenized, ovariectomized mouse. Endocrinology 1978; 103:1894–1898.
22. Hashimoto K, Noguchi M, Nakatsuji N. Mouse offspring derived from fetal ovaries or reaggregates which were cultured and transplanted into adult females. Develop Growth Differ 1992; 34:233–238.
23. Kazusa K, Namiki Y, Asano A, Kon Y, Endoh D, Agui T. Differences in spermatogenesis in cryptorchid testes among various strains of mice. Comp Med 2004; 54:179–184.
24. Ogonuki N, Mori M, Shinmen A, Inoue K, Mochida K, Ohta A, Ogura A.

The effect on intracytoplasmic sperm injection outcome of genotype, male germ cell stage and freeze-thawing in mice. PLoS One 2010; 5:e11062.
25. Ogura A, Matsuda J, Yanagimachi R. Birth of normal young after electrofusion of mouse oocytes with round spermatids. Proc Natl Acad Sci U S A 1994; 91:7460–7462.
26. Wakayama T, Perry AC, Zuccotti M, Johnson KR, Yanagimachi R. Full-term development of mice from enucleated oocytes injected with cumulus cell nuclei. Nature 1998; 394:369–374.
27. Goddard MJ, Pratt HP. Control of events during early cleavage of the mouse embryo: an analysis of the ''2-cell block.'' J Embryol Exp Morphol 1983; 73:111–133.
28. Chuva de Sousa Lopes SM, Roelen BA. On the formation of germ cells: the good, the bad and the ugly. Differentiation 2010; 79:131–140.
29. Novak I, Lightfoot DA, Wang H, Eriksson A, Mahdy E, Höög C. Mouse embryonic stem cells form follicle-like ovarian structures but do not progress through meiosis. Stem Cells 2006; 24:1931–1936.
30. Klinger FG, De Felici M. In vitro development of growing oocytes from fetal mouse oocytes: stage-specific regulation by stem cell factor and granulosa cells. Dev Biol 2002; 244:85–95.
31. Eppig JJ, O'Brien MJ. Development in vitro of mouse oocytes from primordial follicles. Biol Reprod 1996; 54:197–207.
32. Honda A, Hirose M, Inoue K, Hiura H, Miki H, Ogonuki N, Sugimoto M, Abe K, Kanatsu-Shinohara M, Kono T, Shinohara T, Ogura A. Large-scale production of growing oocytes in vitro from neonatal mouse ovaries. Int J Dev Biol 2009; 53:605–613.
33. Abir R, Orvieto R, Raanani H, Feldberg D, Nitke S, Fisch B. Parameters affecting successful transplantation of frozen-thawed human fetal ovaries into immunodeficient mice. Fertil Steril 2003; 80:421–428.
34. Demeestere I, Simon P, Emiliani S, Delbaere A, Englert Y. Orthotopic and heterotopic ovarian tissue transplantation. Hum Reprod Update 2009; 15: 649–665.