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CHAPTER TWO

GERMLINE MODIFICATION USING MOUSE SPERMATOGONIAL STEM CELLS

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Abstract

Spermatogonial stem cells (SSCs) in the testes are a new target for germline modification. With the development of an *in vitro* culture system and spermatogonial transplantation technique, SSCs can now be manipulated and used as an

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alternative to embryonic stem cells for knockout mice production. The genetic and epigenetic stability of SSCs provide new possibilities for the application of germline mutagenesis in a wide range of animals.

1. Introduction

Spermatogonial stem cells (SSCs) provide a foundation for spermatogenesis. SSCs, which constitute a fraction of spermatogonia in the testes, self-renew and differentiate to produce sperm throughout adult life. While female germline cells stop proliferating during the fetal period and isolation of oocyte/eggs is limited, male germ cells can be isolated and expanded in number. These advantages suggest that SSCs are a valuable target for germline modification.

Recently, several SSC manipulation techniques have been developed. First, a breakthrough was made in the establishment of a germ cell transplantation technique. When dissociated donor testicular cells are injected into seminiferous tubules of infertile recipient testes lacking endogenous spermatogenesis, they colonize, differentiate into sperm, and produce normal offspring (Brinster and Avarbock, 1994; Brinster and Zimmermann, 1994). Spermatogenesis also occurs with cryopreserved SSCs and with SSCs in xenogeneic host. Rat SSCs undergo spermatogenesis in immunodeficient nude mouse testis and produce normal offspring, indicating a significant flexibility of spermatogenesis (Clouthier *et al.*, 1996; Shinohara *et al.*, 2006).

Second, a long-term SSC culture technique was established. In 2000, glial cell line-derived neurotrophic factor (GDNF) was found to induce spermatogonial proliferation (Meng *et al.*, 2000). Homozygous GDNF knockout mice die perinatally, whereas heterozygous knockout mice exhibit reduced spermatogenesis and eventually become infertile due to germ cell depletion. In contrast, GDNF-transgenic mice possess clumps of undifferentiated spermatogonia, suggesting that GDNF stimulates the self-renewal division of SSCs. Considering this finding, our group succeeded in the long-term culture of SSCs in 2003, and we designated these cells as germline stem (GS) cells (Kanatsu-Shinohara *et al.*, 2003a). In the presence of GDNF, GS cells produce uniquely shaped germ cell colonies. Although GS cells were originally established from neonatal testis, similar cells were subsequently established from adult testis, demonstrating that GS cells can be derived from SSCs at various stages (Kanatsu-Shinohara *et al.*, 2004a; Kubota *et al.*, 2004; Ogawa *et al.*, 2004). GS cells can be used to produce transgenic and knockout animals through genetic transduction and drug selection (Kanatsu-Shinohara *et al.*, 2005c, 2006a). Because the transgene is transmitted to half of the haploid cells, the efficiency of transgenesis is about

50%, and it is 5–10 times higher than that achieved by conventional methods using eggs or oocytes (Nagano *et al.*, 2001). Moreover, the frequency of homologous recombination is comparable to that achieved in embryonic stem (ES) cells (Kanatsu-Shinohara *et al.*, 2006a). Most importantly, GS cells possess a very stable germline potential, retain a normal karyotype and DNA methylation patterns, and produce normal fertile offspring even after 2 years of culture (Kanatsu-Shinohara *et al.*, 2005b). This is in contrast to ES cells, which often change DNA methylation patterns and lose their germ cell potential due to trisomy (Liu *et al.*, 1997; Longo *et al.*, 1997). Thus, SSCs may serve as a new target for animal transgenesis, which may provide an alternative to ES cells.

We also found that the developmental potential of SSCs is not limited to spermatogenesis. Although primordial germ cells (PGCs), the fetal precursors of SSCs, can give rise to ES-like pluripotent cells (Matsui *et al.*, 1992; Resnick *et al.*, 1992), germline cells were believed to be fully committed to the germline by the middle of gestation and that such ES-like potential was missing from postnatal germ cells (Labosky *et al.*, 1994). Unexpectedly, however, ES-like cells rarely appear in GS cell cultures of neonatal testes during culture initiation (Kanatsu-Shinohara *et al.*, 2004a). These cells, referred to as multipotent GS (mGS) cells, not only differentiate into somatic cells, but also differentiate into germ cells. Several groups have reported the derivation of similar pluripotent/multipotent cells from mouse and human postnatal testes, including adults (Golestaneh *et al.*, 2009; Guan *et al.*, 2006; Kossack *et al.*, 2009). Although the origin of these pluripotent/multipotent cells is unclear, we recently discovered that GS cells may be converted directly into mGS cells *in vitro* (Kanatsu-Shinohara *et al.*, 2008a). mGS cells behave like ES cells and are capable of producing knockout animals in a manner similar to ES cells (Takehashi *et al.*, 2007b). Although the efficiency of establishing mGS cells (1 of 30 testes) is low and needs improvement, these results suggest an alternative use for SSCs in germline modification.

In this chapter, we describe the methods associated with the SSC culture technique. Although several groups have reported alternative SSC culture methods (Guan *et al.*, 2006; Kubota *et al.*, 2004; Seandel *et al.*, 2007), our method allows for the genetic selection and production of knockout mice from SSCs.

2. Establishing and Maintaining a GS Cell Culture

GS cell culture may be initiated from both neonatal/pup and adult testes (Kanatsu-Shinohara *et al.*, 2003a, 2004a; Kubota *et al.*, 2004; Ogawa *et al.*, 2004), but establishment is quicker and more efficient using neonatal/

pup testes. Immature testes are useful because the germ cell/somatic cell ratio is relatively high, and germ cells are easily separated from somatic cells based on their differential ability to attach to a gelatin-coated dish; therefore, antibody-mediated purification is not necessary. Furthermore, immature germ cells proliferate more actively than adult germ cells. In contrast, one must purify SSCs from adult testes because only 0.02–0.03% of all germ cells in the testis are stem cells (Meistrich and van Beek, 1993; Tegelenbosch and de Rooij, 1993). In both cases, removing as many somatic cells as possible is advisable.

The efficiency of establishing GS cells is affected by the mice strain. While DBA/2, ICR, and C57BL/6 × DBA/2F1(B6D2F1) are efficient, C57BL/6 is less efficient. GS cells can also be established from ddy, C3H, A, and AKR with variable efficiencies. Here, we describe a protocol for establishing a GS cell culture from P0-3 testes of DBA/2 or the ICR strain.

2.1. GS cell culture medium

GS cell culture medium is prepared by modifying commercial medium (StemPro®-34 serum-free medium (SFM); Invitrogen, Carlsbad, CA). Although other conditions with defined medium can also support SSC proliferation, a modified StemPro®-34 medium currently provides the most efficient proliferation of GS cells in our laboratory.

SSC proliferation is maintained by a combination of several cytokines. GS cell culture medium contains GDNF, and fibroblast growth factor-2 (FGF2). Although epidermal growth factor (EGF) was included in the original protocol, it is dispensable, and GS cell proliferation can be maintained with GDNF + FGF2 only. Leukemia inhibitory factor (LIF) enhances colony formation from gonocytes, while it is dispensable for spermatogonia culture (Kanatsu-Shinohara *et al.*, 2007). It is also dispensable for the maintenance of GS cells.

Complete GS cell culture medium is prepared by adding several factors to basal medium (Table 2.1). Basal medium is made by adding 16 components to StemPro®-34 SFM, followed by filtration through a 0.22-μm bottle-top filter. It can be stored in a refrigerator for at least 3 weeks. The six additives listed in Table 2.2 should be added before use to make the complete medium. Complete medium with growth factors can be stored in a refrigerator for up to 3 days.

2.2. Step 1. Dissociation of testis cells

Dissolve collagenase (#C5138; Sigma, St. Louis, MO) at 1 mg/ml and deoxyribonuclease (DNase, #DN25; Sigma) at 7 mg/ml in Hanks' balanced salt solution (HBSS) and filter. Isolate the testes from the mice, and remove

Table 2.1 Composition of basal medium for GS cell culture

Component	Catalogue #	Final concentration	Volume	Aliquots
Insulin	Nacalai Tesque[a] #19251-24	25 μg/ml	500 μl	Dissolve 100 mg/3.8 ml DDW + 0.2 ml 1 *N* HCl; store at −20 °C
Transferrin	Sigma #T1147	100 μg/ml	1 ml	Dissolve 100 mg/2 ml DDW; store at −20 °C
Putrescine	Sigma #P7505	60 μ*M*	500 μl	Dissolve 96.7 mg/10 ml DDW; store at −20 °C
Sodium selenite	Sigma #S1382	30 n*M*	500 μl	Dissolve 5.2 mg/1000 ml DDW; store at −20 °C
D-(+)-glucose	Sigma #G7021	6 mg/ml	3g	Dissolve in 10 ml DDW and add all
Pyruvic acid	Sigma #P2256	200 μg/ml	100 mg	
DL-Lactic acid	Sigma #L4263	1 μl/ml	500 μl	
Bovine albmin	MP Biomedicals[b] #810661	5 mg/ml	2.5 g	
L-Glutamine	Sigma #G7513	2 m*M*	5 ml	100×; store at −20 °C
2-Mercaptoethanol	Sigma #M3148	5×10^{-5} *M*	5 ml	5×10^{-3} *M*
MEM vitamin solution	Invitrogen #11120-052		5 ml	Store at 4 °C
Nonessential amino acids	Invitrogen #11140-050		5 ml	Store at 4 °C
Ascorbic acid	Sigma #A4544	10^{-4} *M*	500 μl	Dissolve 17.6 mg/ml DMSO; use immediately
d-Biotin	Sigma #B4501	10 μg/ml	500 μl	Dissolve 10 mg/ml DMSO; use immediately
β-Estradiol	Sigma #E2758	30 ng/ml	750 μl	Dissolve 1 mg/ml ethanol; add 49 ml sterile medium. Store at −20 °C
Progesterone	Sigma #P8783	60 ng/ml	1.5 ml	Dissolve 1 mg/ml ethanol;, add 49 ml sterile medium. Store at −20 °C

StemPro®-34 SFM (Invitrogen #10639) is modified by addition of the following components. The amounts to be added to 500 ml StemPro®-34 SFM are shown.
[a] Nacalai Tesque, Inc., Kyoto, Japan.
[b] MP Biomedicals, Inc., Irvine, CA.

Table 2.2 Components added to basal medium immediately before use

Component	Catalog number	Final	Volume	Aliquot
StemPro®-34 supplement	Invitrogen #10639		20 μl	50× Supplement is supplied with StemPro®-34SFM.
Mouse EGF	BD Biosciences 354010	20 ng/ml	2 μl	Optional; Dissolve 100 mg/10 ml PBS+BSA; store at −20 °C
Human FGF2	Peprotech Inc.[a] #100-18B	10 ng/ml	5 μl	Dissolve 10 mg/5 ml PBS+BSA; store at −20 °C
Rat GDNF	Peprotech Inc.[a] #450-51	15 ng/ml	15 μl	Dissolve 10 mg/10 ml PBS+BSA; store at −20 °C
FBS	Hyclone[b] #SH30396.03	1%	10 μl	
ESGRO (murine LIF)	Millipore[c] #ESG1107	10^3 units/ml	10 μl	Optional; enhances GS cell establishment when added to the initiation of neonatal testis culture. Dissolve 10^6 units/10 ml PBS+BSA; store at −20 °C

The amounts to be added to 1 ml basal medium are shown.
[a] Peprotech Inc. Rocky Hill, NJ.
[b] Hyclone Laboratories, Inc. South Logan, UT.
[c] Millipore, Billerica, MA.

the tunica with fine forceps in cold HBSS. Wash two to three times with HBSS and transfer the tissue to 1–2 ml of collagenase and incubate at 37 °C for 15 min. Agitate the tube several times during the incubation. Wash twice with HBSS and add 0.8 ml of 0.25% trypsin + 0.2 ml of DNase, shake the tube several times to dissociate the seminiferous tubules, and incubate at 37 °C for 10 min. Add 5 ml of Iscove's modified Dulbecco's medium (IMDM) + 2% fetal bovine serum (FBS) and repeat pipetting until the cells are dissociated. Centrifuge and remove the supernatant.

2.3. Step 2. Initiation of GS cell culture

Dissolve 1 g gelatin in 500 ml phosphate-buffered saline (PBS) and autoclave to make a 0.2% gelatin solution. Coat a 12-well culture plate with the 0.2% gelatin/PBS and incubate at room temperature for more than 20 min. Remove the gelatin solution, suspend the cells in complete culture medium, and transfer them to a gelatin-coated culture plate. The density should be $\sim 2 \times 10^5$ cells/0.8 ml medium per well of a 12-well culture plate. Incubate in 5% CO_2 at 37 °C overnight.

Many cells attach to the plate after the overnight incubation, but a significant number of germ cells, as distinguished by their large size and characteristic pseudopod, remain floating. The floating cells should be transferred to a second culture plate after vigorous pipetting (use P1000-pipette tips, 10–15 times; Gilson, Middleton, WI). The second culture plate does not need to be treated with gelatin. Very few germ cells are left on the original gelatin-coated plate, and cells transferred to secondary plates are relatively germ cell-enriched (Fig. 2.1A).

Three to four days later, remove half of the culture medium and add the same volume. Within 1 week, the transferred cells will proliferate and spread on the bottom of the well; round proliferating cells will form germ cell colonies on top of the flat cell layer. Most of these primary colonies consist of compact clusters of cells with unclear borders.

The timing of the first passage depends on colony growth, but between 10 and 14 days after culture initiation (DIV, days *in vitro*) is recommended. Wash twice with PBS, add 0.25% trypsin, and incubate at 37 °C for 4 min. Add IMDM + 2% FBS to stop the reaction. Replate at 1× dilution. The colonies will grow to their original size in about 10 days, when the cells are passed again (1/2× dilution).

2.4. Step 3. Maintenance

After the third or fourth passage, the cells should be transferred onto mitomycin-C treated mouse embryonic fibroblasts (MEFs) (Fig. 2.1B). MEF feeders should be prepared according to the conventional ES cell

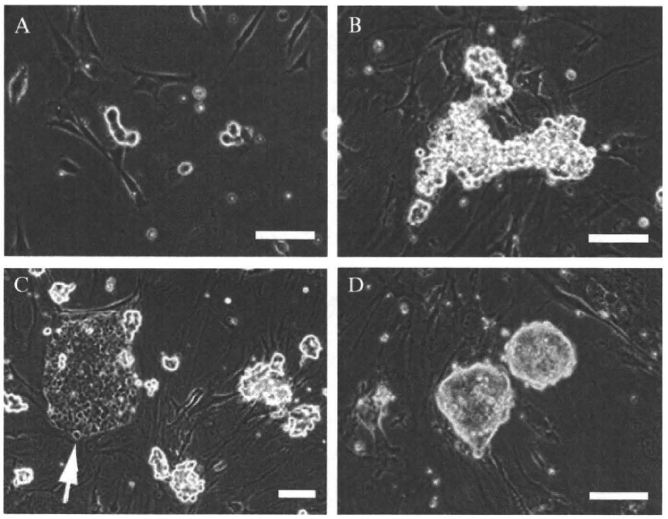

Figure 2.1 Culture appearances. (A) Gonocytes at 2 days *in vitro*. Gonocytes have large cell bodies and attach loosely to the plate, whereas fibroblasts and other somatic cells attach strongly to the plate. (B) Established GS cells. GS cell colonies produce a grape-like cluster. (C) Epiblast-like cell sheet, which appears in GS cell cultures. An epiblast-like cell sheet (arrow) is often observed when mGS cells appear in the culture. While distinguishing clearly between GS cell and mGS cell colonies is difficult, this is a clear sign indicating that mGS cells are starting to appear in the culture. However, this structure disappears after mGS cells are established, and whether it is an mGS cell precursor or a differentiated mGS cell state that exists only transiently is unclear. (D) mGS cells have similar appearances to ES cells. Bar = 100 μm.

culture method, except they should be plated at a lower density (7.5×10^4 per well in 6-well culture plates). Briefly, treat MEF with 10 μg/ml mitomycin-C (#M053; Sigma; dissolve in PBS at 2 mg/4 ml and filter) for 2 h at 37 °C and dissociate with trypsin. Suspend the MEF cells in Dulbecco's modified Eagle's medium (DMEM) + 10% FBS and plate onto a gelatin-coated culture dish. MEF feeders should be used within 10 days (it is not necessary to change medium). Immediately before transferring the GS cells remove the medium from the MEF feeders and wash once (or twice) with PBS.

GS cell growth becomes stable after about 30 DIV. The established GS cells should be plated at a density of 3×10^5 cells/well in 6-well culture plates. Cultures should be passed every 4–6 days depending on proliferation. The medium should be changed every 3 days (half medium change). Established GS cells continue to proliferate for more than 2 years without losing stem cell activity.

2.5. Trouble shooting

One of the problems in culture initiation is overgrowth of somatic cells. Somatic cells grow faster than SSCs *in vitro* and overwhelm the culture. This can be alleviated, in part, by reducing the serum concentration and/or enhancing the GDNF concentration. However, SSCs, at present, cannot grow in the complete absence of serum. Although two different "serum-free" culture systems have been reported (Kanatsu-Shinohara *et al.*, 2005a; Kubota *et al.*, 2004), one of these cultures were maintained using serum to stop the trypsin reaction at each passage in one study (Kubota and Brinster, 2008). Because the same medium cannot support feeder-free GS cell culture without serum, we also cannot exclude the possibility that residual serum remaining with the feeder cells promote GS cell propagation (Kanatsu-Shinohara *et al.*, 2005a). Besides somatic cells, loss or reduced GDNF activity often causes problems. Because GDNF is essential for SSC self-renewal, low concentrations of GDNF are detrimental to GS cell culture.

When the culture reaches confluency, c-Kit expression, a marker of differentiating spermatogonia, is occasionally upregulated, suggesting that some levels of differentiation occur in culture. At the same time, GS cell growth is suppressed. In this situation, remove the medium and replace with fresh medium. In most cases, a decrease in the number of differentiating cells is observed, and the stem cells revive by GDNF action. If the GS cells look unhealthy, change the medium or add GDNF. GS cells are very tough, and in most cases will recover.

2.6. Optional: Feeder-free culture and GS cell suspension culture

GS cells can be maintained under a feeder-free condition or in suspension culture (Kanatsu-Shinohara *et al.*, 2005a, 2006b). For feeder-free culture, the culture plates should be coated with 20 μg/ml laminin (#354232; BD Biosciences, Franklin Lakes, NJ) for 1–2 h at room temperature (0.8 ml laminin solution per well of a 6-well culture plate). Remove the laminin solution and immediately plate the GS cells at 3×10^5 per well. The culture can be passed with trypsin. For the suspension culture, plate 2×10^5 GS cells directly onto a Petri dish. GS cells aggregate to make clumps, but continue to proliferate slowly. These cultures can be passed without trypsin, and GS cell clumps can be disaggregated using simple pipetting with P1000 tips. Although the proliferation rates are lower than MEF culture (doubling time, 2.7 days for MEF culture, 5.6 days for feeder-free culture, and 4.7 days for suspension culture), GS cells maintained feeder-free or in suspension continue to proliferate for more than 6 months without losing SSC activity.

2.7. Optional: Establishing GS cells from adult testes

GS cells can also be established from adult testes, but enrichment using gelatin-coated plates is not effective. Because the SSC frequency is low in adult testes (about 2–3 in 10^4 cells; Tegelenbosch and de Rooij, 1993; Meistrich and van Beek, 1993), purification using either magnetic beads or a fluorescence-activated cell sorter is necessary. Several SSC markers are useful for SSC enrichment. For example, a relatively high rate of enrichment has been achieved in our lab using magnetic beads sorted with α6- or β1-integrins and CD9 (Kanatsu-Shinohara *et al.*, 2004b; Shinohara *et al.*, 1999). Thy-1 is also expressed in SSCs, but seems to work better after Percoll separation (Ryu *et al.*, 2004). A higher enrichment efficiency (about 166-fold) can be achieved with cell sorting using a combination of several markers (Ryu *et al.*, 2004; Shinohara *et al.*, 2000), but enrichment by magnetic beads is sufficient for culture initiation in many cases.

Purified cells are transferred to gelatin- or laminin-coated plates and cultured in GS cell culture medium. In a manner similar to the initiation of GS cell culture from neonatal testes, germ cell colonies are formed on top of the flat somatic cell layer. However, because adult germ cells proliferate more slowly than neonatal or pup germ cells in the initiation of culture, more time is required to establish a GS cell culture from adult testes than from neonatal testes. Retrieving GS cell colonies is sometimes helpful using glass needles in cases of somatic cell overproliferation.

2.8. Optional: Derivation of mGS cells and its application in gene targeting

mGS cells were initially discovered in our attempt to produce knockout GS cells. Unusual colonies may rarely occur during the initiation of a GS cell culture; typical GS cell colonies show grape-like clumps, whereas pluripotent cell colonies appear like ES cells, or as an epiblast cell sheet (Fig. 2.1C), a derivative of the inner cell mass. Once these colonies develop in culture, they outgrow GS cells after several passages because they proliferate faster. Although they can grow in GS cell culture medium, changing the medium (DMEM + 15% FBS) and supplementing it with LIF is advisable to maintain their undifferentiated state. GDNF is no longer necessary after mGS cells are established, and established mGS cells can be maintained in the same manner as ES cells (Fig. 2.1D).

Although both GS and mGS cells are derived from testicular germ cells, they have different characteristics. GS cells are unipotent and committed to spermatogenesis, whereas mGS cells are pluripotent and can differentiate into various cell types. They behave exactly like ES cells, except they have a partial androgenetic imprinting pattern and characteristic centromeric DNA hypomethylation, reflecting their postnatal male germ cell origin (Kanatsu-Shinohara *et al.*, 2004a; Yamagata *et al.*, 2007). Because SSC activity is lost

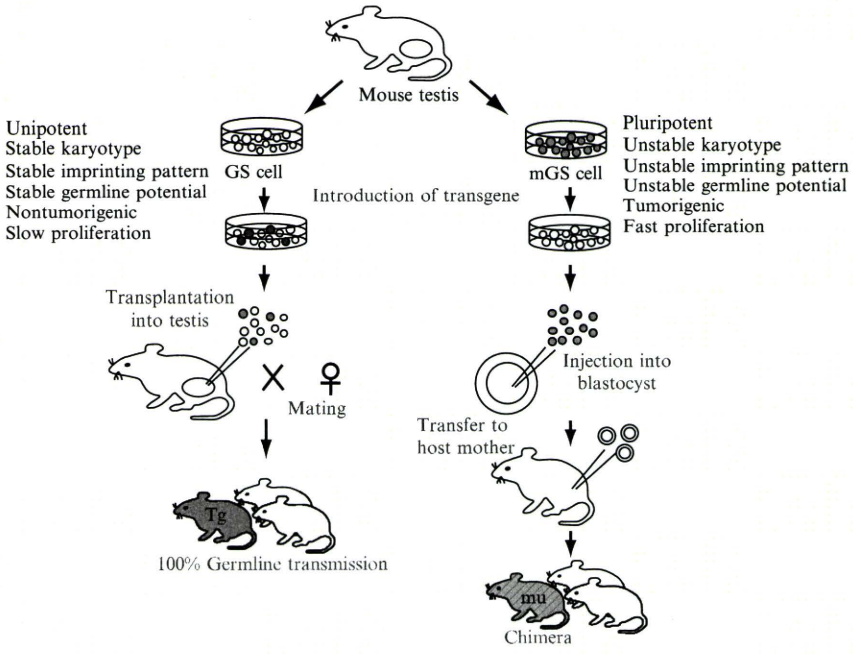

Figure 2.2 GS and mGS cell differences in their utility for animal transgenesis.

in mGS cells, they produce teratomas instead of sperm when transplanted into testes (Fig. 2.2). They produce germline chimeras following injection into blastocysts, in a manner similar to ES cells. The application of conventional methods used for ES cells allows for the production of knockout mice or double-knockout cells from mGS cells (Takehashi *et al.*, 2007b).

3. Gene Transduction and Genetic Selection of GS Cells

SSCs can be transduced with a retrovirus (Nagano *et al.*, 2000), lentivirus (Nagano *et al.*, 2002), adenovirus (Takehashi *et al.*, 2007a), or adeno-associated virus vector (Honoramooz *et al.*, 2008), and with plasmid vectors by various methods, including the calcium phosphate method, lipofection, and electroporation (Kanatsu-Shinohara *et al.*, 2005c). Knockout mice were produced with either a virus-based gene trap vector or with a gene-targeting plasmid vector. However, because viral origin promoters are repressed in germ cells and early embryonic cells, choosing promoters that are not suppressed in germline cells is necessary. According to our experience, most expression vectors used in ES cells will express foreign genes in SSCs.

Genetic selection with drugs is relatively difficult with GS cells. While ES cells grow robustly like transformed cells and can proliferate from single cells, GS cell proliferation is modest and is influenced by cell density. Therefore, one should avoid an extremely low cell density during culture. When only a few cells are recovered after drug selection, we add nontransfected "carrier" cells to enhance the growth of transfected cells (Kanatsu-Shinohara *et al.*, 2005c). These carrier cells are removed by repeated drug selection, which usually takes about 2 months.

Here, we describe the protocol for gene transduction of a gene targeting vector and the genetic selection of GS cells using a neomycin-resistant gene.

3.1. Step 1. Gene transduction to GS cells

GS cells can be transduced using the Cell Line T Nucleofector Kit (#VCA-1002; Lonza, Münster, Germany). Briefly, dissociate the GS cells with trypsin and suspend them in IMDM + 2% FBS. Remove as much of the MEF as possible because MEF abrogates GS cell survival. Cells ($4–8 \times 10^6$) should be transferred to a tube and centrifuged. Wash the cells twice with PBS, and suspend the pellet in 100 μl of Nucleofector solution (#VCA-1002; Lonza). Then add 5 μg of the gene targeting vector. The gene targeting vector is constructed following the conventional method used for ES cells, and we are able to isolate clones with a homologous recombination using both circular and linearized vectors. Plasmid DNA is suspended in sterile water or Tris–EDTA (TE). Transfer the cell suspension to a cuvette and treat with an electroporator (Nucleofector II device; Lonza) using program A-23. GS cells can also be efficiently transduced using an electroporator (Bio-Rad, Hercules, CA); the cells are suspended in PBS and treated with a single 500 μF pulse. After electroporation, culture the cells with conventional GS cell medium on MEF. The cells are passed in fresh MEF (1:1) the next day.

3.2. Step 2. Drug selection

Start the drug selection after the cells have recovered from the damage of electroporation (about 7–10 days). At 2–3 days after passage, add 40–120 μg/ml G418 (Geneticin, #83-5027; Invitrogen). If the culture reaches confluency, regardless of the G418, the cells should be passed (1:1). Puromycin (#P7255; Sigma; dissolve 1 mg/ml water) can also be used as a selection marker at 0.1–0.2 μg/ml. In contrast, if the drug efficiently reduces cell density, leave the culture plate for about 3 weeks and only change the medium, until transduced cell colonies have formed.

Treat the culture with 0.25% trypsin, and pass to fresh MEF at a 1:1 dilution; add nontransfected GS cells at a density of about 3×10^4 cells/cm^2. Restart the drug selection 2–3 days after the passage. Repeat the same procedure until a sufficient number of transfected GS cells is obtained at 100% purity. Overall, about 2–3 months is needed to obtain about 10^6 cells of an established clone.

3.3. Step 3. DNA isolation and detection of homologous recombination

Genomic DNA is isolated from cultured cells. More than about 20 μg DNA can be obtained from 2×10^6 GS cells. The clones inserted with a homologous recombination are screened with the polymerase chain reaction (PCR) or Southern blotting.

4. Spermatogonial Transplantation and Offspring Production

SSCs will migrate into the germline niche after microinjecting them into the seminiferous tubules and create germ cell colonies. This method is conceptually similar to hematopoietic stem cell transplantation (Till and McCulloch, 1961), in that donor stem cells are reintroduced into a microenvironment that lacks endogenous stem cells. This technique provided the first functional SSC assay. Furthermore, because the recipient animals eventually produce offspring from donor SSCs, this technique also allowed the possibility to manipulate SSCs to produce offspring.

4.1. Donor cell preparation

Having a donor marker during germ cell transplantation is advisable. Because single germ cell colonies originate from single SSCs (Kanatsu-Shinohara *et al.*, 2006c; Nagano *et al.*, 1999), a marker allows for the quantitative assessment of donor SSC colonization. Moreover, it also helps identify abnormal germ cell development.

Several transgenes, including LacZ or enhanced green fluorescent protein (EGFP), are used. Each has its advantage. For example, EGFP allows for offspring production using microinsemination or serial transplantation, whereas LacZ allows for better histological presentation of donor cell colonization.

Single cell suspensions are prepared using an enzymatic digestion as described above. The reaction can be stopped by adding DMEM + 10–20% FBS. Cells must be filtered though a 40-μm nylon mesh (#BD352340; BD Biosciences) before transplanting, so that cell aggregates do not clog the injection needle. Cells are usually suspended in DMEM + 10% FBS. The concentration of cells can be as high as 10^8 cells/ml.

Although fresh testicular cells can be transplanted relatively easily, freeze–thawed cells or cultured cells often clog the injection needle, which may occasionally be resolved by adding a small amount of DNase (about 0.7 mg/ml; #DN25; Sigma) to the cell suspension. Although the cells can be stored on ice for several hours, injecting the cells as soon as the cell suspension is made is better.

4.2. Recipient preparation

Donor SSCs must be histocompatible with the recipient animal. Although testes are immune-privileged organs, allogeneic SSCs can be rejected despite successful transplantation (Kanatsu-Shinohara *et al.*, 2003b). Although several cases of successful transplantation have been reported, using immunodeficient nude/scid mice is advisable when the donor cells are not histocompatible with the recipients. This allows for a xenogeneic transplantation and produces fertile spermatozoa (Clouthier *et al.*, 1996; Shinohara *et al.*, 2006).

Furthermore, elimination of endogenous SSCs is prerequisite. Transplanted SSCs are believed to compete for available niches with endogenous SSCs, and reducing the number of endogenous SSCs is thought to increase the transplantation efficiency (Shinohara *et al.*, 2002). This can be achieved by busulfan or radiation treatment. Additionally, one can use congenitally infertile mutants such as WBB6F1-W/W^{v} (designated as W) mice (Brinster and Zimmermann, 1994). While local radiation of testis specifically eliminates germ cells (Creemers *et al.*, 2002), busulfan treatment sometimes induces anemia caused by its systemic side effect on bone marrow. This can be a problem with nude mice that are relatively weak to busulfan-induced damage. However, most germ cell transplantation experiments depend on busulfan-treated recipients as described in the following protocol.

4.2.1. Step 1. Busulfan preparation

Busulfan (#B2635; Sigma) is first dissolved in dimethyl sulfoxide (DMSO) at 8 mg/ml. Because of its strong toxicity, one must wear gloves and a mask for protection. Once it is dissolved in DMSO, an equal amount of distilled water is added to the busulfan solution at 4 mg/ml. Upon mixing, the solution will generate heat. It is advisable to use the busulfan solution before it starts to form precipitates.

4.2.2. Step 2. Injection of the busulfan solution into recipient animals

Busulfan solution is injected intraperitoneally into animals. The amount of busulfan varies according to the animal background. However, we generally use doses ranging from 44 to 50 mg/kg. Inbred mice are more sensitive to busulfan than F_1 hybrids or animals in closed colonies. Although spermatogenesis can be suppressed at a lower dose, it may regenerate from endogenous SSCs after several weeks, which interferes with SSC colonization and the subsequent development of germ cell colonies. In addition, one must consider that the complete elimination of endogenous spermatogenesis is undesirable to successfully produce offspring in both mice and rats (Brinster and Avarbock, 1994; Ryu *et al.*, 2007).

4.3. Transplantation

A single donor cell suspension can be introduced into the seminiferous tubules via three routes: the seminiferous tubules, rete testis, or efferent duct (Ogawa *et al.*, 1997). In the original report (Brinster and Zimmermann, 1994), the cells were injected directly into the seminiferous tubules by exposing the tubules to air via tunica albuginea removal. However, this method is time-consuming and is the most difficult. The most popular method is efferent duct injection. The efferent duct provides passage for mature spermatozoa from the testis to the epididymis. After microinjecting the donor cell suspension, the seminiferous tubules are filled in a retrograde manner. Therefore, transplanted cells migrate in the opposite direction to normal sperm transport.

The injection can be performed manually with a 1-ml syringe equipped with a glass needle. This allows for easier guidance of the injection pipette and is recommended for the novice. Alternatively, automatic microinjection equipment used commonly for producing transgenic offspring (model 5242; Eppendorf, Hamburg, Germany) can be used. This has an advantage in that it provides a more accurate quantitative injection and better pressure control.

Here, we describe the method used to inject into the efferent duct.

4.3.1. Step1. Glass needle preparation

We regularly use glass needles (1 mm in diameter using a Sutter puller; model P-97 and #BF200-156-10; Sutter Instruments, Novato, CA) for transplantation. However, the needle edge does not need to be sharpened, as is often necessary for pronuclear DNA injection. The glass needle can be of any size or shape as long as cells can go pass through the needle tip into the seminiferous tubules.

4.3.2. Step 2. Efferent duct exposure

After anesthetizing the animal, make a straight midline incision (1.5–2 cm) in the center of the abdomen. Using blunt forceps, expose the testis by pulling out the fat attached to the epididymis. Dissect out the efferent duct using fine forceps. Finding the efferent duct is sometimes difficult in aged, fat animals. Excessive dissection will damage the duct and is not advisable.

4.3.3. Step 3. Insertion of the donor cell suspension into the glass needle

Insert the donor cell suspension from the rear end of the glass needle. Optionally, add trypan blue to the donor cell suspension to help visualize the fate of the transplanted cells. However, trypan blue may cause inflammation in some cases (Kanatsu-Shinohara *et al.*, 2008b), and including the dye in the suspension solution is not advisable for regular experiments.

The transplantation volume varies depending on the recipient type. In busulfan-treated testis, as much as 10 μl of donor cell suspension can be injected to fill 80–90% of the tubules. For a W testis, we regularly inject 4 μl because it is smaller than a busulfan-treated testis.

4.3.4. Step 4. Injection into the efferent duct

Insert the glass needle into the efferent duct (Fig. 2.3). Placing the epididymis on the medial side of the testis generally helps expose the duct. If the needle is injected too deeply along the efferent duct through the rete testis, cells will leak out into the interstitial tissue and will not colonize the seminiferous tubules.

If clogging occurs during the injection, pull out the injection needle, and after removing the clog by breaking the pipette tip, reinsert it into the efferent duct multiple times as long as the efferent duct maintains its original shape and rigidity. It is advisable not to fill 100% of the tubules because this can limit blood supply or induce inflammation in the recipient testis. High pressure in the testis can be relieved by making a small incision in the tunica using a 26–30-gauge needle. The accidental injection of air does not seem to cause a problem, but should be avoided.

Pull out the needle and move the testis back into the abdominal cavity. One need not place the testis back in the scrotum. Pull out another testis for transplantation. After the injection, return the testis to the body cavity. Muscle layers as well as skin must be sutured. Typically, transplantation into

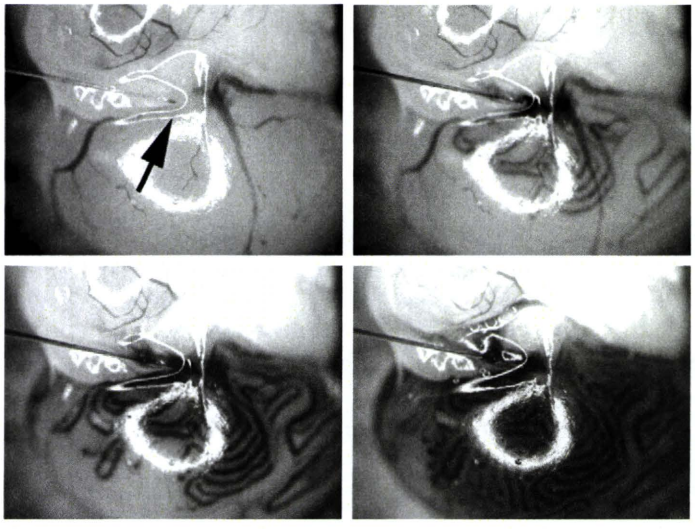

Figure 2.3 Spermatogonial transplantation. A glass needle is inserted into the efferent duct of a W mouse. The donor cell suspension is visualized by adding trypan blue solution, which gradually fills up the seminiferous tubules by increasing the injection pressure. Arrow indicates the glass needle tip.

one animal (with two testes) takes about 5–10 min for an experienced manipulator. Researchers are encouraged to refer to a detailed protocol if necessary (Ogawa *et al.*, 1997).

4.4. Optional: Measurement of SSC activity by analyzing the recipient testes

According to the donor cell marker type, colonization can be visualized by X-gal staining (LacZ) or under a UV light (EGFP). Within 2–3 weeks following transplantation, donor SSCs form a network or chains of spermatogonia on the basement membrane (Nagano *et al.*, 1999). By 1 month, the cells start to differentiate vertically toward the tubule center. By 3 months posttransplantation, donor-derived spermatozoa appear in the seminiferous tubules. Although the distribution of SSCs in the colony remains unknown, SSCs are believed to preferentially undergo self-renewal divisions at both ends of the colony and start differentiation in the center (Nagano *et al.*, 1999). During the course of colony development, the length of the colony gradually increases (1.73 mm/month), and colonies tend to merge after the long term. Colonies can be safely determined at least 6 weeks after transplantation.

An important issue here is the definition of germ cell colony because numerous donor cell clusters are present in the seminiferous tubules and one must set a criteria to define a "germ cell colony." We slightly modified the criterium of Nagano *et al.* (1999) and define a colony as when the donor cells occupy the entire seminiferous tubule and the colony is larger than 0.1 mm.

Note that not all transplanted SSCs make germ cell colonies: 5–10% of the SSCs are thought to have a repopulating ability (Nagano *et al.*, 1999; Ogawa *et al.*, 2003). In the case of GS cells, about 1–2% of the transplanted cells repopulate seminiferous tubules (Kanatsu-Shinohara *et al.*, 2005b).

4.5. Offspring production from recipient mice

Offspring are produced from donor-derived cells either by crossing the recipient mice with wild-type females via natural mating or microinsemination. Both busulfan-treated recipients and W mice will produce offspring from donor SSCs. Transplantation into pup testes results in a more rapid fertility recovery, possibly due to the absence of tight junctions between Sertoli cells in immature testes (Shinohara *et al.*, 2001). Theoretically, about 50% of the F_1 offspring from founders are heterozygous because the transgene is transmitted to half of the haploid germ cells after meiosis. Unlike ES and mGS cells, the F_1 offspring from the founder are not chimeras, and offspring are directly produced from the transplanted donor cells. However, it is necessary to confirm donor cell origin by PCR or Southern blotting because endogenous SSCs may regenerate to produce fertile sperm. Offspring were produced using primary testis cells by transplanting a few

hundred SSCs (Ogawa *et al.*, 2000; Shinohara *et al.*, 2001). Single GS cells can also produce offspring after drug selection and *in vitro* expansion (Kanatsu-Shinohara *et al.*, 2005c, 2006a).

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