Published in final edited form as:

Methods Cell Biol. 2008 ; 86: 59–84. doi:10.1016/S0091-679X(08)00004-6.

Culture of Rodent Spermatogonial Stem Cells, Male Germline Stem Cells of the Postnatal Animal

Hiroshi Kubota* and Ralph L. Brinster†

*Laboratory of Cell and Molecular Biology Department of Animal Science School of Veterinary Medicine Kitasato University Towada, Aomori 034-8628, Japan

†Department of Animal Biology School of Veterinary Medicine University of Pennsylvania Philadelphia, Pennsylvania 19104

Abstract

Spermatogonial stem cells (SSCs), postnatal male germline stem cells, are the foundation of spermatogenesis, during which an enormous number of spermatozoa is produced daily by the testis throughout life of the male. SSCs are unique among stem cells in the adult body because they are the only cells that undergo self-renewal and transmit genes to subsequent generations. In addition, SSCs provide an excellent and powerful model to study stem cell biology because of the availability of a functional assay that unequivocally identifies the stem cell. Development of an *in vitro* culture system that allows an unlimited supply of SSCs is a crucial technique to manipulate genes of the SSC to generate valuable transgenic animals, to study the self-renewal mechanism, and to develop new therapeutic strategies for infertility. In this chapter, we describe a detailed protocol for the culture of mouse and rat SSCs. A key factor for successful development of the SSC culture system was identification of *in vitro* growth factor requirements for the stem cell using a defined serum-free medium. Because transplantation assays using immunodeficient mice demonstrated that extrinsic factors for self-renewal of SSCs appear to be conserved among many mammalian species, culture techniques for SSCs of other species, including farm animals and humans, are likely to be developed in the coming 5–10 years.

I. Introduction

Germ cells are specialized cells that pass the genetic information of an individual to the next generation. Production of functional germ cells is essential for continuation of the germline of the species. Spermatogenesis, the process of male germ cell production, takes place in the seminiferous tubules of the postnatal testis and is a highly productive system in the body. In the mammalian testis, more than 20 million sperms per gram of tissue are produced daily (Amann, 1986). The high productivity relies on spermatogonial stem cells (SSCs). Like other types of stem cells in adult tissues, SSCs self-renew and produce daughter cells that commit to differentiate throughout life of the male (Meistrich and van Beek, 1993). Furthermore, in mammals, SSCs are unique among stem cells in the adult body, because they are the only cells that undergo self-renewal and transmit genes to subsequent generations.

Stem cells are defined by their biological function; therefore, unequivocal identification of a stem cell requires a functional assay (Weissman *et al.*, 2001). A functional transplantation assay for SSCs was developed in mice a decade ago and made it possible to study the biological function of SSCs, including self-renewal and differentiation (Brinster and Avarbock, 1994; Brinster and Zimmermann, 1994). In the transplantation assay, donor cells are harvested from the testes of fertile mice and are microinjected into seminiferous tubules of recipient mice. A small subpopulation of transplanted testis cells, the SSCs, colonize the basement membrane of the seminiferous tubules and begin to proliferate. Individual donor SSCs eventually form spermatogenic colonies in the seminiferous tubules of the recipients. When testicular cells from transgenic mice that express the *Escherichia coli LacZ* gene, which encodes a β -galactosidase, or the green fluorescence protein (GFP) gene are transplanted, donor colonies can be identified and counted readily because of the transgene expression in the donor cells (Fig. 1). About 2 months after transplantation of mouse SSCs, differentiated germ cells in a colony have progressed to spermatozoa in the seminiferous tubules of the recipient animal. The reconstituted spermatogenic colonies continuously produce spermatozoa throughout the remaining life of the recipient males. Several lines of evidence indicate that each donor-derived spermatogenic colony arises from a single SSC (Dobrynski *et al.*, 1999b; Kanatsu-Shinohara *et al.*, 2006b; Zhang *et al.*, 2003), and the colonization efficiency of transplanted SSCs is estimated to be 5–12% (Nagano, 2003; Ogawa *et al.*, 2003). Following transplantation, the recipient males can become fertile and produce progeny with donor cell haplotype, demonstrating normal function of the spermatozoa originating from transplanted germ cells (Brinster and Avarbock, 1994). Thus, it is clear that the spermatogenic colony-forming cells are SSCs, and the spermatogonial transplantation technique is a functional assay that allows quantitative evaluation of SSCs from a variety of sources.

Existence of a definitive functional assay to unequivocally identify SSCs provides an ideal experimental system to study stem cell biology. Using the functional assay, SSCs and the surrounding microenvironment, or the stem cell niche, in the seminiferous tubules have been studied (Brinster, 2002). Furthermore, by means of genetic modification of SSCs isolated from testes followed by transplantation, it has been shown that an SSC is a valuable vehicle to generate genetically modified animals (Hamra *et al.*, 2002; Nagano *et al.*, 2001a).

Because of the enormous potential of SSCs in basic research and applied science, including agriculture and medicine, development of an *in vitro* culture system for stem cells is extremely important. An early study demonstrated that SSCs could survive on STO (SIM mouse embryo-derived thioguanine and ouabain resistant) mouse embryonic fibroblast feeder layers for several months in culture (Nagano *et al.*, 1998). Recently, several methods to culture rodent SSCs for long periods have been reported (Hamra *et al.*, 2005; Kanatsu-Shinohara *et al.*, 2003; Kubota *et al.*, 2004b; Ryu *et al.*, 2005). To develop a long-term culture system for SSCs, one of the most crucial objectives is identification of extrinsic factors essential to promote self-renewal and expansion of SSCs *in vitro*. Previous studies using transgenic mice with gain-of-function and loss-of-function of glial cell line-derived neurotrophic factor (GDNF) indicated that this ligand is a key growth factor to control survival and proliferation of undifferentiated spermatogonia and perhaps SSCs *in vivo*.

(Meng *et al.*, 2000). Using a serum-free culture system, we clearly demonstrated that GDNF is indeed the primary growth factor for SSC self-renewal (Kubota *et al.*, 2004b). In the presence of GDNF, SSCs formed tightly packed clumps of cells and continuously proliferated. Clump-forming germ cells kept on expanding for more than 6 months in the defined serum-free medium supplemented with GDNF, and reconstituted long-term spermatogenesis following transplantation into recipient testes (Fig. 2). Furthermore, GDNF was found to be the crucial extrinsic factor for rat SSC self-renewal and proliferation (Ryu *et al.*, 2005). With slight modifications of the culture condition for mouse SSCs, rat SSCs can be expanded *in vitro*, and progeny derived from cultured rat SSCs could be generated. In this chapter, we describe a detailed method to culture mouse and rat SSCs. Since this is a technical and methodological chapter, neither the developmental biology of male germ cells nor the physiology of spermatogenesis will be reviewed. Excellent publications have described these processes in detail (de Rooij, 1998; Nagy *et al.*, 2003; Russell *et al.*, 1990; Zhao and Garbers, 2002).

II. Rationale

A. Basic Concept of SSC Culture

Development of new culture conditions for animal cells has been largely empirical. At present no unifying culture technique applicable to various tissue-specific stem cells has been developed. Despite early hopeful expectations by stem cell biologists, recent studies have indicated that characteristics of tissue-specific stem cells do not appear to be conserved (Kubota *et al.*, 2003). However, because the general process of spermatogenesis is believed to be conserved among many species, the self-renewal machinery of SSCs in different species might be similar. In fact, xenotransplantation experiments demonstrated that SSCs from all mammals examined, including rats, rabbits, dogs, pigs, cattle, horses, baboons, and humans, colonized and proliferated or were maintained in the seminiferous tubules of immunodeficient mice (Clouthier *et al.*, 1996; Dobrinski *et al.*, 1999a, 2000; Nagano *et al.*, 2001b, 2002; Oatley *et al.*, 2004). These results suggest that critical exogenous factors to promote self-renewal of SSCs are conserved among various species. Therefore, we employed a systematic approach to develop a SSC culture system using the mouse as a model because once a defined culture condition for mouse SSCs was developed, it would form the basis for other species.

Stem cells generally divide rarely in normal physiological conditions (Meistrich and van Beek, 1993; Morrison *et al.*, 1997; Potten and Morris, 1988). The micro-environment surrounding stem cells, which is called the stem cell niche, controls the timing of proliferation and differentiation of the stem cells (Spradling *et al.*, 2001). To maintain stem cells *in vitro*, reconstitution of the stem cell niche would be ideal, but likely not completely attainable. However, if one can maintain self-renewing stem cells *in vitro*, the culture condition probably provides essential signals that promote self-renewal found endogenously in the stem cell niche *in vivo*. Clearly, any knowledge about stem cell niche factors provides valuable information for the development of an *in vitro* culture system for maintaining stem cells.

To develop a defined culture condition for mouse SSCs, we chose a culture system that consists of a germ cell population enriched for stem cells, serum-free hormonally defined culture medium, and mitotically inactivated STO feeder cells (Kubota *et al.*, 2004a). Originally this culture system was developed for hepatic progenitors, hepatoblasts, in the rat (Kubota and Reid, 2000). A previous study clearly demonstrated that the culture system is useful to investigate stem/progenitor cells and the surrounding microenvironment, because the condition minimized unknown components in the culture (Kubota and Reid, 2000). Such an *in vitro* system will allow controlled and detailed investigation of factors involved in cell fate decisions. We first optimized the basic culture condition to allow mouse SSCs to survive for a short period by modifying the serum-free medium for hepatoblasts. The modified culture condition was able to maintain mouse SSCs without loss of the stem cell activity assessed by the transplantation assay for at least 1 week (Kubota *et al.*, 2004a). The availability of a functional assay made possible the evaluation of various conditions for survival of SSCs. Subsequently, we sought factors that increase stem cell number in the culture. After extensive screening utilizing the serum-free culture technique and the transplantation assay, we determined extrinsic factors essential for self-renewal proliferation of SSCs *in vitro* (Kubota *et al.*, 2004b). As described earlier, the culture system consists of three components, enrichment of SSCs, serum-free medium, and feeder cells. Because each element was important for the systematic development of the mouse SSC culture system, we describe the three components in the following sections.

B. Components of SSC Culture System

1. Spermatogonial Stem Cells—The number of SSCs in the testis is very low, presumably as few as 1 in 3000–4000 cells in the adult mouse testis (Tegelenbosch and de Rooij, 1993). Therefore, unfractionated testis cell suspensions are not ideal as a starting cell population for SSC culture. The great majority of unfractionated testicular cells in adult testes are diVerentiated or diVerentiating germ cells. These germ cells have limited proliferative activity; therefore, they die and disappear gradually in culture although the cell number is high at the beginning. In addition, since diVerentiating germ cells are nonadherent cells, they can be removed easily by changing the medium, while SSCs and spermatogonia generally adhere to the stromal feeder cells or extracellular matrices (Shinohara *et al.*, 1999). Although the diVerentiated germ cells showed limited proliferative activity in culture, there are two reasons to remove them from starting cell populations. First, the stem cells are rare in the testis cell suspension; therefore, it is almost impossible to identify the stem cells in culture. Because microscopic observation of cell divisions in culture is a valuable indication of stem cell proliferation, a large number of diVerentiated cells are an impediment to accurate assessment of stem cell behavior. Second, it is thought that diVerentiating germ cells may provide growth-inhibitory signals to undiVerentiated spermatogonia, including stem cells (Bootsma and Davids, 1988; de Rooij *et al.*, 1985; Meistrich and van Beek, 1993). Existence of such inhibitory signals or negative feedback systems is believed to occur in other stem cell systems as well (LoeZer and Potten, 1997). Although the identity of these signals is not known, it is desirable to eliminate any possibility of a detrimental eVect on SSCs.

Somatic cells, such as Sertoli cells, Leydig cells, myoid cells, and fibroblasts, also exist in the testis, although they are not major cell populations in adult testes. It is believed that terminally differentiated cells do not proliferate; therefore, most somatic cells will disappear in culture during subculturing. However, because they could produce a variety of hormones, growth factors, or extracellular matrices, it is important to remove as many somatic cells as possible to minimize the complexity of the culture conditions. In addition, fibroblastic cells generally can proliferate in regular culture medium containing animal serum; even if they are isolated from adult tissues (see below). Based on our experience, SSCs from testes of immature animals are easier to culture, but removal of testicular somatic cells from these cell suspensions is even more critical. Rapid overgrowth of fibroblastic cells can be a major problem in primary cultures, and fibroblastic cells from young animals have a high proliferative ability. In the adult, the number of testicular fibroblastic cells is fewer. However, they can gradually become dominant if serum is added to the culture medium. Serum-supplemented culture conditions selectively expand fibroblasts, because serum contains a variety of trophic factors for fibroblasts (Sato *et al.*, 1960). Use of a germ cell population enriched for SSCs is crucial to diminish the detrimental effects of fibroblastic cells and other somatic cells on SSC survival and proliferation.

2. Serum-Free Defined Medium

In general, addition of serum facilitates survival and proliferation of animal cells *in vitro*; however, several crucial drawbacks exist. First, serum contains complex materials, which have been as yet poorly defined or characterized. In addition, there is considerable batch variation depending on the physiological conditions, sex, and age of donors. Second, serum contains inhibitors of certain tissue-specific cells (Barnes and Sato, 1980b; Enat *et al.*, 1984). Third, serum enriches growth factors for mesenchymal cells, such as platelet-derived growth factor or fibroblast growth factors; therefore, mesenchymal cells, particularly fibroblasts, selectively overgrow in serum-supplemented medium (Sato *et al.*, 1960). These fibroblasts produce factors to inhibit proliferation of other cell types.

Serum-free hormonally defined medium was developed by Gordon Sato's group in the 1970s (Barnes and Sato, 1980b; Bottenstein *et al.*, 1979; Hayashi and Sato, 1976). This series of studies revealed that one of the major functions of serum is to provide hormones or growth factors which stimulate replication of cells. In serum-free media supplemented with specific hormones or growth factors, many mammalian cells were able to be maintained without loss of the cell lineage-specific and developmental stage-specific characteristics (Barnes and Sato, 1980a,b; Bottenstein *et al.*, 1979). Although there was no report of long-term cultures to maintain functional germ cells in such serum-free hormonally defined medium, early studies culturing testicular cells in serum-free medium clearly demonstrated that using defined medium is a powerful approach to study the physiology of testicular somatic cells, such as Sertoli cells or Leydig cells (Mather, 1980; Mather *et al.*, 1981). Serum-free culture is a prerequisite to identify hormones or growth factors essential for self-renewal of SSCs.

3. Feeder Cells

Generally, when a small number of cells are placed in a culture dish, they do not grow well. Conditioned media or feeder cells commonly have been used to culture cells at low cell

densities (Ham, 1963). While conditioned media support cell growth by soluble factors, feeder cells are able to stimulate cells cocultured not only by soluble factors but also by insoluble signals through direct cell–cell contact or via extracellular matrices. Originally, nonmultiplying irradiated feeder cells were used to supply conditioning factors for colony formation from single HeLa cells (Puck and Marcus, 1955). The most commonly used coculture system with a feeder layer technique was first developed for human epidermal keratinocyte culture (Rheinwald and Green, 1975). The original culture system consisted of irradiated 3T3 cells as mesenchymal feeders with serum-supplemented medium, and it supported colony formation and serial cultivation of human keratinocytes (Rheinwald and Green, 1975). A coculture system for clonal growth of rat hepatic progenitors using STO feeder cells and a defined serum-free medium (Kubota and Reid, 2000) is an evolved form of the human keratinocyte culture.

Although SSCs can be enriched by several methods (Kubota *et al.*, 2003, 2004a; Shinohara *et al.*, 1999, 2000), it is still laborious to obtain a large number of stem cells. In addition, the slow proliferation rate is a common characteristic of tissue stem cells. Therefore, a coculture system using feeder cells is reasonable for cultivation of stem cells. Early studies with hematopoietic stem cell culture have shown that coculture using stromal monolayers derived from hematopoietic tissues was able to support self-renewal of hematopoietic stem/progenitor cells for several months (Dexter *et al.*, 1977). It is worthwhile to point out that primary mouse embryonic fibroblast (MEF) feeders or STO feeder cells have generally been used for culture of germline-derived pluripotent stem cells, such as embryonic carcinoma (EC) stem cells, embryonic stem (ES) cells, or embryonic germ (EG) cells (Evans and Kaufman, 1981; Martin, 1981; Martin and Evans, 1975; Matsui *et al.*, 1992; Resnick *et al.*, 1992).

One drawback to using feeder cells is that they produce factors that are not defined. Although our culture system uses feeder cells, they were prepared from a well-established mouse cell line, STO cells, instead of primary MEFs. Recent studies have demonstrated that fibroblasts from different anatomic locations are significantly different, and substantial heterogeneity of embryonic fibroblasts exists (Chang *et al.*, 2002). Using feeders of an established cell lines minimizes the variability of the unknown contribution of the feeder cells to the culture conditions.

III. Methods

A. Protocol for Mouse SSC Culture

1. Preparation of Testis Cell Suspension and Enrichment of SSCs—

Identification of surface antigens on SSCs is necessary for immunoselection. Using fluorescence-activated cell sorting (FACS) in conjunction with the transplantation assay, the surface phenotype of mouse SSC was determined to be Thy-1⁺ α V-integrin^{−/dim} α 6-integrin⁺ c-kit[−] and major histocompatibility complex class I (MHC-I)[−] (Kubota *et al.*, 2003). Thy-1 is a surface marker expressed on SSCs in neonatal, pup, and adult testes of the mouse. Following identification of the surface antigen for SSC by FACS, a method to enrich mouse SSCs using microbeads conjugated with a Thy-1 antibody was developed (Kubota *et al.*, 2004a). The method, magnetic-activated cell sorting (MACS), using Thy-1 antibody

cannot completely purify the Thy-1⁺ α V-integrin^{-dim} α 6-integrin⁺ c-kit⁻ MHC-I⁻ cell population, but the technique requires less time and less tissues to obtain an SSC-enriched cell population. The SSC content of the cell population (MACS Thy-1 cells) depends on the age of animal. Usually, testis cells from younger mice result in cell populations with a greater percentage of SSCs than do cells from adult testes, because fewer Thy-1⁺ somatic cells are present in younger testes. Gonocytes (prespermatogonia) can be enriched by MACS from neonatal testes; however, they are quiescent in the testis and appeared to take more time to start proliferating at the beginning of the SSC culture than do MACS Thy-1 cells from pup testes (unpublished observation). Based on our experience, MACS Thy-1 cells from pup testis at 5–8 days postpartum (dpp; day of birth is 0 dpp) are the best population for SSC culture in the mouse. The following procedure is for cell preparation of MACS Thy-1⁺ cells from 4 to 8 pup testes (2–4 pups) at the age of 5–8 dpp. The procedure consists of three steps: digestion of pup testes; Percoll fractionation; and MACS separation. The procedure to enrich Thy-1⁺ cells by MACS was performed according to the manufacture's protocol. Details for preparing each reagent in the following procedures are described in the *Materials* section.

Testis cell preparation

1. Remove testes from pups with fine forceps using sterile procedures and collect the testes in a 35-mm petri dish in 3 ml of Hank's balanced salt solution (HBSS).
2. Transfer the testes to a second dish of HBSS and remove tunica albuginea under a dissecting microscope.
3. Using a p200 pipette, transfer the testis tissue without tunica to a 15 ml conical centrifuge tube containing 0.5 ml of 7 mg/ml DNase I solution and 4.5 ml of 0.25% Trypsin-EDTA.
4. Pipette up and down with p1000 pipette to disperse seminiferous tubules.
5. Incubate the tissues at 37 °C for 5 min.
6. After pipetting with p1000 pipette several times, incubate the tube at 37 °C for an additional 3 min. At this point, the cell suspension will be viscous.
7. Add 0.7 ml of fetal bovine serum (FBS) (one tenth volume) to stop enzymatic digestion, also add 0.5 ml of 7 mg/ml DNase to digest genomic DNA from dead cells.
8. Pipette well to make a single cell suspension. If cells remain clumped, add another 0.5 ml of 7 mg/ml DNase and pipette again.
9. Filter the cell suspension through a 40- μ m pore nylon cell-strainer (BD Biosciences 352340) followed by washing the cell-strainer with HBSS.
10. Centrifuge the cell suspension at 600 $\times$ g for 7 min at 4 °C.
11. Remove supernatant and resuspend cells in 10 ml of PBS-S (Dulbecco's PBS supplemented with 1% FBS, 10 mM HEPES, 1 mg/ml glucose, 1 mM pyruvate, 50 units/ml penicillin, and 50 μ g/ml of streptomycin).

12. Count cells. Typical testicular cell number obtained by this method is 1×10^6 to 1.3×10^6 cells/testis at 5 dpp and 1.7×10^6 to 2×10^6 cells/testis at 8 dpp.

Percoll fractionation

1. Slowly overlay 5 ml of cell suspension on 2 ml of 30% Percoll solution in a 15 ml conical centrifuge tube. Do not put more than 2×10^7 cells in the 15 ml tube.
2. Centrifuge at $600 \times g$ for 7 min at 4 °C without using the centrifuge brake.
3. Carefully remove the cells and debris at the interface between the HBSS and the 30% Percoll solution. Then, remove all aqueous phases containing HBSS and 30% Percoll solution. Leave the pellet at the bottom of the tube.
4. Resuspend the pellet of cells in 2 ml of PBS-S and transfer the cell suspension into a 5 ml polypropylene tube (BD Biosciences 352063). Count cell number. The range of cell recovery is 40–70%.
5. Centrifuge cell suspension at $600 \times g$ for 7 min at 4 °C.
6. Resuspend the pellet in 90 μ l of PBS-S and pipette well to make a single cell suspension.

MACS separation

1. Add 10 ml of magnetic microbeads conjugated with anti-Thy-1 antibody (Miltenyi Biotec 130-049-101, Auburn, CA) into the 90 μ l of the cell suspension and mix well. Ten microliter of Thy-1 microbeads is for one separation column. The typical range of the cell number for 10 μ l of Thy-1 microbeads is 3×10^6 to 6×10^6 cells. Use 20 μ l of Thy-1 microbeads when cell number is more than 6×10^6 cells.
2. Incubate the cell suspension containing Thy-1 microbeads for 20 min at 4 °C. Mix gently by tapping every 10 min. Longer incubation (for 30–40 min) occasionally increases the recovery rate of Thy-1⁺ cells.
3. Add 2 ml of PBS-S to the tube to dilute Thy-1 microbeads and centrifuge at $600 \times g$ for 7 min at 4 °C. Remove the supernatant completely and resuspend in 1 ml of PBS-S.
4. Place a separation column (MS Column; Miltenyi Biotec 130-042-201) in the magnetic field of the mini MACS Separation Unit (Miltenyi Biotec 130-142-102) and rinse with 0.5 ml of PBS-S.
5. Apply the cell suspension to the column. After the cell suspension has passed through the column and the column reservoir is empty, wash the column with 0.5 ml of PBS-S three times.
6. Remove the column from the MACS Separation Unit and elute the magnetically retained cells slowly into a 5 ml polypropylene tube (BD Biosciences 352063) with 1 ml of serum-free culture medium (see below) using the plunger supplied with the column.

7. Centrifuge the tube containing the cells at $600 \times g$ for 7 min at 4 °C and resuspend the cell pellet with 1 ml of mouse serum-free medium (SFM) for rinsing. Repeat this step once.
8. After the final rinsing step, resuspend cells in 0.5 ml of SFM and count the cell number. The recovery of Thy-1⁺ cells from one MS Column is 1.6×10 to 2.2×10^5 cells.

2. Serum-Free Medium

The composition of SFM for mouse SSCs is shown in Table I. To prepare mouse SFM, bovine serum albumin (BSA) powder and antibiotics are added to MEM α medium and stored at 4 °C overnight, because it takes several hours to dissolve BSA completely. The following day, stock solutions of transferrin, free fatty acid (FFA) mixture (Chessebeuf and Padieu, 1984, see Table II), selenium (Na₂SeO₃), freshly prepared 2-mercaptoethanol (2-ME), insulin, HEPES, and putrescine are added to the MEM α containing BSA and antibiotics. Preparation of stock solutions for each supplemental component is described in the *Materials* section. SFM is sterilized by filtration using a 0.2 μ m membrane filter and stored at 4 °C.

3. Preparation of Feeder Cells

STO cell s (SNL76/7 cell s) (McMahon and Bradley, 1990) were obtained from Dr. A. Bradley (The Wellcome Trust Sanger Institute, London). STO cells are routinely grown in 10 cm dishes in Dulbecco's modified Eagle's medium (DMEM) supplemented with 7% FBS (serum-supplemented medium for STO cells, SSM/STO). Although the culture methods are based on previous reports using newborn calf serum for the growth medium (Robertson, 1987), STO cells in the medium with FBS appeared to grow faster. Cultures of STO cells should be passaged promptly when they reach confluency because it is possible to accumulate noncontact-inhibited cells in the population. In addition, continuous passage of STO cells may also select clones that cannot support SSC culture. Therefore, it is advisable to expand STO cells at early passages and cryopreserve immediately in several low-passage aliquots.

1. Add mitomycin C at a final concentration of 10 μ g/ml in culture medium for confluent 10 cm dishes of STO cells. Incubate the dishes for 3–4 h at 37 °C. Avoid more than 5 h exposure to mitomycin C.
2. Remove the medium containing mitomycin C from the STO cells and wash plates three times with 10 ml of HBSS.
3. Digest the cells with trypsin-EDTA solution for 3 min at 37 °C and mix with SSM/STO to stop the trypsin activity. Collect digested cells and centrifuge at $600 \times g$ for 7 min at 4 °C. Resuspend the cells in SSM/STO at 12×10^6 cells/ml. Add to the cell suspension an equal volume of 2 $\times$ freeze medium [20% dimethyl sulfoxide (DMSO) in SSM/STO] slowly at 4 °C. The final cell concentration will be 6×10^6 cells/ml in 10% DMSO in SSM/STO.

4. Aliquot 1 ml of the STO cell suspension into each cryotube and store at -70°C using a freezing container (Nalgene 5100–0001).
5. For preparation of monolayers of STO cell feeders, thaw one vial of mitomycin C-treated STO cells at 37°C and put the cell suspension into a 50 ml conical centrifugation tube.
6. Add slowly 9 ml of SSM/STO on ice to dilute the freeze medium and mix gently. Centrifuge at $600 \times g$ for 7 min at 4°C .
7. Resuspend the cell pellet with SSM/STO to make a cell suspension at a cell concentration of 2×10^5 cells/ml. The cell viability is usually more than 90%.
8. Seed the cells on to gelatinized plates at a concentration of 5×10^4 cells/cm². Typically put 1 ml of the cell suspension (2×10^5 cells/ml) into one well of a 12-well plate. Gelatinized plates are prepared by precoating plates with 0.1% gelatin for 1 h at 37°C . STO monolayers can be created with fresh mitomycin-C treated STO cells as well.
9. Use the STO feeder cells within 4 days. If older STO feeder cells (5–7 days after seeding) are used for SSC culture, change the medium on the fourth day.

4. Culture of Mouse SSCs

While the culture of SSCs is dependent on the presence of appropriate growth factors, other elements can influence cell proliferation. After placing freshly isolated MACS Thy-1 cells in the primary culture, initiation of germ cell clump formation is an important indication of likely successful continuous culture of SSC (Fig. 3A) (Kubota *et al.*, 2004b). Each culture shows some difference in the number of germ cell clumps and the growth speed. Probably the age of the testes, purity of Thy-1⁺ cells, and damage caused by cell preparation are factors influencing initial and continuous clump formation. One of the most influential factors observed is the mouse strain used. While SSCs from DBA/2 mice proliferate easily in the presence of GDNF alone, stem cells from other mouse strains such as C57BL/6 or 129/SvCP require soluble GDNF family receptor alpha 1 (GFR α 1) and basic fibroblast growth factor (bFGF or FGF2) for continuous proliferation *in vitro* (Kubota *et al.*, 2004b). The growth advantage of DBA/2-derived SSCs was reported earlier (Kanatsu-Shinohara *et al.*, 2003). Cellular responses to GDNF are mediated by a multicomponent receptor complex consisting of RET receptor tyrosine kinase and a glycosyl phosphatidylinositol-anchored ligand-binding subunit, GFR α 1 (Sariola and Saarma, 2003). Because RET stimulation by soluble GFR α 1 potentiates downstream signaling (Paratcha *et al.*, 2001), addition of soluble GFR α 1 may play an important role for *in vitro* proliferation of SSCs in most mouse strains. bFGF has been shown to be a potent growth factor for *in vitro* proliferation of primordial germ cells (Matsui *et al.*, 1992; Resnick *et al.*, 1992). Therefore, bFGF may also provide a critical stimulus to support SSC replication in culture. Moreover, recent studies suggest that bFGF may play an important role for SSC proliferation in human testes (Goriely *et al.*, 2003).

From our experience, SSCs from DBA/2 appear to proliferate faster in the SFM supplemented with GDNF, GFR α 1, and bFGF than in GDNF alone. In the presence of three

factors, SSCs expand in culture easily from DBA/2 mice, with more diYculty from C57BL/6 mice and the most diYculty from 129/SvCP mice.

Primary culture of Thy-1⁺ germ cells

1. Remove culture medium from STO monolayer cultures and rinse the plates with HBSS twice to wash out residual medium containing serum.
2. Place 5×10^4 to 10×10^4 MACS Thy-1 cells in wells of 12-well plates containing STO monolayers. Two to four days after seeding of STO feeder cells in gelatin-coated wells is optimal for SSC primary culture.
3. Add recombinant human GDNF, rat GFR α 1, and human bFGF at a final concentration of 20 ng/ml, 150 ng/ml, and 1 ng/ml, respectively.
4. Maintain cells at 37 °C in a humidified 5% CO₂ atmosphere. Change the medium every other day. By 48 h in culture, Thy-1⁺ germ cells form small clumps with tight intercellular contacts (Fig. 3A). They continuously proliferate and form large clumps (Fig. 3A).

Recently, several studies reported SSC culture using feeder cells. Kanatsu-Shinohara *et al.* used MEF for long-term culture of SSCs derived from DBA/2 gonocytes (Kanatsu-Shinohara *et al.*, 2003). Hamra *et al.* reported that feeder cells from a mouse Sertoli cell line (MSC-1) to be superior to STO feeder cells, although germ cells were cultured on the feeder cells for only a short period (Hamra *et al.*, 2004, 2005). Both culture conditions contain FBS. MSC-1 was established from transgenic mice carrying a fusion gene composed of human Müllerian inhibitory substance transcriptional regulatory sequences linked to the SV40 T-antigen gene (Peschon *et al.*, 1992). Using our culture system, MEF feeders supported initiation of germ cell clumps; however, the growth was not as fast as STO cell feeders (Fig. 3B). MSC-1 feeder cells maintained germ cell survival and proliferation poorly (Fig. 3B). By 9 days after initial plating, most germ cells on MSC-1 feeder layers died.

The first subculture is performed 6–9 days after initial culture of MACS Thy-1 cells on STO feeders. The second and subsequent subcultures also are performed at a similar interval (6–9 days). Timing of subculture must be determined subjectively on the basis of the number of proliferating germ cells and testicular fibroblasts.

1. Remove culture medium and add 0.5 ml of trypsin-EDTA solution per well of a 12-well plate to digest the germ cell clumps.
2. Incubate cultures for 3–5 min at 37 °C and add 0.1 ml of FBS to stop digestion. Pipette digested cells gently with p1000 pipette several times. In some instances, genomic DNA from dead cells may cause cell aggregation and clumping, especially when many germ cells are cultured for more than 1 week. In that case, add 0.1 ml of 7 mg/ml DNase solution with the FBS.
3. Collect digested cells from a well of the 12-well plate in a 15 ml conical centrifugation tube and dilute the cell suspension with 2 ml of mouse SFM. Centrifuge at $600 \times g$ for 7 min at 4 °C.

4. Resuspend the cell pellet with 2 ml of mouse SFM and centrifuge at $600 \times g$ for 7 min at 4 °C. Cells are resuspended in 3 ml of SFM and plated onto fresh STO cell feeders. The split ratio of the first subculture is 1:2.
5. Sometimes testicular fibroblastic cells outgrow clump-forming germ cells. Because clump-forming germ cells are attached on STO feeders weakly, they can be removed from the feeders by pipetting gently with p1000 pipette (Ryu *et al.*, 2005). Following gentle pipetting on the surface of feeder cells, collect the culture medium containing the detached germ cell clumps and centrifuge at $600 \times g$ for 7 min at 4 °C.
6. Resuspend the pellet in mouse SFM and pipette gently several times to break up cell clumps. Place cells onto fresh STO feeder cells in the same size well as the original culture (1:1).

Usually, 1 month after initial plating, clump-forming germ cells constantly proliferate on the feeder in mouse SFM supplemented with GDNF, GFR α 1, and bFGF (Fig. 4A, phase contrast). Once cultures reach that stage, subculture can be conducted every 4–7 days at the split ratio of 1:2–1:4.

5. Characteristics of Cultured Mouse SSCs

The clump-forming germ cells express germ cell markers, such as germ cell nuclear antigen 1 (GCNA1) or mouse vasa homologue (MVH) (Enders and May, 1994; Fujiwara *et al.*, 1994; Kubota *et al.*, 2004b; Oatley *et al.*, 2006). More importantly, they show very similar characteristics with freshly isolated SSCs. Previous studies using transplantation assays and FACS demonstrated that the phenotype of SSCs in pup testes is α V-integrin^{-dim} α 6-integrin⁺ Thy-1^{lo/+} (Kubota *et al.*, 2004a). Flow cytometric analysis showed that continuously cultured germ cells are also α V-integrin^{-dim} α 6-integrin⁺ Thy-1^{lo/+}, and the surface phenotype of cultured germ cells was constant during a 3-month culture period (Kubota *et al.*, 2004b). Transplantation assays indicated that the stem cell activity of clump-forming germ cells during a 3-month culture period and fresh α V-integrin^{-dim} α 6-integrin⁺ Thy-1^{lo/+} cells isolated by MACS was similar (Kubota *et al.*, 2004b). Usually, the stem cell activity is represented by donor-derived colony number generated in the recipient testes per 10^5 transplanted cells. In our studies, the values of stem cell activity of freshly isolated cells and cultured cells were both ~500 colonies per 10^5 α V-integrin^{-dim} α 6-integrin⁺ Thy-1^{lo/6} cells transplanted (Kubota *et al.*, 2004b). Since the colonization efficiency is ~5%, 1 in 10 clump-forming germ cells was an SSC. These results indicate that the culture conditions efficiently support proliferation of α V-integrin^{-dim} α 6-integrin⁺ Thy-1^{lo/+} cells. All clump-forming germ cells expressed RET tyrosine kinase, which is the signal transducer of GDNF (Fig. 4A) (Kubota *et al.*, 2004b). Although soluble GFR α 1 was added to the culture medium, cultured germ cells expressed GFR α 1 on the cell surface (Fig. 4B) (Kubota *et al.*, 2004b). In addition to RET and GFR α 1 expression, clump-forming germ cells are positive for several spermatogonia markers including promyelocytic leukaemia zinc finger (PLZF) protein, epithelial cell adhesion molecule (EpCAM, CD326), and E-cadherin (Fig. 4A and B) (Buaas *et al.*, 2004; Costoya *et al.*, 2004; Tokuda *et al.*, 2007; van der Wee *et al.*, 2001).

The cultured SSCs shared several characteristics of undifferentiated ES cells, such as POU5F1 (previously known as Oct-3/4) expression and alkaline phosphatase activity (Kubota *et al.*, 2004b). However, significant differences exist. For example, although FBS supported ES cells in culture, the constituents of FBS are detrimental to SSC proliferation (Kubota *et al.*, 2004b). When SSCs were cultured in medium containing only 0.1% FBS, proliferation of SSCs was dramatically decreased compared with that of SSCs in SFM (Kubota *et al.*, 2004b). Cultured SSCs do not generate tumors when transplanted to immunocompromised mice (Kubota *et al.*, 2004b), whereas ES cells produce teratocarcinomas when injected into mice (Evans and Kaufman, 1981; Martin, 1981). In addition, SSCs are negative for Nanog, which is highly expressed in undifferentiated ES cells (Chambers *et al.*, 2003; Mitsui *et al.*, 2003; Oatley *et al.*, 2006).

B. Protocol for Rat SSC Culture

1. Enrichment of Rat SSCs—SSCs from rat pup testis can be enriched by immunoselection using an EpCAM antibody (Ryu *et al.*, 2004). In the rat pup testis (8 dpp), ~8% of testis cells are EpCAM⁺. The transplantation assay indicated that almost all SSCs express EpCAM (Ryu *et al.*, 2004). EpCAM is a homophilic cell–cell adhesion molecule and has been used for identification and purification of primordial germ cells or spermatogonia in rodents (Anderson *et al.*, 1999; Moore *et al.*, 2002; van der Wee *et al.*, 2001). Although rat SSCs express Thy-1 antigen, a subpopulation of the testicular somatic cells also express this surface molecule; therefore, MACS using Thy-1 antibody did not enrich rat SSCs efficiently. To obtain an SSC-enriched cell population for culture, we developed a protocol for isolation of EpCAM⁺ cells using MACS with anti-EpCAM antibody (MACS EpCAM cells). The protocol is for 4–8 testes (2–4 pups) from 8–12 dpp rat pups.

Testis cell preparation and MACS separation

1. Prepare testes for digestion as described in the mouse section.
2. Put testes into 10 ml of 1 mg/ml collagenase solution and incubate 5–8 min. Tap the bottom of the tube gently every 2–3 min.
3. Centrifuge at $600 \times g$ for 1 min at 4 °C to collect loose seminiferous tubules.
4. Remove supernatant and add 10 ml of HBSS to loosen tubules by inverting the tube. Repeat steps 3 and 4 for rinsing.
5. Remove supernatant and add 1.5 ml of 7 mg/ml DNase and 6 ml of trypsin-EDTA.
6. Digest the tissues as described in the mouse protocol.
7. Add 0.9 ml of FBS and 1 ml of 7 mg/ml DNase and pipette well using a p1000 pipette.
8. Filter the cells through a 40 mm pore nylon cell strainer and spin down cells at $600 \times g$ for 7 min at 4 °C.
9. Resuspend cells in 14 ml of MEMa containing 1% FBS and count the cell number. Typical cell recovery is 10×10^6 to 13×10^6 cells/testis at 9–10 dpp.

10. Employ Percoll fractionation as described in the mouse protocol.
11. For MACS separation, resuspend 50×10^6 cells of Percoll-fractionated cells in 5 ml of PBS-S.
12. Add 0.3–0.5 μg of anti-rat EpCAM antibody (Clone: GZ1) per 10^6 cells for labeling with primary antibody and incubate for 20 min on ice.
13. After rinsing the cells twice with 10 ml of PBS-S, resuspend cells in 0.4 ml of PBS-S. Add 0.1 ml of goat anti-mouse IgG microbeads (Miltenyi Biotec, 130–048–402) to label magnetically EpCAM⁺ cells. Incubate cells for 20 min on ice.
14. Add 10 ml of PBS-S and centrifuge at $600 \times g$ for 7 min at 4 °C.
15. Remove the supernatant and resuspend in 2 ml of PBS-S.
16. Separate EpCAM⁺ cells using two MS column according to the manufacture's protocol. For detail, see the mouse protocol described earlier.

Approximately 4–6% of cells applied to MS columns are recovered. For example, 2×10^6 to 3×10^6 EpCAM⁺ cells are obtained from 50×10^6 cells (see step 11).

2. Culture of Rat SSCs—The basic culture condition for rat SSC culture consists of SFM, STO feeder cells, and a growth factor combination that are the same as for mouse SSC culture. Although components of SFM for rat SSCs are identical to those of mouse SFM, the concentrations of several components in the SFM for rat SSCs were increased. The SFM that contains increased concentrations of several supplements was designated modified mouse SFM. The concentration of each supplement in the modified mouse SFM is indicated in Table I. In addition, reduction of medium osmolarity by the addition of water resulted in an increase in clump-forming germ cells (Ryu *et al.*, 2005). The modified mouse SFM diluted with 10% (vol/vol) distilled water was designated rat SFM.

To prepare rat SFM, BSA powder, transferrin, and antibiotics are added to MEM α , and the medium is stored at 4 °C overnight. The next day, stock solutions of FFA, Na₂SeO₃, freshly prepared 2-ME, insulin, HEPES, and putrescine are added to the MEM α containing BSA and transferrin followed by sterilization by filtration through a membrane filter of 0.2 μm pore size.

Because the concentration of several nutrients is increased in the rat SFM, the medium supports somatic cell growth better than mouse SFM. As a result, we observed during cultures a gradual increase in the number of testicular somatic cells contaminating the original MACS EpCAM cell population. Because testicular somatic cells interfered with SSC maintenance and replication (Kubota *et al.*, 2004a), their number was decreased when necessary by removing germ cell clumps at the time of subculture using gentle pipetting of medium across the surface of the feeder layer and recovery of the detached clumps with the culture medium. The collected clumps are digested with a 1:25 dilution of trypsin/EDTA (final 0.01% trypsin/40 nM EDTA) for 1 min or longer if necessary. Enzymatic digestion is stopped by FBS. Following gentle pipetting, clump-forming germ cells become small clumps, but not single cells. After rinsing the germ cells twice, place them on fresh STO feeder layers. During the first 2 months after initial plating of MACS EpCAM cells, the split

ratio for subculture should be 1:1 or 1:1.5 at an interval of every 7–10 days. When testicular fibroblasts outgrow germ cells in culture, germ cell clumps can be collected by gentle pipetting, even though the sizes of clumps are small. In that case, enzymatic digestion is not necessary, because trypsin treatment damages clump-forming cells and results in a decrease in cell recovery. Once clump-forming germ cells constantly proliferate in culture, subculture can be conducted at the split ratio of 1:2. Low atmospheric oxygen (5%) was used for long-term culture of rat SSCs (Ryu *et al.*, 2005). Low atmospheric oxygen has shown a beneficial effect on long-term proliferation of several types of mammalian cells in vitro (Ezashi *et al.*, 2005; Parrinello *et al.*, 2003).

3. Characteristics of Cultured Rat SSCs—The surface phenotype of freshly isolated rat SSCs in pup testis is EpCAM⁺ Thy-1^{lo} β 3-integrin[−] (Ryu *et al.*, 2004). The surface antigen profile of rat SSCs cultured for more than 10 months was basically similar to that of fresh SSCs (Ryu *et al.*, 2005). However, the surface expression of these antigens increased slightly in cultured cells. POU5F1 expression and alkaline phosphatase activity are present in cultured rat SSCs, indicating that mouse and rat SSCs share these characteristics found in ES cells or PGCs. Rat SSCs also express RET and GFR α 1, which are the receptors for GDNF (Ryu *et al.*, 2005).

IV. Materials

A. Reagents for Cell Preparation

DNase: DNase I (Sigma DN25) solution is prepared at 7 mg/ml in HBSS and sterilized by filtration.

Collagenase: Collagenase type IV (Sigma C5138) solution is prepared at 1 mg/ml in HBSS and sterilized by filtration.

Trypsin-EDTA: 0.25% trypsin and 1 mM EDTA (Gibco/Invitrogen 25200). **Antibiotics:** 10,000 units/ml penicillin and 10,000 μ g/ml of streptomycin (Gibco/Invitrogen 15140). **FBS:** FBS (Hyclone) is treated at 56 °C for 30 min. Aliquots are stored at −20 °C.

Pyruvate: Stock solution is prepared at 100 mM sodium pyruvate (Sigma P2256) in distilled deionized water (DDW), sterilized by filtration using 0.2 μ m membrane filter, and stored at 4 °C.

PBS-S: Dulbecco's PBS supplemented with 1% FBS, 10 mM HEPES (Sigma H0887), 1 mg/ml glucose (Sigma G6152), 1 mM pyruvate, 50 units/ml penicillin, and 50 μ g/ml streptomycin sterilized by filtration using 0.2 μ m membrane filter, and stored at 4 °C.

Percoll solution: 30% (vol/vol) Percoll (Sigma P4937) is prepared in Dulbecco's PBS containing 1% FBS, 50 units/ml penicillin, and 50 mg/ml streptomycin. Percoll solution is sterilized by filtration using a 0.2 μ m membrane filter and stored at 4 °C.

B. Stock Solution of Reagents for SFM and Cell Culture

Transferrin: Stock solution is prepared at 10 mg/ml in Dulbecco's PBS and stored at −20 °C.

FFA mixture: Detailed information is described in Table II. Make a small volume aliquot (e.g., 50–100 μ l/0.5 ml tube) of FFA mixture. For storage of stock solutions of each FFA or FFA mixture aliquot, it is advised to flush vials or tubes with nitrogen before closing to prevent oxidation of FFAs. The tubes are sealed with parafilm and stored at -20°C .

Na_2SeO_3 : Stock solution is prepared at 3×10^{-4} M in DDW and stored at -20°C .

2-ME: 100 mM solution is prepared freshly each time medium is made. 100 mM solution is prepared by adding 7 μ l of 14.4 M 2-ME (Sigma M7522) in 1 ml of MEM α medium.

Insulin: Stock solution is prepared at 10 mg/ml in 10 mM HCl and stored at -20°C .

Putrescine: Stock solution is prepared at 100 mM in Dulbecco's PBS and stored at -20°C .

GDNF: Human recombinant GDNF (R&D Systems, 212-GD-010) stock solution is prepared at 20 μ g/ml in Dulbecco's PBS containing 0.1% BSA and stored at -70°C .

GFR α 1: Rat recombinant GFR α 1/Fc Chimera (R&D Systems, 560-GR-100) stock solution is prepared at 100 μ g/ml in Dulbecco's PBS containing 0.1% BSA and stored at -70°C .

bFGF: Human recombinant bFGF (BD Biosciences, 354060) stock solution is prepared at 10 μ g/ml in Dulbecco's PBS containing 0.1% BSA and stored at -70°C .

C. Reagents for STO Feeder Layers

SSM/STO (serum-supplemented medium for STO cells): The medium for STO cell culture is Dulbecco's modified Eagle's medium (DMEM, Gibco/Invitrogen 11965) supplemented with 7% FBS, 100 μ M 2-ME, 10 mM HEPES, 2 mM glutamine, 50 units/ml penicillin, and 50 μ g/ml streptomycin. SSM/STO is sterilized by filtration using a 0.2 μ m membrane filter and stored at 4°C .

Mytomycin C: Stock mitomycin C (Sigma M4287) solution is prepared at 200 μ g/ml in Dulbecco's PBS and stored at -70°C .

Freezing medium (2 $\times$): 20% dimethyl sulfoxide (DMSO, Sigma D2650) in SSM/STO.

Gelatin: 0.1% gelatin (porcine skin type A, Sigma G2500) in DDW is autoclaved for sterilization and stored at room temperature.

V. Discussion

An exciting practical aspect of the development of culture systems for SSCs is that the technique establishes a foundation for sophisticated genetic manipulation, including targeted modification, of the species from which the stem cells were isolated (Kanatsu-Shinohara *et al.*, 2006a). The conservation of GDNF signaling in mouse and rat SSCs as the essential pathway to stimulate *in vitro* self-renewal and the previously demonstrated ability of SSC of many species to maintain and proliferate in mouse seminiferous tubules suggest that a similar culture system can be developed to obtain continuous proliferation of SSCs of many mammalian species, including humans. In addition, because a large number of SSCs can be

generated in culture, they represent a powerful resource for gene analysis to elucidate the mechanisms governing self-renewal and differentiation of the stem cells (Oatley *et al.*, 2006). Moreover, continuous *in vitro* proliferation of SSCs of any species lays the foundation for the development of systems to support germ cell differentiation *in vitro*. Modulating culture conditions that support differentiation processes of male germ cells resulting in production of functional gametes *in vitro* will create a valuable model for studying the molecular and cellular biology of male germ cell differentiation. Such an *in vitro* experimental system may allow development of new therapeutic strategies for infertility (Brinster, 2007; Kubota and Brinster, 2006). The progressive development of culture systems for SSCs of other mammalian species including humans is imminent and will provide the basis for a wide range of studies on the biology of the stem cell, *in vitro* differentiation of germ cells, and modification of germlines.

Acknowledgments

Research support has been provided by the National Institutes of Health and Robert J. Kleberg, Jr. and Helen C. Kleberg Foundation.

References

- Amann RP. Detection of alterations in testicular and epididymal function in laboratory animals. *Environ. Health Perspect.* 1986; 70:149–158. [PubMed: 3830100]
- Anderson R, Schaible K, Heasman J, Wylie C. Expression of the homophilic adhesion molecule, EPCAM, in the mammalian germ line. *J. Reprod. Fertil.* 1999; 116:379–384. [PubMed: 10615264]
- Barnes D, Sato G. Methods for growth of cultured cells in serum-free medium. *Anal. Biochem.* 1980a; 102:255–270. [PubMed: 6999941]
- Barnes D, Sato G. Serum-free cell culture: A unifying approach. *Cell.* 1980b; 22:649–655. [PubMed: 7460009]
- Bootsma AL, Davids JA. The cell cycle of spermatogonial colony forming stem cells in the CBA mouse after neutron irradiation. *Cell Tissue Kinet.* 1988; 21:105–113. [PubMed: 3203373]
- Bottenstein J, Hayashi I, Hutchings S, Masui H, Mather J, McClure DB, Ohasa S, Rizzino A, Sato G, Serrero G, Wolfe R, Wu R. The growth of cells in serum-free hormone-supplemented media. *Methods Enzymol.* 1979; 58:94–109. [PubMed: 423795]
- Brinster RL. Germline stem cell transplantation and transgenesis. *Science.* 2002; 296:2174–2176. [PubMed: 12077400]
- Brinster RL. Male germline stem cells: From mice to men. *Science.* 2007; 316:404–405. [PubMed: 17446391]
- Brinster RL, Avarbock MR. Germline transmission of donor haplotype following spermatogonial transplantation. *Proc. Natl. Acad. Sci. USA.* 1994; 91:11303–11307. [PubMed: 7972054]
- Brinster RL, Zimmermann JW. Spermatogenesis following male germ-cell transplantation. *Proc. Natl. Acad. Sci. USA.* 1994; 91:11298–11302. [PubMed: 7972053]
- Buaas FW, Kirsh AL, Sharma M, McLean DJ, Morris JL, Griswold MD, de Rooij DG, Braun RE. Plzf is required in adult male germ cells for stem cell self-renewal. *Nat Genet.* 2004; 36:647–652. [PubMed: 15156142]
- Chambers I, Colby D, Robertson M, Nichols J, Lee S, Tweedie S, Smith A. Functional expression cloning of Nanog, a pluripotency sustaining factor in embryonic stem cells. *Cell.* 2003; 113:643–655. [PubMed: 12787505]
- Chang HY, Chi JT, Dudoit S, Bondre C, van de Rijn M, Botstein D, Brown PO. Diversity, topographic differentiation, and positional memory in human fibroblasts. *PNAS.* 2002; 99:12877–12882. [PubMed: 12297622]

- Chessebeuf M, Padieu P. Rat liver epithelial cell cultures in a serum-free medium: Primary cultures and derived cell lines expressing differentiated functions. *In Vitro*. 1984; 20:780–795. [PubMed: 6151543]
- Clouthier DE, Avarbock MR, Maika SD, Hammer RE, Brinster RL. Rat spermatogenesis in mouse testis. *Nature*. 1996; 381:418–421. [PubMed: 8632797]
- Costoya JA, Hobbs RM, Barna M, Cattoretti G, Manova K, Sukhwani M, Orwig KE, Wolgemuth DJ, Pandolfi PP. Essential role of Plzf in maintenance of spermatogonial stem cells. *Nat. Genet.* 2004; 36:653–659. [PubMed: 15156143]
- de Rooij DG. Stem cells in the testis. *Int. J. Exp. Pathol.* 1998; 79:67–80. [PubMed: 9709376]
- de Rooij DG, Lok D, Weenk D. Feedback regulation of the proliferation of the undifferentiated spermatogonia in the Chinese hamster by the differentiating spermatogonia. *Cell Tissue Kinet.* 1985; 18:71–81. [PubMed: 3971417]
- Dexter TM, Allen TD, Lajtha LG. Conditions controlling the proliferation of hemopoietic stem cells in vitro. *J. Cell. Physiol.* 1977; 91:335–344. [PubMed: 301143]
- Dobrinski I, Avarbock MR, Brinster RL. Transplantation of germ cells from rabbits and dogs into mouse testes. *Biol. Reprod.* 1999a; 61:1331–1339. [PubMed: 10529282]
- Dobrinski I, Avarbock MR, Brinster RL. Germ cell transplantation from large domestic animals into mouse testes. *Mol. Reprod. Dev.* 2000; 57:270–279. [PubMed: 11013435]
- Dobrinski I, Ogawa T, Avarbock MR, Brinster RL. Computer assisted image analysis to assess colonization of recipient seminiferous tubules by spermatogonial stem cells from transgenic donor mice. *Mol. Reprod. Dev.* 1999b; 53:142–148. [PubMed: 10331452]
- Enat R, Jefferson DM, Ruiz-Opazo N, Gatmaitan Z, Leinwand LA, Reid LM. Hepatocyte proliferation in vitro: Its dependence on the use of serum-free hormonally defined medium and substrata of extracellular matrix. *Proc. Natl. Acad. Sci. USA.* 1984; 81:1411–1415. [PubMed: 6584889]
- Enders GC, May II. Developmentally regulated expression of a mouse germ cell nuclear antigen examined from embryonic day 11 to adult in male and female mice. *Dev. Biol.* 1994; 163:331–340. [PubMed: 8200475]
- Evans MJ, Kaufman MH. Establishment in culture of pluripotential cells from mouse embryos. *Nature*. 1981; 292:154–156. [PubMed: 7242681]
- Ezashi T, Das P, Roberts RM. Low O₂ tensions and the prevention of differentiation of hES cells. *Proc. Natl. Acad. Sci. USA.* 2005; 102:4783–4788. [PubMed: 15772165]
- Fujiwara Y, Komiya T, Kawabata H, Sato M, Fujimoto H, Furusawa M, Noce T. Isolation of a DEAD-family protein gene that encodes a murine homolog of Drosophila vasa and its specific expression in germ cell lineage. *Proc. Natl. Acad. Sci. USA.* 1994; 91:12258–12262. [PubMed: 7991615]
- Goriely A, McVean GA, Rojmyr M, Ingemarsson B, Wilkie AO. Evidence for selective advantage of pathogenic FGFR2 mutations in the male germ line. *Science*. 2003; 301:643–646. [PubMed: 12893942]
- Ham RG. An improved nutrient solution for diploid Chinese hamster and human cell lines. *Exp. Cell Res.* 1963; 29:515–526. [PubMed: 13952250]
- Hamra FK, Chapman KM, Nguyen DM, Williams-Stephens AA, Hammer RE, Garbers DL. Self renewal, expansion, and transfection of rat spermatogonial stem cells in culture. *Proc. Natl. Acad. Sci. USA.* 2005; 102:17430–17435. [PubMed: 16293688]
- Hamra FK, Gatlin J, Chapman KM, Grellhesl DM, Garcia JV, Hammer RE, Garbers DL. Production of transgenic rats by lentiviral transduction of male germ-line stem cells. *Proc. Natl. Acad. Sci. USA.* 2002; 99:14931–14936. [PubMed: 12391306]
- Hamra FK, Schultz N, Chapman KM, Grellhesl DM, Cronkhite JT, Hammer RE, Garbers DL. Defining the spermatogonial stem cell. *Dev. Biol.* 2004; 269:393–410. [PubMed: 15110708]
- Hayashi I, Sato GH. Replacement of serum by hormones permits growth of cells in a defined medium. *Nature*. 1976; 259:132–134. [PubMed: 813153]
- Kanatsu-Shinohara M, Ikawa M, Takehashi M, Ogonuki N, Miki H, Inoue K, Kazuki Y, Lee J, Toyokuni S, Oshimura M, Ogura A, Shinohara T. Production of knockout mice by random or targeted mutagenesis in spermatogonial stem cells. *Proc. Natl. Acad. Sci. USA.* 2006a; 103:8018–8023. [PubMed: 16679411]

- Kanatsu-Shinohara M, Inoue K, Miki H, Ogonuki N, Takehashi M, Morimoto T, Ogura A, Shinohara T. Clonal origin of germ cell colonies after spermatogonial transplantation in mice. *Biol. Reprod.* 2006; 75:68–74. [PubMed: 16598026]
- Kanatsu-Shinohara M, Ogonuki N, Inoue K, Miki H, Ogura A, Toyokuni S, Shinohara T. Long-term proliferation in culture and germline transmission of mouse male germline stem cells. *Biol. Reprod.* 2003; 69:612–616. [PubMed: 12700182]
- Kubota H, Avarbock MR, Brinster RL. Spermatogonial stem cells share some, but not all, phenotypic and functional characteristics with other stem cells. *Proc. Natl. Acad. Sci. USA.* 2003; 100:6487–6492. [PubMed: 12738887]
- Kubota H, Avarbock MR, Brinster RL. Culture conditions and single growth factors affect fate determination of mouse spermatogonial stem cells. *Biol. Reprod.* 2004a; 71:722–731. [PubMed: 15115718]
- Kubota H, Avarbock MR, Brinster RL. Growth factors essential for self-renewal and expansion of mouse spermatogonial stem cells. *Proc. Natl. Acad. Sci. USA.* 2004b; 101:16489–16494. [PubMed: 15520394]
- Kubota H, Brinster RL. Technology insight: In vitro culture of spermatogonial stem cells and their potential therapeutic uses. *Nat. Clin. Pract. Endocrinol. Metab.* 2006; 2:99–108. [PubMed: 16932264]
- Kubota H, Reid LM. Clonogenic hepatoblasts, common precursors for hepatocytic and biliary lineages, are lacking classical major histocompatibility complex class I antigen. *Proc. Natl. Acad. Sci. USA.* 2000; 97:12132–12137. [PubMed: 11050242]
- LoeZer, M.; Potten, CS. Stem cells and cellular pedigrees—a conceptual introduction.. In: Potten, CS., editor. *Stem Cells*. Academic Press; San Diego: 1997. p. 1-27.
- Martin GR. Isolation of a pluripotent cell line from early mouse embryos cultured in medium conditioned by teratocarcinoma stem cells. *Proc. Natl. Acad. Sci. USA.* 1981; 78:7634–7638. [PubMed: 6950406]
- Martin GR, Evans MJ. Differentiation of clonal lines of teratocarcinoma cells: Formation of embryoid bodies in vitro. *Proc. Natl. Acad. Sci. USA.* 1975; 72:1441–1445. [PubMed: 1055416]
- Mather JP. Establishment and characterization of two distinct mouse testicular epithelial cell lines. *Biol. Reprod.* 1980; 23:243–252. [PubMed: 6774781]
- Mather JP, Saez JM, Haour F. Primary cultures of Leydig cells from rat, mouse and pig: Advantages of porcine cells for the study of gonadotropin regulation of Leydig cell function. *Steroids.* 1981; 38:35–44. [PubMed: 6270852]
- Matsui Y, Zsebo K, Hogan BL. Derivation of pluripotential embryonic stem cells from murine primordial germ cells in culture. *Cell.* 1992; 70:841–847. [PubMed: 1381289]
- McMahon AP, Bradley A. The Wnt-1 (int-1) proto-oncogene is required for development of a large region of the mouse brain. *Cell.* 1990; 62:1073–1085. [PubMed: 2205396]
- Meistrich, ML.; van Beek, MEAB. Spermatogonial stem cells.. In: Desjardins, C.; Ewing, LL., editors. *Cell and Molecular Biology of the Testis*. New York; Oxford University Press: 1993. p. 266-295.
- Meng X, Lindahl M, Hyvonen ME, Parvinen M, de Rooij DG, Hess MW, Raatikainen-Ahokas A, Sainio K, Rauvala H, Lakso M, Pichel JG, Westphal H, et al. Regulation of cell fate decision of undifferentiated spermatogonia by GDNF. *Science.* 2000; 287:1489–1493. [PubMed: 10688798]
- Mitsui K, Tokuzawa Y, Itoh H, Segawa K, Murakami M, Takahashi K, Maruyama M, Maeda M, Yamanaka S. The homeoprotein Nanog is required for maintenance of pluripotency in mouse epiblast and ES cells. *Cell.* 2003; 113:631–642. [PubMed: 12787504]
- Moore TJ, Boer-Brouwer M, Dissel-Emiliani FM. Purified gonocytes from the neonatal rat form foci of proliferating germ cells in vitro. *Endocrinology.* 2002; 143:3171–3174. [PubMed: 12130583]
- Morrison SJ, Shah NM, Anderson DJ. Regulatory mechanisms in stem cell biology. *Cell.* 1997; 88:287–298. [PubMed: 9039255]
- Nagano M, Avarbock MR, Leonida EB, Brinster CJ, Brinster RL. Culture of mouse spermatogonial stem cells. *Tissue Cell.* 1998; 30:389–397. [PubMed: 9787472]
- Nagano M, Brinster CJ, Orwig KE, Ryu BY, Avarbock MR, Brinster RL. Transgenic mice produced by retroviral transduction of male germ-line stem cells. *Proc. Natl. Acad. Sci. USA.* 2001a; 98:13090–13095. [PubMed: 11606778]

- Nagano M, McCarrey JR, Brinster RL. Primate spermatogonial stem cells colonize mouse testes. *Biol. Reprod.* 2001b; 64:1409–1416. [PubMed: 11319145]
- Nagano M, Patrizio P, Brinster RL. Long-term survival of human spermatogonial stem cells in mouse testes. *Fertil. Steril.* 2002; 78:1225–1233. [PubMed: 12477516]
- Nagano MC. Homing efficiency and proliferation kinetics of male germ line stem cells following transplantation in mice. *Biol. Reprod.* 2003; 69:701–707. [PubMed: 12700185]
- Nagy, A.; Gertsenstein, M.; Vintersten, K.; Behringer, R. *Manipulating the Mouse Embryo: A Laboratory Manual*. Cold Spring Harbor Laboratory Press; Cold Spring Harbor, New York, USA: 2003.
- Oatley JM, Avarbock MR, Telaranta AI, Fearon DT, Brinster RL. Identifying genes important for spermatogonial stem cell self-renewal and survival. *Proc. Natl. Acad. Sci. USA.* 2006; 103:9524–9529. [PubMed: 16740658]
- Oatley JM, Reeves JJ, McLean DJ. Biological activity of cryopreserved bovine spermatogonial stem cells during in vitro culture. *Biol. Reprod.* 2004; 71:942–947. [PubMed: 15151932]
- Ogawa T, Ohmura M, Yumura Y, Sawada H, Kubota Y. Expansion of murine spermatogonial stem cells through serial transplantation. *Biol. Reprod.* 2003; 68:316–322. [PubMed: 12493728]
- Paratcha G, Ledda F, Baars L, Couplier M, Besset V, Anders J, Scott R, Ibanez CF. Released GFRalpha1 potentiates downstream signaling, neuronal survival, and differentiation via a novel mechanism of recruitment of c-Ret to lipid rafts. *Neuron.* 2001; 29:171–184. [PubMed: 11182089]
- Parinello S, Samper E, Krtolica A, Goldstein J, Melov S, Campisi J. Oxygen sensitivity severely limits the replicative lifespan of murine fibroblasts. *Nat. Cell Biol.* 2003; 5:741–747. [PubMed: 12855956]
- Peschon JJ, Behringer RR, Cate RL, Harwood KA, Idzerda RL, Brinster RL, Palmiter RD. Directed expression of an oncogene to Sertoli cells in transgenic mice using mullerian inhibiting substance regulatory sequences. *Mol. Endocrinol.* 1992; 6:1403–1411. [PubMed: 1331774]
- Potten CS, Morris RJ. Epithelial stem cells in vivo. *J. Cell. Sci. Suppl.* 1988; 10:45–62. [PubMed: 3077942]
- Puck T, Marcus P. A rapid method for viable cell titration and clone production with hela cells in tissue culture: The use of X-irradiated cells to supply conditioning factors. *Proc. Natl. Acad. Sci. USA.* 1955; 41:432–437. [PubMed: 16589695]
- Resnick JL, Bixler LS, Cheng L, Donovan PJ. Long-term proliferation of mouse primordial germ cells in culture. *Nature.* 1992; 359:550–551. [PubMed: 1383830]
- Rheinwald JG, Green H. Serial cultivation of strains of human epidermal keratinocytes: The formation of keratinizing colonies from single cells. *Cell.* 1975; 6:331–343. [PubMed: 1052771]
- Robertson, EJ. Embryo-derived stem cell lines.. In: Robertson, EJ., editor. *Teratocarcinomas and Embryonic Stem Cells: A Practical Approach*. IRS Press; Oxford, England: 1987. p. 71-112.
- Russell, LD.; Ettlin, RA.; Sinha-Hikim Amiya, P.; Clegg, ED. *Histological and Histopathological Evaluation of the Testis*. Cache River Press; Clearwater, FL.: 1990.
- Ryu BY, Kubota H, Avarbock MR, Brinster RL. Conservation of spermatogonial stem cell self-renewal signaling between mouse and rat. *Proc. Natl. Acad. Sci. USA.* 2005; 102:14302–14307. [PubMed: 16183739]
- Ryu BY, Orwig KE, Kubota H, Avarbock MR, Brinster RL. Phenotypic and functional characteristics of spermatogonial stem cells in rats. *Dev. Biol.* 2004; 274:158–170. [PubMed: 15355795]
- Sariola H, Saarma M. Novel functions and signalling pathways for GDNF. *J. Cell Sci.* 2003; 116:3855–3862. [PubMed: 12953054]
- Sato G, Zaroff L, Mills SE. Tissue culture populations and their relation to the tissue of origin. *Proc. Natl. Acad. Sci. USA.* 1960; 46:963–972. [PubMed: 16590700]
- Shinohara T, Avarbock MR, Brinster RL. beta1- and alpha6-integrin are surface markers on mouse spermatogonial stem cells. *Proc. Natl. Acad. Sci. USA.* 1999; 96:5504–5509. [PubMed: 10318913]
- Shinohara T, Orwig KE, Avarbock MR, Brinster RL. Spermatogonial stem cell enrichment by multiparameter selection of mouse testis cells. *Proc. Natl. Acad. Sci. USA.* 2000; 97:8346–8351. [PubMed: 10900001]

- Spradling A, Drummond-Barbosa D, Kai T. Stem cells find their niche. *Nature*. 2001; 414:98–104. [PubMed: 11689954]
- Tegelenbosch RA, de Rooij DG. A quantitative study of spermatogonial multiplication and stem cell renewal in the C3H/101 F1 hybrid mouse. *Mutat. Res.* 1993; 290:193–200. [PubMed: 7694110]
- Tokuda M, Kadokawa Y, Kurahashi H, Marunouchi T. CDH1 is a specific marker for undifferentiated spermatogonia in mouse testes. *Biol. Reprod.* 2007; 76:130–141. [PubMed: 17035642]
- van der Wee KS, Johnson EW, Dirami G, Dym TM, Hofmann MC. Immuno-magnetic isolation and long-term culture of mouse type A spermatogonia. *J. Androl.* 2001; 22:696–704. [PubMed: 11451367]
- Weissman IL, Anderson DJ, Gage F. Stem and progenitor cells: Origins, phenotypes, lineage commitments, and transdifferentiations. *Annu. Rev. Cell Dev. Biol.* 2001; 17:387–403. [PubMed: 11687494]
- Zhang X, Ebata KT, Nagano MC. Genetic analysis of the clonal origin of regenerating mouse spermatogenesis following transplantation. *Biol. Reprod.* 2003; 69:1872–1878. [PubMed: 12904317]
- Zhao GQ, Garbers DL. Male germ cell specification and differentiation. *Dev. Cell.* 2002; 2:537–547. [PubMed: 12015962]

Fig. 1.

Colony formation of donor-derived spermatogenesis in infertile recipient mouse testes.

Testicular germ cells were isolated from transgenic mice that express reporter genes (*LacZ* or GFP) and injected into testes of mice treated with busulfan. Two months after transplantation, donor-derived spermatogenesis is reconstituted. Left: testis transplanted with *LacZ* expressing SSCs from transgenic mouse line B6.129S-Gt(ROSA)26Sor/J (ROSA, Jackson Laboratory). The testis was stained with 5-bromo-4-chloro-3-indolyl β -D-galactoside (X-gal). Right: testis transplanted with GFP-expressing SSCs from transgenic mouse line C57BL/6-TgNACTB-EGFP)1Os/J (Jackson Laboratory). Each blue stretch or green stretch of cells in the testes represents a colony of spermatogenesis that arises from a single SSC.

Fig. 2.

Histological cross sections of seminiferous tubules of recipient W^{54}/W^r testes transplanted with cultured SSCs. Histological sections of testes transplanted with ROSA SSCs that were cultured for 6 months (left, stained with X-gal, counter stain; Nuclear Fast Red) and 7.5 months (right, stain; Hematoxylin-Eosin).

Fig. 3.

Microscopic observation of cultured SSCs. (A) development of germ cell clumps from 129/SvCP Thy-1⁺ pup testis cells isolated by MACS. MACS Thy-1 cells were placed on STO feeder cells in serum-free defined medium supplemented with GDNF. Single Thy-1⁺ cells at 5 h in culture (left). Initiation of cell clump formation at 2 days (middle). Growth of germ cell clumps at 5 days (right). (B) Comparison of feeder cells for their ability to support germ-cell clump formation and growth. C57BL/6-derived SSCs were placed on STO, MEF, or MSC-1 feeders in the presence of GDNF, GFR α 1, and bFGF, and cultured for 9 days.

Fig. 4. Phenotypic characteristics of cultured SSCs. (A) SSCs from C57BL/6 pup testes continuously proliferate on STO feeder cells in serum-free defined medium supplemented with GDNF, GFR α 1, and bFGF. A phase contrast image of SSCs cultured for 1 month (left) and immunocytochemistry of RET (middle) and PLZF (right). Clump-forming germ cells were stained with antibodies against RET or PLZF. (B) Cell surface molecules on clump-forming germ cells were analyzed by flow cytometry. Cultured SSCs express GFR α 1, EpCAM, and E-cadherin. Filled histograms represent stained cells with antibodies indicated. Open histograms indicate unstained cells.

Table I

Components in Mouse Serum-Free Medium (SFM) and Modified Mouse SFM

	Manufacturer	Catalog number	Mouse SFM ^a	Modified mouse SFM ^b
MEMa	Gibco/Invitrogen	12561	Basal media	Basal media
Penicillin	Gibco/Invitrogen	15140	50 units/ml	50 units/ml
Streptomycin	Gibco/Invitrogen	15140	50 µg/ml	50 µg/ml
BSA	MP Biomedicals/ICN	See Note ^c	0.2%	0.6%
Transferrin	Sigma	T 1283	10 µg/ml	100 µg/ml
FFA mixture	Sigma	See Table II	7.6 µeq/L	15.2 µeq/L
Na ₂ SeO ₃	Aldrich/Sigma	481815	3 × 10 ⁻⁸ M	6 × 10 ⁻⁸ M
L-glutamine	Gibco/Invitrogen	25030	2 mM	2 mM
2-ME	Sigma	M 7522	50 µM	100 µM
Insulin	Sigma	I 5500	5 µg/ml	25 µg/ml
HEPES	Sigma	H 0887	10 mM	10 mM
Putrescine	Sigma	P 5780	60 µM	120 µM

^aThe concentration of each component in serum-free medium for mouse SSC culture is indicated.

^bModified mouse SFM is made by increasing several components of mouse SFM. Serum-free medium for rat SSC culture is modified mouse SFM plus 10% DDW (10 ml of water is added to 100 ml of modified mouse SFM). Thus, the final concentrations of each supplemental component in rat SFM is about 90% of that in modified mouse SFM.

^cNote: The source and lot of BSA are important. BSA of MP Biochemicals (formerly ICN), catalog number 810661 (lot number 2943C) and 194774 (lot number R14550), supported long-term *in vitro* proliferation of mouse SSCs. BSA from Sigma, catalog number A3803 (lot numbers 064K0720, 025K1497, and 124K0729), could be used for mouse SSC culture. For rat SSC culture, BSA of MP Biochemicals, catalog number 810661 (lot number 2943C and 4561H), was effective.

Table II

Preparation of Free Fatty Acid Mixture

Free fatty acid (FFA)	Manufacturer	Catalog number	Stock solution ^a	100 meq/l FFA mixture ^b
Linolenic acid	Sigma	L 2376	1 M	5.6 μ l (5.6 mM)
Oleic acid	Sigma	O 1008	1 M	13.4 μ l (13.4 mM)
Palmitoleic acid	Sigma	P 9417	1 M	2.8 μ l (2.8 mM)
Linoleic acid	Sigma	L 1012	1 M	35.6 μ l (35.6 mM)
Palmitic acid	Sigma	P 0500	1 M	31.0 μ l (31.0 mM)
Stearic acid	Sigma	S 4751	151 mM	76.9 μ l (11.6 mM)
Absolute ethanol				834.7 μ l
Final volume				1000 μ l (100 meq/l)

^a Ethanol is used to make stock solutions of each FFA. One molar stock solutions of linolenic acid, oleic acid, palmitoleic acid, and linoleic acid are liquid at room temperature. One molar palmitic acid and 151 mM stearic acid are solid. These two FFA stock solution need to be heated to 45–50 °C to dissolve.

^b Seventy-six microliter of 100 meq/l FFA mixture is added to 1000 ml of serum-free medium. This makes 7.6 μ eq/l FFA mixture at the final concentration in the medium. Numbers in parentheses indicate final concentration in serum-free medium.