

# Spermatogenesis from epiblast and primordial germ cells following transplantation into postnatal mouse testis

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## Summary

Primordial germ cells (PGCs) are derived from a population of pluripotent epiblast cells in mice. However, little is known about when and how PGCs acquire the capacity to differentiate into functional germ cells, while keeping the potential to derive pluripotent embryonic germ cells and teratocarcinomas. In this investigation, we show that epiblast cells and PGCs can establish colonies of spermatogenesis after transfer into postnatal seminiferous tubules of surrogate infertile mice. Furthermore, we obtained normal fertile offspring by microinsemination using spermatozoa or spermatids derived from PGCs harvested from fetuses as early as 8.5 days post coitum.

Thus, fetal male germ cell development is remarkably flexible, and the maturation process, from epiblast cells through PGCs to postnatal spermatogonia, can occur in the postnatal testicular environment. Primordial germ cell transplantation techniques will also provide a novel tool to assess the developmental potential of PGCs, such as those manipulated *in vitro* or recovered from embryos harboring lethal mutations.

Key words: Germ cell, Epiblast, Primordial Germ Cell (PGC), Pro-spermatogonia, Spermatogonia, Testis, Spermatogenesis, Transplantation, Microinsemination

## Introduction

Mammalian germ cells undergo unique genetic and cellular changes as they develop and differentiate to form functional gametes. A population of pluripotent epiblast cells at around 6.5 days post coitum (dpc) gives rise to primordial germ cells (PGCs), which become identifiable as a cluster of cells at the base of the allantois at 7.25 dpc (Ginsburg et al., 1990; Tam and Zhou, 1996; Tsang et al., 2001). During development, the number of PGCs increases from 40 cells at 7.5 dpc to 25,000 cells at 13.5 dpc, and they migrate through the developing hindgut and mesentery to reach the urogenital ridge (UGR) at around 10.5 dpc. By 13.5 dpc, PGCs in the male genital ridge enter into mitotic arrest and become pro-spermatogonia, while germ cells in the female arrest at meiotic prophase I (reviewed by McLaren, 2003). Primordial germ cells show different features at different developmental stages. For example, migratory-stage PGCs exhibit a higher frequency of conversion into embryonic germ cells, pluripotent cells that resemble blastocyst-derived embryonic stem cells, than do PGCs in the gonads (Matsui et al., 1992; Resnick et al., 1992; Labosky et al., 1994). In addition, epigenetic changes characteristic to germline cells also occur in PGCs. Erasure of parental genomic imprints on both paternal and maternal alleles in PGCs commences near the time of their settlement in the UGR at 10.5 dpc, and new imprints are imposed in pro-spermatogonia before birth (Szabo and Mann, 1995; Ueda et al., 2000; Surani,

2001; Hajkova et al., 2002). Therefore, the characteristics of PGCs change during development before they mature into postnatal germ cells.

Spermatogenesis is initiated shortly after birth (Russell et al., 1990; Meistrich and van Beek, 1993). Pro-spermatogonia resume mitosis as spermatogonia, at around postnatal day 5, then enter into meiosis as spermatocytes and produce spermatids, which develop into spermatozoa. Spermatogonial stem cells are a subpopulation of spermatogonia and have the unique ability to self-renew as well as to differentiate to produce spermatozoa (Meistrich and van Beek, 1993; de Rooij and Russell, 2000). These cells continue to divide throughout the life of the animal, and can be identified by their ability to generate and maintain colonies of spermatogenesis following transplantation into the seminiferous tubules of infertile recipient testes (Brinster and Zimmermann, 1994). Using this assay, several groups have shown that pro-spermatogonia in developing fetal testes can differentiate into spermatogonial stem cells when transferred into the adult testis (Ohta et al., 2004; Jiang and Short, 1998). However, it is unknown if germline cells at earlier stages of development can produce spermatogonial stem cells or spermatogenic colonies after transplantation.

In this investigation, we sought to determine the potential of germline cells from earlier embryos to develop into spermatogonial stem cells, using immature recipient animals.

Epiblast cells or PGCs were transplanted into infertile mouse testes and examined for their ability to re-populate the seminiferous tubules.

## Materials and methods

### Collection of donor cells

Donor cells were collected from pregnant C57BL/6 mice that were maintained in a controlled environment with 12:12 light:dark cycles from 08.00 h to 20.00 h (SLC, Shizuoka, Japan). The day when a copulation plug was found was designated as 0.5 dpc. In some experiments, we used a transgenic mouse line C57BL/6 Tg14 (act-EGFP) OsbY01 (designated Green) provided by Dr M. Okabe (Osaka University, Osaka, Japan) (Okabe et al., 1997). The spermatogonia, spermatocytes and round spermatids of these mice express the enhanced green fluorescent protein (EGFP) gene, which gradually decreases after meiosis. The sex of embryos was determined using genotyping based on the *Ube1* PCR method (Chuma and Nakatsuji, 2001), and only male embryos were used in this study. Due to the limitations of cell recovery and the number of animals that could be injected per day, the sex check was not performed in experiments using 6.5 dpc embryos. Cells for transplantation were obtained from whole embryonic ectoderm with primitive endoderm at 6.5 dpc, or germ-cell-containing tissues by dissection of the posterior thirds of 8.5 dpc embryos, the mesenteries and guts of 10.5 dpc embryos, the UGRs of 10.5 dpc embryos, and the genital ridges of 11.5, 12.5, 14.5 and 16.5 dpc embryos. Tissues from each developmental stage were dissociated by enzymatic digestion using 0.25% trypsin with 1 mmol/l EDTA (Invitrogen, Carlsbad, CA) for 10 minutes. Cells were suspended in Dulbecco's modified Eagle's medium, supplemented as previously described (Ogawa et al., 1997).

### Transplantation into recipient testes

Donor cells were transplanted in histocompatible  $W/W^u$  or  $W^u/W^u$  mice ( $W$  mice, obtained from SLC, Shizuoka, Japan). Only 5- to 10-day-old male mice were used as recipients.  $W$  mutants lack endogenous spermatogenesis (Silvers, 1979), because of mutations in the *Kit* gene (Nocka et al., 1990; Hayashi et al., 1991). Recipient animals were placed on ice to induce hypothermic anesthesia, and returned to their dams after surgery (Shinohara et al., 2001). Approximately 2  $\mu$ l of cell suspension were introduced into each testis by injection via the efferent duct (Ogawa et al., 1997).

### Histological analysis

Three to four months after transplantation, the recipient testes were fixed in 10% neutral-buffered formalin (Wako Pure Chemical Industries, Osaka, Japan) and processed for paraffin sectioning. Sections were stained with hematoxylin and eosin. Two histological sections were prepared from the testes of each animal and viewed at 400 $\times$  magnification to determine the extent of spermatogenesis. The numbers of tubule cross-sections with or without spermatogenesis (defined as the presence of multiple layers of germ cells in the seminiferous tubule) were recorded for one histological section from each testis. Meiosis was detected by immunofluorescence staining using anti-synaptonemal complex protein 3 (SCP3) antibody (Chuma and Nakatsuji, 2001) and Alexa 488-conjugated anti-rabbit immunoglobulin G antibody (Molecular Probes, Eugene, USA). Periodic acid Schiff (PAS) staining (Muto Pure Chemicals, Tokyo, Japan) was carried out to examine acrosome formation in spermatids. In experiments using Green mice, recipient testes were recovered 10 to 11 weeks after donor cell transplantation, and analyzed by observing EGFP signals under fluorescence microscopy. Donor cells were identified specifically because host testis cells had no endogenous fluorescence. A cluster of germ cells was defined as a colony when it occupied the entire circumference of the tubule and was at least 0.1 mm long (Nagano et al., 1999). Cryosections of the

testes fixed in 4% paraformaldehyde in PBS were stained with Rhodamine-conjugated Peanut agglutinin (PNA) (Vector, Burlingame, CA) for acrosomes, and with Hoechst 33258 (Sigma, St Louis, MO) for nuclei.

### Microinsemination

Microinsemination was undertaken by intracytoplasmic injection into C57BL/6  $\times$  DBA/2 F1 oocytes (Kimura and Yanagimachi, 1995). Embryos that were constructed using spermatozoa or elongated spermatids derived from 8.5 dpc or 12.5 dpc PGCs were transferred into the oviducts of pseudopregnant ICR females after 24 or 48 hours in culture, respectively. Live fetuses retrieved on day 19.5 were raised by lactating ICR foster mothers.

### Genotyping of offspring and bisulfite sequencing of imprinted genes

PCR fragments of the *Kit* gene encompassing the  $W$  point mutation or the  $W^u$  mutation (Nocka et al., 1990; Hayashi et al., 1991) were amplified using genomic DNA from mice derived from PGC transplantation, or from a  $W/W^u$  mouse as a control heterozygote for both mutations. PCR primers were 5'-CATTTATCTCCTCGACAAC-CTTCC-3' and 5'-GCTGCTGGCTCACAATCATGGTTC-3' for  $W$  genotyping, and 5'-AGATGGCAACTCGAGACTCACCTC-3' and 5'-TGCCCCACGCTTTGTTTTGCTAA-3' for  $W^u$  genotyping. Amplified products were gel extracted and directly sequenced.

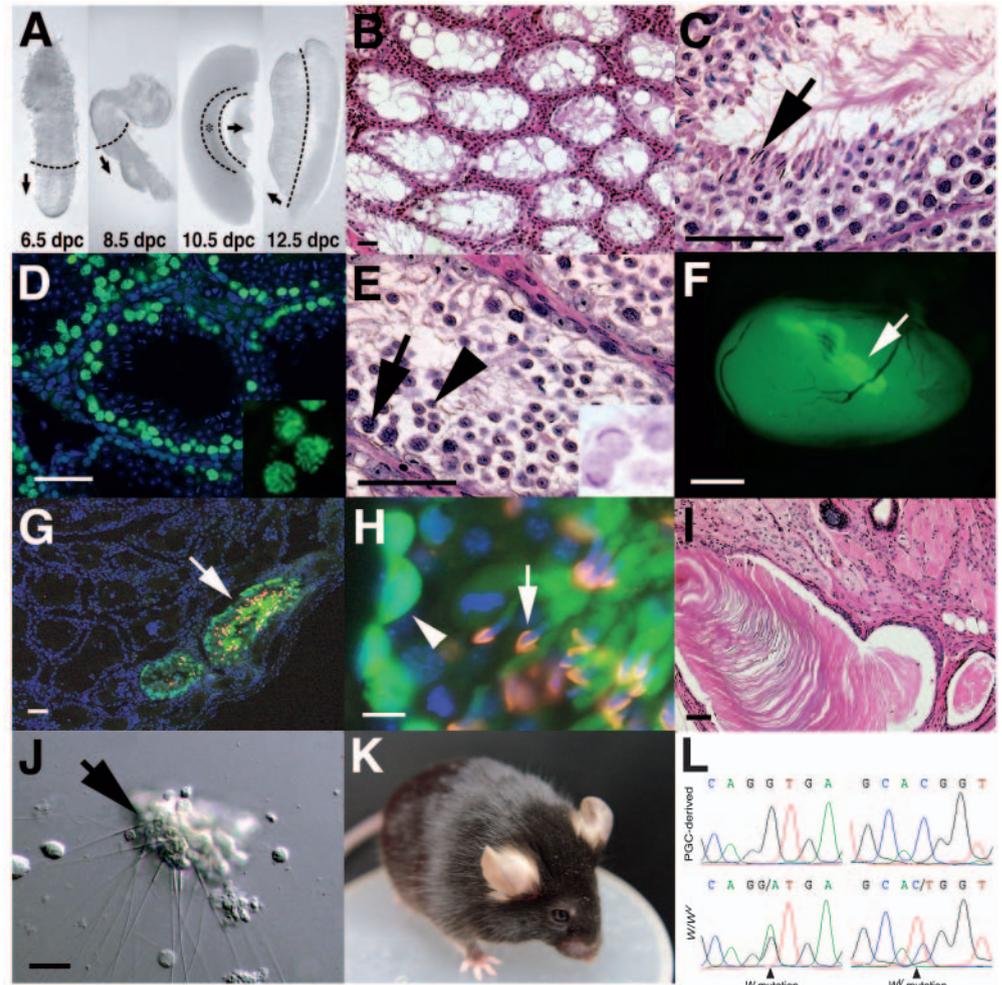
Bisulfite genomic sequencing of differentially methylated regions (DMRs) of the *Igf2r* and *H19* imprinted genes was carried out as described (Ueda et al., 2000; Lee et al., 2002; Lucifero et al., 2002). Briefly, genomic DNAs were isolated from the offspring derived from PGC transplantation, and treated with sodium bisulfite, which deaminates unmethylated cytosines to uracils, but does not affect 5-methylated cytosines. Polymerase chain reaction amplification of each DMR from bisulfite-treated genomic DNAs was carried out using primer sets as described (Ueda et al., 2000; Lucifero et al., 2002), and DNA sequences were determined.

## Results

Epiblasts with primitive endoderms (6.5 dpc) or tissues containing fetal germ cells were collected from different stages of embryos (posterior third of 8.5 dpc embryos, mesenteries and guts of 10.5 dpc embryos, or gonads of 10.5 to 16.5 dpc embryos) (Fig. 1A). The cells were dissociated enzymatically and single cell suspensions were transplanted into the seminiferous tubules of recipient immature  $W$  mice. Although  $W$  mice have a very small number of spermatogonia (Ohta et al., 2003), spermatogenesis is arrested at the point of undifferentiated type A spermatogonia and no differentiating germ cells are found due to defects in the *Kit* gene (Nocka et al., 1990; Hayashi et al., 1991) (Fig. 1B). Therefore, any spermatogenesis detected in the recipient testis must be derived from the donor cells. Although the concentration of cells injected varied due to the more limited recovery of cells from early-stage fetuses, the cell viability, or percentage of tubules filled with donor cells, was similar in all experiments. The recipient mice were sacrificed 3 to 4 months after transplantation, and the testes were examined histologically for the presence of spermatogenesis. This time period represents three to four spermatogenic cycles in mice (Meistrich and van Beek, 1993; de Rooij and Russell, 2000), which would allow sufficient time for the development of sperm from spermatogonial stem cells.

At least three experiments were performed using cells harvested from each stage of embryonic development, and the

**Fig. 1.** Spermatogenesis and teratogenesis from fetal germ cells and epiblast cells. (A) Embryos at 6.5 and 8.5 dpc, a mid-part of 10.5 dpc embryo, and a male gonad and mesonephros at 12.5 dpc. Dotted lines demarcate regions used for transplantation. At 10.5 dpc, the urogenital ridges (asterisk) and mesentery with gut (arrow) were dissected separately. (B) A section of a *W* male testis (control recipient) stained with HE. Spermatogenesis is absent. (C) *W* mouse testis after transplantation of 8.5 dpc PGCs. Spermatozoa (arrow) are present in the center of the seminiferous tubule. (D) Anti-SCP3 immunostaining (green) of *W* testis after transplantation of 8.5 dpc PGCs, counterstained with Hoechst 33258 dye (blue). Inset, higher magnification view of the same sample. (E) Transplantation of epiblast cells at 6.5 dpc. Spermatocyte (arrow) and round spermatid (arrow head) were found. Inset shows acrosomes stained with PAS (red) in round spermatids. (F) *W* mouse testis transplanted with 8.5 dpc PGCs from Green mice embryos. Colonization of the recipient seminiferous tubule by EGFP (+) donor cells (green) was observed (arrow). (G) A section of the same testis as in (F), stained with Rhodamine-PNA (red) for acrosomes and with Hoechst 33258 dye (blue) for nuclei. Spermatogenic colonies derived from EGFP (+) donor cells are present (arrow). (H) Higher magnification view of (G), showing spermatogonia (arrowhead) residing at the base of the seminiferous tubule, and elongated spermatids (arrow) with acrosomes (red), shedding the EGFP (+) cytoplasm. (I) Teratoma from epiblast cells at 6.5 dpc. Muscle, dermoid cyst and neuronal tissue are observed. (J) Spermatozoa (arrow) clustered around a Sertoli cell, released from a recipient testis of 8.5 dpc PGCs. (K) An offspring developed from an oocyte injected with a sperm derived from 8.5 dpc PGCs. (L) DNA sequences of the *Kit* gene around the *W* (left panels) and *W<sup>v</sup>* (right panels) point mutations of an offspring derived from transplantation of 8.5 dpc PGCs (upper panels) and a *W/W<sup>v</sup>* mouse as a control heterozygote (lower panels). Scale bars: 1 mm in F; 10  $\mu$ m in H; 20  $\mu$ m in J; 50  $\mu$ m in others.



**Table 1. Donor cell colonization in *W* recipient mice**

Donor age (dpc)	Number of recipient testes	Number of transplanted cells ( $\times 10^4$ /testis)	Number of testes with spermatogenesis (%)	% tubule cross-section with spermatogenesis*	Number of testes with teratoma (%)
6.5	27	0.4 $\pm$ 0.2	2 (7.4)	0.1 $\pm$ 0.1	4 (14.8)
8.5	13	7.2 $\pm$ 0.6	9 (69.2)	3.8 $\pm$ 0.1	4 (30.8)
10.5 (mes+gut)	12	12.8 $\pm$ 1.4	4 (33.3)	0.9 $\pm$ 0.5	0
10.5 (UGR)	15	11.4 $\pm$ 2.4	11 (73.3)	7.2 $\pm$ 0.1	0
11.5	13	2.4 $\pm$ 1.0	8 (61.5)	2.4 $\pm$ 0.7	0
12.5	13	9.0 $\pm$ 1.6	11 (84.6)	15.7 $\pm$ 4.5	0
14.5	12	20	12 (100)	56.5 $\pm$ 10.8	0
16.5	12	20	12 (100)	59.3 $\pm$ 7.5	0

Values are mean $\pm$ s.e.m. Results from at least three separate experiments. Testes were analyzed 3-4 months after transplantation. Mes+gut, mesentery and gut; UGR, urogenital ridge.

\*Percentage of tubule cross-sections containing spermatogenesis/total tubule cross-sections examined in each testis.

results are summarized in Table 1. Overall, 69 of 117 (59%) recipient testes showed spermatogenesis, but no differentiating germ cells were found in control testes that did not receive an

injection of donor cells (Fig. 1B). Spermatogenesis observed in the recipient testes originated from spermatogonial stem cells, because other spermatogenic cells do not have the

**Table 2. Development of oocytes injected with spermatogenic cells derived from PGCs**

Donor age (dpc)	Number of reconstituted eggs	In vitro development (%)			Number of eggs transferred	Implantation sites (%)	Number of offspring (%)
		One cell	Two cell	Four cell			
8.5*	186	78 (41.9)	105 (56.5)	NA <sup>†</sup>	105	47 (25.2)	20 (10.8)
12.5	182	8 (4.4)	25 (13.7)	130 (71.4)	130	88 (48.3)	29 (15.9)

Combined results using elongated spermatids and spermatozoa.

\*Combined results from two different recipient testes.

<sup>†</sup>NA, not applicable because cells were transferred at the two-cell stage.

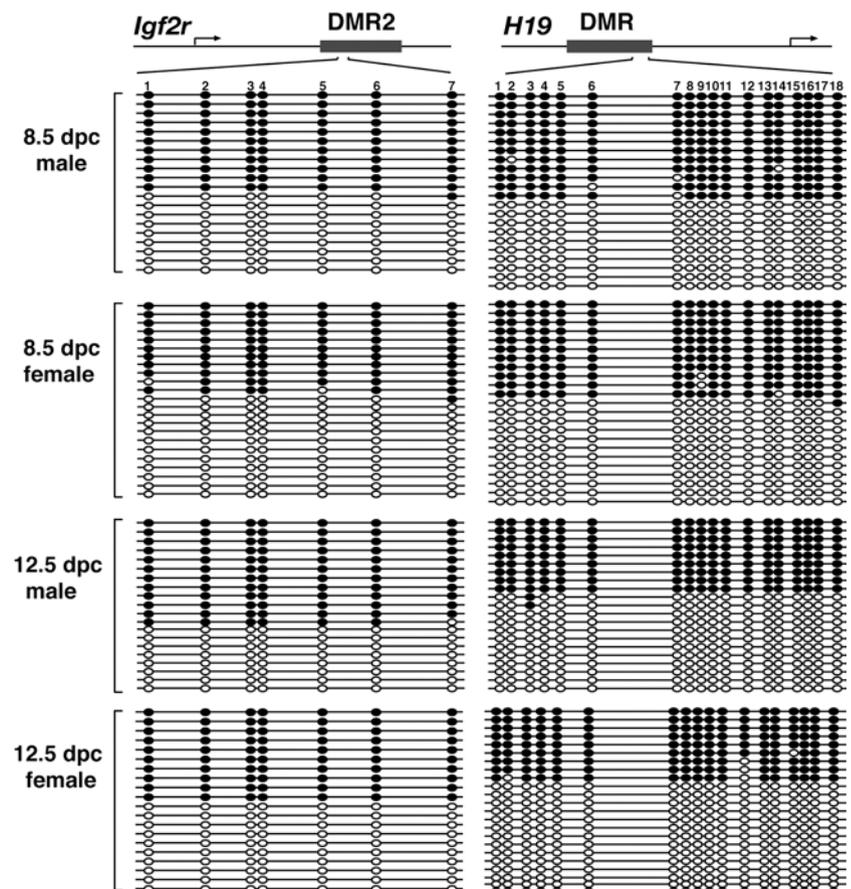
capacity for self-renewal and disappear by 35 days after transplantation (Russell et al., 1990; Brinster and Zimmermann, 1994; Shinohara et al., 2001). Spermatogenesis in the recipient testes that received fetal germ cells (8.5-16.5 dpc) was morphologically normal (Fig. 1C), and the kinetics of spermatogenic colonization were generally comparable to results obtained after transplantation of postnatal spermatogonial stem cells (Shinohara et al., 2001). All stages of spermatogenic cells, including mature spermatozoa, were found in recipient testes. Synchrony of meiosis in seminiferous tubules was confirmed by immunostaining for SCP3, a component of the synaptonemal complex (Fig. 1D).

The age of the donors had a significant effect on the number of recipient seminiferous tubules with observable spermatogenesis. Whereas only 0 to 11% of the tubules showed spermatogenesis in testes that received 8.5 dpc PGCs, spermatogenesis derived from donor cells at later stages of gonadal development was generally more extensive, and recipient testes grew larger due to the increased production of germ cells. In one case, transplantation of 16.5 dpc pro-spermatogonia resulted in 96% of the tubules with evident spermatogenesis, and spermatozoa were transported to the epididymis (data not shown). Spermatogenesis was also found in the recipients that were injected with cells from epiblasts with primitive endoderms. Although we could not identify mature spermatozoa histologically, spermatogenesis was found in two different testes that received injections of 6.5 dpc epiblast and primitive endoderm cells. The spermatogonia, spermatocytes and round spermatids that developed in the recipient seminiferous tubules appeared morphologically normal (Fig. 1E), and acrosome formation in round spermatids was confirmed by PAS staining (Fig. 1E, inset).

To confirm the donor origin of spermatogenesis, we used Green mice that ubiquitously express the *EGFP* transgene. Donor cells were collected from the posterior thirds of 8.5 dpc embryos that showed EGFP signals. This

allowed specific identification of donor cells, because endogenous host testis cells had no detectable fluorescence. Four experiments were performed, and a total of 16 testes were microinjected with donor cells. Approximately 6 to 12 × 10<sup>3</sup> cells were transplanted into each testis. When the recipients were analyzed 10 to 11 weeks after transplantation, 4 of 16 (25%) testes had spermatogenic colonies that showed EGFP signals (Fig. 1F). The average number of colonies per testis was 0.3 ± 0.1 (mean ± s.e.m.). Histological analysis of the EGFP (+) colonies showed the presence of apparently normal spermatogenesis (Fig. 1G,H).

While these results indicate that all types of donor cells differentiated into spermatogonial stem cells, differentiation of donor cells was not restricted to the germline lineage. Consistent with findings of previous studies (Illmensee and Stevens, 1979), testes receiving epiblast cells or 8.5 dpc PGCs formed teratomas (Fig. 1I). Epiblast cells produced larger tumors than did 8.5 dpc PGCs, and some of the seminiferous



**Fig. 2.** DMR methylation of the *Igf2r* and *H19* genes in offspring derived from transplantation of 8.5 and 12.5 dpc PGCs. DNA methylation was analyzed by bisulfite genomic sequencing. Both male and female offspring of each stage of donor PGCs were analyzed. Individual lines represent sequenced clones. Black ovals indicate methylated cytosine-guanine sites (CpGs) and white ovals indicate unmethylated CpGs.

tubules were dilated and broken. Cells from three germ layers, including ciliated epithelium, muscle, neuron and bone, were found in these testes. Interestingly, spermatogenesis was occasionally observed in tubules not affected by tumorigenesis, indicating that the same population of donor cells could cause spermatogenesis and teratogenesis. No teratomas were found in the recipients of gonadal PGCs or pro-spermatogonia.

To examine whether germ cells generated from PGCs were fully functional, we performed microinsemination, a technique commonly used to produce offspring from infertile animals and humans (Kimura and Yanagimachi, 1995; Palermo et al., 1992). Donor PGCs were collected from 8.5 or 12.5 dpc embryos, and transplanted into the testes of *W* mice. Four months after transplantation, spermatozoa or elongated spermatids were collected from tubule fragments by mechanical dissociation (Fig. 1J), and microinjected into oocytes. The results of the microinsemination experiments are summarized in Table 2. Spermatogenic cells derived from 8.5 dpc PGCs appeared to be less competent for egg activation, because a significant number of eggs receiving elongated spermatid/spermatozoa from 8.5 dpc PGCs stayed at metaphase II or the metaphase II-anaphase transition and did not develop into the 2-cell stage after 24 hours. However, offspring were obtained from oocytes inseminated with spermatid/spermatozoa derived from both stages of PGCs (Fig. 1K). Genotyping of offspring showed that they did not carry *W* or *W'* mutations in the *Kit* gene (Nocka et al., 1990; Hayashi et al., 1991), demonstrating the offspring were derived from wild-type donor PGCs that had been transplanted into the testis (Fig. 1L). No apparent abnormality was seen in any of the offspring, and they were fertile. Bisulfite sequencing analysis of the offspring showed no obvious fluctuations in the methylation status of the DMRs of paternally methylated *H19* and maternally methylated *Igf2r* genes (Fig. 2).

## Discussion

This study demonstrates that not only migrating PGCs but also epiblast cells can differentiate into spermatogonial stem cells and produce spermatogenesis after transfer into postnatal testis. The ability to initiate and maintain spermatogenesis after transfer into the infertile testis fulfills the criteria for the identification of spermatogonial stem cells (Brinster and Zimmermann, 1994). Developmental processes that occur during differentiation of spermatogonial stem cells from epiblast cells include induction of PGCs among epiblast cells, migration and proliferation of PGCs, erasure of parental genomic imprints, and G1 (G0) arrest in the developing male gonad. Nonetheless, our results demonstrate that these developmental events do not necessarily require embryonic somatic environments, and most processes of male germline differentiation can take place in postnatal testis. This flexibility of fetal germ cell differentiation may be related to, and partly account for, the recent success in the derivation of sperm from embryonic stem cells in vitro (Toyooka et al., 2003; Geijsen et al., 2004).

The important factor that contributed to the results of our experiments is the use of immature postnatal testes as recipients. Recently, Ohta et al. (Ohta et al., 2004) showed that pro-spermatogonia from 14.5 dpc embryos completed spermatogenesis when transplanted in mature seminiferous

tubules, while spermatogenesis did not occur from PGCs from 12.5 dpc embryos. Another group reported that germ cells from day 0-3 mouse pups did not show spermatogenic colonies after transplantation into adult testes (McLean et al., 2003). In our study, however, epiblast cells at 6.5 dpc and PGCs at 8.5 to 16.5 dpc produced spermatogenesis after transplantation into immature seminiferous tubules at postnatal day 5 to 10. This difference might simply be ascribed to structural differences; immature Sertoli cells lack tight junctions and may allow migrating transplanted cells easier access to the stem cell niches, which are distributed nonrandomly in the seminiferous tubules (Chiarini-Garcia et al., 2001). Alternatively, immature testis may express factors that support survival and differentiation of epiblast cells and PGCs, while mature testis may not. Because PGCs have chemotactic activity (Godin et al., 1990), we speculate that some of the transplanted cells migrated into the niches of immature seminiferous tubules, where they could survive. PGCs and epiblast cells may have then switched their cell cycle fate to function as spermatogonial stem cells. Both male and female PGCs enter into meiosis in the absence of the male gonadal environment, but do not if they lodge there (McLaren and Southee, 1997; Chuma and Nakatsuji, 2001). Somatic cells in the fetal testis are assumed to produce a substance that inhibits the meiotic transition of PGCs. Given our results, it appears that seminiferous tubules of newborn mice may also have similar meiosis-inhibiting activity.

Erasure of parental genomic imprints commences in PGCs at around the time of their arrival in the UGR (Szabo and Mann, 1995; Surani, 2001; Hajkova et al., 2002). However, it has not been clear whether this epigenetic event depends on induction from the UGR or is programmed autonomously in PGCs. As the offspring from PGC transplantations were viable and apparently healthy, epigenetic modifications, including erasure of parental genomic imprints, should have occurred appropriately in transplanted PGCs. This was corroborated by the normal methylation patterns exhibited in the DMRs of the *Igf2r* and *H19* genes. Because the establishment of paternal methylation proceeds after birth in the normal testis (Ueda et al., 2000), it seems unlikely that the postnatal testis has the ability to erase parental methylation. Therefore, our results suggest that PGCs may have the autonomous program for the erasure of parental methylation before reaching the UGR.

Primordial germ cell transplantation will provide a new experimental approach for the study of PGC development. Primordial germ cells harvested from embryonic lethal mutants as early as gastrulation can be traced for their differentiation capacities by transplantation into recipient testis. Similarly, PGCs manipulated in vitro, such as those cultured with growth factors or transfected with vectors (De Miguel et al., 2002; Watanabe et al., 1997), can now be assessed for their effects on subsequent differentiation in vivo. Such functional studies would help elucidate factors that regulate male germline development in mammals.

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