Transplantation and Culture of Spermatogonial Stem Cells

11

Hiroshi Kubota and Ralph L. Brinster

Abstract

Spermatogonial stem cells (SSCs), also called postnatal male germline stem cells, continuously undergo self-renewal and produce daughter spermatogonia that commit to differentiation to spermatozoa, thereby maintaining spermatogenesis and fertility throughout postnatal life. Development of the SSC transplantation technique, in which donor testis cells from a fertile male are microinjected into the seminiferous tubules of an infertile male where SSCs reconstitute donor-derived spermatogenesis and restore fertility, provides a powerful means to unequivocally identify SSCs in a quantitative manner. SSC transplantation is a remarkable breakthrough for SSC research and has established a crucial foundation to study the biology of SSCs. In this chapter, we first describe the transplantation technique that allows characterization of SSCs and their niche, cryopreservation of the germline, and transgenesis. We subsequently describe SSC culture systems that establish a platform for studying SSCs in vitro and enormously enhance their biological value. SSC transplantation, culture, and cryopreservation were originally developed in mice and subsequently in rats, and have since then been extending to other species including domestic animals, endangered or rare species, and primates. Therefore, in the final section, we discuss potential applications of SSCs, for example, the transplantation technique and SSC culture, in human medicine.

Keywords

Spermatogonial stem cell • Germline stem cell • Transplantation • Stem cell culture • Germline modification • Stem cell niche • Fertility • Spermatogonium • Sertoli cell • Spermatogenesis

H. Kubota (✉)
Department of Animal Science, School of Veterinary Medicine, Kitasato University, 35-1, Higashi 23-bancho, Towada, Aomori 034-8628, Japan
e-mail: hiroshi@vmas.kitasato-u.ac.jp

R.L. Brinster
Department of Biomedical Sciences, School of Veterinary Medicine, University of Pennsylvania, Philadelphia, PA 19104, USA

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11.1 Introduction

Spermatogenesis is the process in which male diploid germ cells undergo meiosis and produce a number of haploid germ cells, spermatozoa, which eventually fertilize female haploid germ cells to generate zygotes. The spermatogenesis process, which takes place in seminiferous tubules in testes, is complex, but well organized, and known as one of the most productive systems in mammalian tissues (Clermont 1972; Potten and Morris 1988; Russell et al. 1990). In the testes, millions (rodents) to hundreds of millions (primates and farm animals) of spermatozoa are produced daily from the onset of spermatogenesis at puberty until death (Amann 1986). In all mammalian species, spermatogenesis consists of three phases, mitotic, meiotic, and maturation phases. In the mitotic phase, diploid spermatogonia on the basal lamina of the seminiferous tubules extensively proliferate to increase cell number before the meiotic phase. There are several types of spermatogonia, which are distinguished by heterochromatin patterns in the nuclei and morphometric analysis with stages of seminiferous epithelium cycles (Russell et al. 1990). Spermatogonia are the initial cell population in spermatogenesis, and the spermatogonial stem cell (SSC) is the foundation cell of spermatogonia and subsequent spermatogenesis. The high productivity of spermatogenesis relies on self-renewal of SSCs and the mitotic phase of spermatogenesis (Clermont and Bustos-Obregon 1968; Huckins 1971; Oakberg 1971).

Although the cell type of the seminiferous epithelium and the process of spermatogenesis are conserved among different species, there exist species differences in the duration of spermatogenesis and subtypes of spermatogonia. The duration from spermatogonia to spermatozoa varies for each mammalian species, and the range in most mammals is approximately 30–75 days (Hess and Renato de Franca 2008; Russell et al. 1990). Murine spermatogonia are the most intensively studied and have become a model for other mammalian species, because spermatogonia of other species including rat, hamster, pig, sheep, and cattle, have many similar characteristics (Russell et al. 1990). On the other hand, primates, including humans, have unique subtypes of spermatogonia (Hermann et al. 2010; Meistrich and van Beek 1993).

During cell divisions associated with differentiation, the daughter spermatogonia do not separate completely, but remain connected by intercellular bridges. Such incomplete cytokinesis is an evolutionally conserved characteristic of germ cells (Greenbaum et al. 2011). This unique cell division is important to synchronize differentiation during spermatogenesis. In the mouse, the most immature spermatogonia that form the initial stages of spermatogenesis are called undifferentiated spermatogonia, which can be further subdivided into type A_{single} (A_s), A_{paired} (A_{pr}), and $A_{aligned}$ (A_{al}) based on the number of cohorts connected by intercellular bridges (Huckins 1971; Oakberg 1971). A_s spermatogonia are single cells and represent a very small number of the undifferentiated spermatogonial population (Tegelenbosch and de Rooij 1993). When A_s spermatogonia divide, there are two possible fates of daughter cells. In one case two A_s spermatogonia result, and in the other case two

A_{pr} spermatogonia are connected by an intercellular bridge. The next division of the A_{pr} spermatogonia forms four A_{al} spermatogonia connected by intercellular bridges. Further cell divisions generate 8, 16, and very rarely 32 A_{al} spermatogonia. The last stages of A_{al} spermatogonia differentiate and sequentially form type A1, A2, A3, and A4 spermatogonia, which then differentiate to intermediate (In) spermatogonia, followed by Type B spermatogonia (Russell et al. 1990). The A1 ~ 4, In, and B spermatogonia are called differentiating spermatogonia (de Rooij and Russell 2000). The timing of cell division of differentiating spermatogonia is relatively fixed, whereas that of undifferentiated spermatogonia is not consistent. Type B spermatogonia differentiate into primary spermatocytes that pass through the blood-testis barrier formed by the tight junctions between Sertoli cells and begin meiotic prophase. The tight junctions of Sertoli cells divide the tubular lumen into the basal compartment containing spermatogonia, which is exposed to blood constituents, and the adluminal compartment containing meiotic and maturation germ cell stages, largely separate from blood cells and large molecules (Mruk and Cheng 2015). Meiosis has two sequential cycles of cell division, and one diploid spermatocyte produces four haploid round spermatids that undergo spermiogenesis. During spermiogenesis, round spermatids morphologically and functionally differentiate into spermatozoa.

Classic studies, using histological sections, whole mounts of seminiferous tubules, and H^3-thymidine labeling experiments for cell-kinetics, suggest that some, perhaps many, A_s spermatogonia are SSCs (de Rooij 1973; Huckins 1971; Oakberg 1971). However, these experimental approaches could not assess the biological activity of the A_s spermatogonia; therefore, it was impossible to determine whether they are indeed SSCs. Stem cells are defined by their biological activity, specifically, they are able to self-renew and produce committed cells that eventually differentiate to functional mature cells. Therefore, to unequivocally conclude which cells in the seminiferous tubules are SSCs, an experimental system that could evaluate the biological function of SSCs is required.

11.2 Spermatogonial Stem Cell Transplantation

11.2.1 Development of Spermatogonial Stem Cell Transplantation

A technique for the transplantation of testis cells into seminiferous tubules of recipient males was developed to identify SSCs using mice in 1994 (Brinster and Avarbock 1994; Brinster and Zimmermann 1994). When donor testis cells from a fertile male are microinjected into the lumen of the seminiferous tubules of an infertile recipient male, some donor germ cells reach the basal lamina passing through the blood–testis barrier of Sertoli cells in the opposite direction of normal spermatogenesis. The recipient mice used are prepared by injection of Busulfan, an alkylating agent, to eliminate endogenous germ cells, or are white spotting (*W*) mutant mice that have

congenital deficiencies of germ cell development due to a mutation of the *Kit* gene. After colonization, the donor cells begin proliferating laterally on the basement membrane during the first month, and then gradually differentiate toward the lumen (Nagano et al. 1999). By 1 month following transplantation, donor-derived spermatocytes appear in the adluminal compartment of the seminiferous tubules. By 2 months after transplantation, donor germ cells fill the tubules, and spermatozoa begin to appear. Although 35 days are required for spermatogenesis in mice (Oakberg 1957), nearly twice as long is necessary to produce donor spermatozoa following transplantation. Some of this additional time likely represents a longer mitotic phase of undifferentiated and differentiating spermatogonia from the colonized cells, because extensive lateral expansion of spermatogonia on the basement membrane occurs 1 month after transplantation (Nagano et al. 1999). Other than this, however, many unknown factors would be involved in these different times to obtain mature spermatozoa following transplantation. The spermatozoa differentiated from donor cells are morphologically normal and are able to fertilize eggs, resulting in production of progeny carrying the donor male haplotype, indicating they are functionally normal (Brinster and Avarbock 1994). The reconstituted spermatogenesis continues throughout the remaining life of the recipients. These characteristics collectively prove that some of the transplanted cells have the ability to colonize the basement membrane and have the differentiation capability for continuous production of functional spermatozoa, clearly indicating that the spermatogenesis-reconstituting cells are SSCs (Fig. 11.1A–F).

Three different methods to introduce donor cells into seminiferous tubules of recipient mice were developed (Ogawa et al. 1997). The first method is to inject cells directly into the seminiferous tubule using a micropipette (Fig. 11.1Ca, inset). This method is the most direct way to introduce germ cells into seminiferous tubules. Donor germ cells are forced into the rete testis from the injected tubule and can then enter other seminiferous tubules, because all seminiferous tubules access the rete testis. The cell suspension entering the rete testis can fill many seminiferous tubules. The second method is to insert a micropipette directly into the rete testis and fill the seminiferous tubules (Fig. 11.1Cb, inset). The third method is to insert a micropipette into one of the efferent ducts and thread it into the rete testis (Fig. 11.1Cc, inset). This method is the most accurate in controlling the injection volume because less cell suspension leaks from the insertion site of the micropipette. All three methods have been used for mice and rats and resulted in successful reconstitution of donor spermatogenesis (Brinster and Avarbock 1994; Brinster and Zimmermann 1994; Ogawa et al. 1997, 1999b). For other animals, including pigs, goats, cattle, sheep, monkeys, and humans, the second method, in which donor cells are directly introduced into the rete testis of recipients, has been used (Hermann et al. 2012; Herrid et al. 2006; Honaramooz et al. 2002, 2003a; Izadyar et al. 2003; Kim et al. 2008; Mikkola et al. 2006; Radford 2003; Rodriguez-Sosa et al. 2009; Schlatt et al. 1999), and successful reconstitution of donor-derived spermatogenesis is also reported.

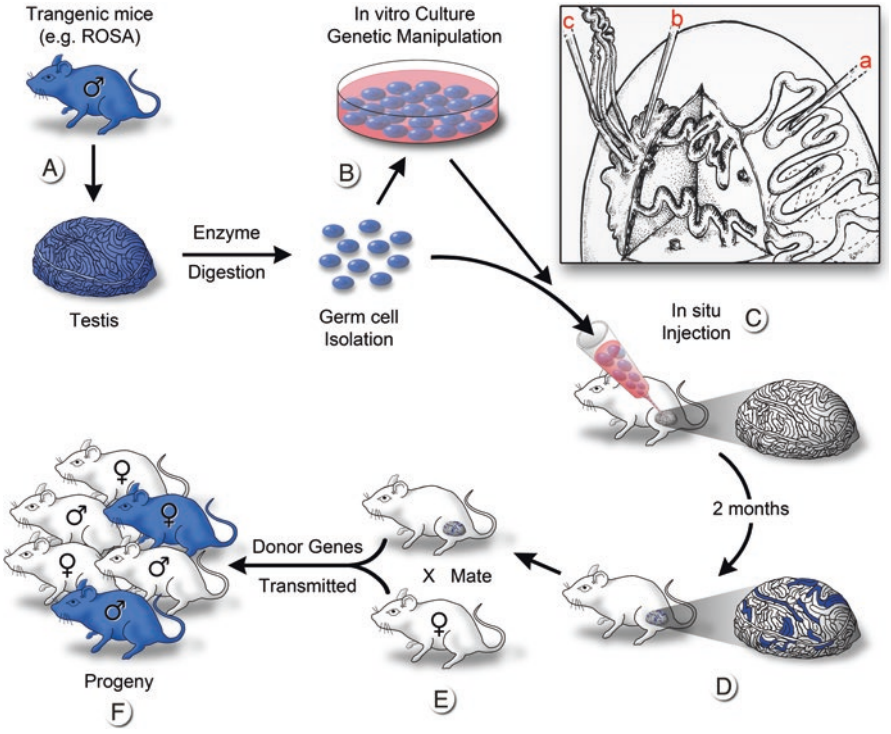

Fig. 11.1 Procedure for testis-cell transplantation as developed in the mouse. (**A**) testis is removed from a fertile male that expresses a reporter transgene, *Escherichia coli lacZ*, and a single-cell suspension is prepared from the testis of the transgenic mouse. (**B**) The testis cells can be cultured with appropriate conditions. Genetic manipulation can be employed during culture. (**C**) Cells are microinjected into the seminiferous tubules of an infertile recipient male. There are three methods for microinjection indicated in the inset: the micropipette can be inserted (*a*) directly into the seminiferous tubules, (*b*) into the rete testis, or (*c*) into an efferent duct. (**D**) Spermatogonial stem cells colonize the basement membrane of the tubules and generate donor-cell-derived spermatogenesis, which can be stained blue using a substrate for the reporter gene product (β-galactosidase). Each blue stretch of cells in the seminiferous tubules of the recipient testis represents a spermatogenic colony derived from a single donor stem cell. (**E**) Mating the recipient male to a wild-type female results in donor-cell-derived spermatozoa fertilizing wild-type oocytes. (**F**) Progeny with the donor haplotype are produced. Modified from Brinster (2002) and Ogawa et al. (1997)

11.2.2 Functional Assay for Spermatogonial Stem Cells

As mentioned above, stem cells are defined by biological function, in which they have the ability to both self-renew and produce large numbers of differentiated functional cells. The transplantation procedure can be used as a quantitative assay for retrospective identification of SSCs. When testis cells from a transgenic mouse that expresses a reporter gene, such as β-galactosidase or green fluorescent protein (GFP), are transplanted into infertile recipient testes, donor-derived spermatogenesis can be unequivocally identified by visualizing the reporter proteins (Brinster

2002; Kubota and Brinster 2008). For example, in recipient testes transplanted with β-galactosidase-expressing germ cells, donor-derived spermatogenesis can be identified as blue colonies after staining with a substrate, X-gal, 2 months after transplantation (Fig. 11.1). These colonies are each derived from a single donor cell (Dobrinski et al. 1999b; Kanatsu-Shinohara et al. 2006c; Zhang et al. 2003), and the colony number does not change from 1 to 4 months, whereas the length of colonies increases (Nagano et al. 1999). Thus, the number of blue colonies represents the number of spermatogenesis-reconstituting cells (SSCs) in donor cell suspensions, which are able to self-renew and continuously produce daughter spermatogonia that eventually differentiate into spermatozoa. Because the colonization efficiency of SSCs into adult recipient testes is 5–12% (Nagano 2003; Ogawa et al. 2003), the actual SSC number in a donor cell suspension is 8- to 20-fold higher than the colony number. The exact reasons of the low colonization efficiency are not clear, but passing through the blood–testis barrier is likely one of the impeding factors.

Although the transplantation technique provides a powerful means to identify SSCs in any donor cell suspension, the technique can be used to identify precursor cells that have the ability to differentiate into SSCs. SSCs are a subpopulation of type A spermatogonia that are derived from gonocytes, or prospermatogonia, a few days after birth in mice (Culty 2013; McCarrey 2013). Gonocytes are mitotically inactive and located in the center of the seminiferous tubules of fetal and newborn testes. When gonocytes from fetal and newborn testes are transplanted into seminiferous tubules of mature testes, donor-derived spermatogenesis occurs, indicating that the donor gonocytes differentiated to SSCs in recipient mature testes (Brinster and Avarbock 1994; Kubota et al. 2004a; Shinohara et al. 2001). Furthermore, when primordial germ cells (PGCs), the precursor of gonocytes, and epiblast cells, the precursor of PGCs, were transplanted into immature testes, but not mature testes, before forming the blood–testis barrier of Sertoli cells, donor-derived spermatogenesis was reconstituted (Chuma et al. 2005; Ohta et al. 2004). Normal fertile progeny were produced by micro-insemination using donor-derived spermatids or spermatozoa, confirming that PGCs and epiblast cells can normally differentiate into SSCs in the postnatal testis (Chuma et al. 2005). This indicates that the transplantation technique can be used for not only a functional assay to identify SSCs, but also for assessing developmental potential of other cell types to produce SSCs.

During the past decade, derivation of spermatozoa from pluripotent stem cells has been attempted in vitro, and several groups reported the successful induction of PGC-like cells from embryonic stem (ES) cells, although no functionally normal haploid germ cells were obtained from the PGC-like cells (Geijsen et al. 2004; Nayernia et al. 2006; Toyooka et al. 2003). However, a recent study reported generation of functionally normal spermatozoa from ES cells and induced pluripotent stem (iPS) cells in the mouse (Hayashi et al. 2011). In this study, initially epiblast-like cells were induced from pluripotent cells, and then further induction to PGC-like cells was carried out in culture. Subsequently, the PGC-like cells were transplanted into the seminiferous tubules of immature infertile mouse testes. Donor-derived spermatogenesis occurred in the recipient testes, and micro-insemination using the spermatozoa from these testes successfully generated fertile offspring without any

defect or abnormality (Hayashi et al. 2011). At present, the transplantation procedure is indispensable for the generation of functional spermatozoa from pluripotent stem cells such as ES cells and iPS cells.

11.2.3 Characterization of Spermatogonial Stem Cells

The availability of a functional assay is essential to identify SSCs based on their biological functions. Because the number of SSCs in the testis is extremely rare, perhaps only 0.03% of all germ cells in mouse testis (Tegelenbosch and de Rooij 1993), determination of the phenotypic characteristics of SSCs is critical to investigate SSCs at the cellular and molecular level, as well as in the context of tissue sections or whole mount analyses. There have been several methods developed that enrich SSCs from postnatal testes, including differential plating, Percoll centrifugation, isolation from cryptorchid testes, and immunoselection (Kubota et al. 2004a; Shinohara et al. 1999, 2000a). Although there are several methods of immunoselection to isolate particular subpopulations from a mixed cell population, fluorescence activated cell sorting (FACS) is the most widely used for identification of stem cell populations from a variety of tissues (Grompe 2012). Originally, an approach using FACS along with a functional transplantation assay was developed for identifying hematopoietic stem cells (HSCs). Although HSCs represent only 0.01% of the cells in bone marrow, the surface characteristics of murine HSCs have been successfully determined by this approach (Osawa et al. 1996; Spangrude et al. 1988). Utilizing a similar experimental approach, the cell surface phenotype of SSCs in rodents was determined. Briefly, testicular cells prepared from testes are stained with fluorochrome-conjugated antibodies against cell surface molecules, followed by flow cytometric analysis to determine cell surface expression of the molecules. When the testicular cell population is divided based on cell surface expression of antigens, each fraction of cells can be isolated by cell sorting and subjected to the transplantation assay. Two months after transplantation of each cell fraction, recipient testes are analyzed to identify donor-derived spermatogenesis. The number of spermatogenic colonies generated by different cell populations indicates the number of SSCs in that population of cells. By repeating this process, surface molecules expressed on SSCs have been determined. Several studies identified the SSC surface phenotype as represented by $ITGA6^{+}$ $ITGB1^{+}$ $THY1^{+}$ $CD9^{+}$ $GFRA1^{+}$ $EPCAM^{+}$ $CD24^{+}$ $MCAM^{+}$ KIT^{-} Major histocompatibility complex class I $(MHC\text{-}I)^{-}$ in mice (Kanatsu-Shinohara et al. 2004b, 2012; Kubota et al. 2003; Shinohara et al. 2000b). At present, no cell surface molecules that are exclusively expressed on SSCs have been identified. However, a combination of surface markers can identify the cell surface phenotype of undifferentiated spermatogonia enriched for SSCs. Although the concentration of SSCs in adult testes is thought to be about 1 in 3000, that of $THY1^{+}$ $ITGA6^{+}$ KIT^{-} $MHC\text{-}I^{-}$ cells in cryptorchid testes or $THY1^{+}$ $ITGA6^{+}$ $ITGAV^{-/lo}$ in normal testes is approximately 1 in 15 ~ 30 (Kubota et al. 2003, 2004a), indicating that FACS in conjunction with the transplantation assay could determine cell surface molecules and facilitate a major enrichment of SSCs. Determination of the antigenic profile of

SSC-enriched cell populations allowed subsequent development of magnetic activated cell sorting (MACS) for the population, which is a simpler and quicker method than FACS (Kubota et al. 2004a). Furthermore, FACS can be used for cell fractionation based on cellular activity, such as efflux pump activity, mitochondrial activity, cell cycle, and intracellular enzymatic activity, which have been used for HSC identification (Ishii et al. 2014; Kanatsu-Shinohara et al. 2013; Kubota et al. 2003; Lo et al. 2005). Using those parameters with cell surface analysis, murine SSCs were further characterized; however, no improvement of SSC enrichment was achieved.

For characterization of SSC-specific genes such as transcription factors, transgenic mice in which a reporter gene, such as GFP, is inserted downstream of the promoter of a putative SSC-specific gene, are useful. Several genes expressed in undifferentiated spermatogonia, including *Pou5f1* (*Oct-3/4*), *Ngn3*, *Nanos2*, and *Id4*, have been investigated (Chan et al. 2014; Ohbo et al. 2003; Ohmura et al. 2004; Sada et al. 2009; Yoshida et al. 2004). Following identification of reporter gene-expressing cells in the testes of the transgenic mice, the cells are isolated by FACS and transplanted into recipient testes. Again, no SSC-specific molecules have been identified, but this approach could allow better SSC enrichment than existing techniques relaying on cell surface markers or physiological characteristics in the future. Furthermore, this approach is important to delineate the ordered expression of gene expression during spermatogonial differentiation from SSCs.

Although A_s spermatogonia have been thought to be SSCs for a long time, recent studies challenge this hypothesis. A live image study of GFP-labeled *Ngn3* spermatogonia indicated a separation or unjoining of A_{al} spermatogonia to A_{pr} or A_s spermatogonia, which then appeared to reinitiate formation of A_{al} spermatogonia from either the A_{pr} or A_s (Hara et al. 2014). Furthermore, a study using FACS and the transplantation assay demonstrated that KIT-expressing differentiating spermatogonia may also possess SSC potential (Barroca et al. 2009). These studies suggest that undifferentiated or differentiating spermatogonia other than A_s spermatogonia could function as stem cells. There is no molecular marker for A_s spermatogonia, and A_s, A_{pr}, and A_{al} spermatogonia can be identified only by whole mount analysis; therefore, identification of each type of undifferentiated spermatogonia by flow cytometry is not feasible. At present, it is not technically easy to determine which undifferentiated spermatogonia possess SSC activity. However, even in a single cohort of A_{al} spermatogonia, it appeared that gene expression patterns in individual spermatogonia might be different (Zheng et al. 2009). If particular spermatogonia within an A_{al} spermatogonia syncytium can be isolated, it would be possible to determine which undifferentiated spermatogonia possess the stem cell activity by the transplantation assay. Assembling all data from whole mount analysis, flow cytometry, and live imaging in conjunction with transplantation assays, we might be able to elucidate cellular and molecular determinants of SSCs, from which our understanding of SSC biology would significantly improve.

11.2.4 Characteristics of the Spermatogonial Stem Cell Niche

The surrounding microenvironment of stem cells is called the stem cell niche, which has been shown to control self-renewal and differentiation of stem cells (Li and Xie 2005; Spradling et al. 2001). The microinjection technique into seminiferous tubules is also useful to investigate the biological function of the SSC niche. When SSCs from adults were transplanted into pups and adult recipient testes, the number of spermatogenic colonies in pup testes was approximately ten times greater than in adult testes (Shinohara et al. 2001). In addition, colonies generated were four times longer in pup testes compared with those in adults (Shinohara et al. 2001). These findings indicated that the SSC niche in pups is more accessible and supportive of transplanted SSCs than that in adults. These transplantation experiments clearly demonstrate that the transplantation assay can be used for evaluating the biological activity of the SSC niche in recipient testes. Components of the stem cell niche comprise neighboring cells, soluble factors, and extracellular matrices. The neighboring cells include Sertoli cells, myoid cells, Leydig cells, potentially other interstitial cells, and spermatogonia. In particular, Sertoli cells play a critical role in establishing the SSC niche, because they produce glial cell line-derived neurotrophic factor (GDNF) and fibroblast growth factor 2 (FGF2), the primary and secondary critical soluble factor for self-renewal and expansion of SSCs (Kubota et al. 2004b; Meng et al. 2000; Mullaney and Skinner 1992). Furthermore, recent studies indicated that the number of Sertoli cells influence the number of niches accessible for colonization of transplanted SSCs in mice (Oatley et al. 2011). In this study, the number of donor-derived spermatogenic colonies significantly increased in the recipient testes with ~50% increase in Sertoli cell numbers following SSC transplantation compared with normal recipient mice. In addition, colony stimulating factor 1 (CSF1), which is produced by Leydig and myoid cells, has been identified as the first specific niche factor that increases the replication and expansion of SSCs (Oatley et al. 2009).

The transplantation of cells into the seminiferous tubules can also be applied to Sertoli cells. In the mouse, expression of the KIT receptor tyrosine kinase by spermatogonia is critical for proliferation and differentiation beyond the undifferentiated spermatogonia stage (Yoshinaga et al. 1991), and the KIT ligand, also known as stem cell factor (SCF), is expressed on and secreted by Sertoli cells in the testes (Ogawa et al. 2000). Steel (*Sl*) mutant mice are infertile due to a congenital defect of the KIT ligand, but transplantation of normal testis cells into seminiferous tubules of *Sl* mice initiates spermatogenesis by transplanted Sertoli cells (Shinohara et al. 2003). Furthermore, infertile *Sl* recipient mice transplanted with Sertoli cells from *W* mice restored endogenous spermatogenesis and fertility, demonstrating the functionality of transplanted Sertoli cells (Kanatsu-Shinohara et al. 2005a). Although the efficiency of Sertoli cell colonization is low (Shinohara et al. 2003; Shinomura et al. 2014), transplantation of Sertoli cells provides an alternative approach to restore fertility in infertile recipients. Moreover, transplantation of xenogeneic Sertoli cells into immunodeficient mice would be able to reconstitute the SSC niche

for foreign species, which provides a foundation for non-rodent SSC studies including human (see below).

11.2.5 Application to Non-mouse Systems

The spermatogonial transplantation technique was initially developed in mice. Following the original mouse system, the technique was extended to other species including rats, monkeys, goats, cattle, pigs, sheep, cats, and dogs (Hermann et al. 2012; Herrid et al. 2006; Honaramooz et al. 2002, 2003a; Izadyar et al. 2003; Jiang and Short 1995; Kim et al. 2006, 2008; Mikkola et al. 2006; Ogawa et al. 1999b; Radford 2003; Rodriguez-Sosa et al. 2009; Schlatt et al. 1999). In rat, the procedure is essentially the same as for mouse. In brief, donor germ cells were collected from transgenic rats that express a β-galactosidase reporter gene and transplanted into infertile recipient rat testes through the efferent duct. Recipient males were prepared by injection of Busulfan to deplete endogenous germ cells (Ogawa et al. 1999b; Ryu et al. 2003). As shown in mice, following transplantation donor SSCs initiate spermatogenesis, and donor-derived spermatogenesis continued during the remaining life of the recipient rats. Donor-derived spermatogenesis was confirmed by X-gal staining. Although the rat system is quite similar to the mouse, there are no rat strains with congenital mutations leading to germ cell deficiency that are suitable for recipient males. Nevertheless, the transplantation system using recipients prepared by Busulfan injection can be used for biological functional assay to identify rat SSCs. Applying the same approach as with mouse SSCs, the surface phenotype of the undifferentiated spermatogonia population enriched for rat SSCs has been determined (Ryu et al. 2004). Furthermore, SSC niche development in postnatal rats was also investigated (Ryu et al. 2003).

In non-rodents, such as farm animals and companion animals, recipient males are prepared by Busulfan injection or local irradiation of testes (Honaramooz et al. 2005; Izadyar et al. 2003; Oatley et al. 2005). Both methods have been shown to be effective for ablation of endogenous germ cells. Although appropriate injection timing and doses for Busulfan must be determined in each species, complete removal of endogenous germ cells is not necessary. Some studies have suggested that residual endogenous spermatogenesis may be helpful to maintain a healthy testicular niche microenvironment to enhance donor spermatogenesis (Ryu et al. 2003). Furthermore, immature pigs and goats without pretreatment can be used as recipients (Honaramooz et al. 2002, 2003a, b). However, recipients with persistence endogenous spermatogenesis can produce both donor-derived and recipient-derived progeny; therefore, genotyping is necessary to determine whether donor or endogenous spermatogenesis produced the progeny. Although there are three methods to inject donor germ cells as described above, donor SSCs in non-rodent system were introduced into the rete testis by ultrasound guidance or surgical dissection (Honaramooz et al. 2002, 2003a; Schlatt et al. 1999). In goat and sheep, progeny with donor SSC-haplotype were successfully generated (Herrid et al. 2009; Honaramooz et al. 2003b, 2008).

11.2.6 Xenotransplantation

Following transplantation of rat SSCs into the seminiferous tubules of infertile immunocompromised mice, rat spermatogenesis was established in recipient testes (Clouthier et al. 1996). The rat spermatozoa in mouse testes are functionally normal, since in a subsequent study normal progeny were generated from the rat spermatozoa developed in the recipient mouse testes (Shinohara et al. 2006). This finding suggests that xenogeneic spermatogenesis could be reconstituted by transplantation of SSCs from variety of species into immunocompromised mice. A striking finding regarding control of timing of germ cell differentiation during spermatogenesis emerged from this rat to mice spermatogonial transplantation experiments (Clouthier et al. 1996). Although the duration time from type A spermatogonia to mature spermatozoa is 35 days in mice, that of rats is 52 days (Russell et al. 1990). It was found that rat spermatogenesis in mouse seminiferous tubules progressed at the rate determined by the transplanted germ cells. Thus, rat spermatogenesis in mouse seminiferous tubules progresses at the slower speed of rat spermatogenesis while supported by mouse Sertoli cells, indicating that the genotype of the germ cell controls this specific timing of spermatogenesis characteristic of different species (França et al. 1998; Russell and Brinster 1996).

In subsequent studies germ cells from various mammalian species, including rabbits, dogs, cats, pigs, cattle, horses, baboon, macaques, and humans, were transplanted into infertile immunocompromised male mice (Dobrinski et al. 1999a, 2000; Hermann et al. 2007; Nagano et al. 2001b, 2002; Oatley et al. 2004). However, complete spermatogenesis from transplanted SSCs was developed only in the combinations between rodents, such as rat to mouse and hamster to mouse (Clouthier et al. 1996; Ogawa et al. 1999a). Although donor-derived spermatogenesis did not occur following transplantation of germ cells from non-rodent mammalian species into the mouse testes, primitive spermatogonia from all mammals examined colonized and proliferated for 1–12 months in the seminiferous tubules of immunocompromised mice (Dobrinski et al. 1999a, 2000; Hermann et al. 2007; Kim et al. 2006; Nagano et al. 2001b, 2002; Oatley et al. 2004). At present, the colony-forming spermatogonia in immunocompromised mice are considered to represent SSCs and perhaps early undifferentiated spermatogonia. The remarkable results of xenogeneic transplantations indicate that factors produced in the mouse SSC niche are able to support proliferation of SSCs from many species. Because the process of spermatogenesis is well conserved among mammalian species, conservation of self-renewing factors for SSCs may not be surprising. On the other hand, species variation in spermatogonial differentiation factors seems to exist among mammalian species. Sertoli cell transplantation from the same species as the donor germ cells may overcome the block in differentiation of xenogeneic donor germ cells into mouse testes. The SSC characteristics in non-rodent mammals including human are largely unknown; however, functional assays to identify these SSCs are under development. Transplantation of Sertoli cells in addition to SSCs from xenogeneic species would reestablish the necessary niche and differentiating factors for foreign species spermatogenesis in immunodeficient mice. If immunocompromised mice with xenogeneic Sertoli cells can

support spermatogenesis from xenogeneic SSCs, such a transplantation system can be used as a functional assay for non-rodent SSCs. It is extremely important to develop functional assays for non-rodent SSCs in which both self-renewal and differentiation can be evaluated.

11.3 Spermatogonial Stem Cell Culture

11.3.1 Mouse

The first demonstration of a long-term culture of murine SSCs was reported in 1998 (Nagano et al. 1998). In this study, testicular cells were cultured on STO (SIM mouse embryo-derived thioguanine and ouabain resistant) feeders in a serum-supplemented medium for approximately 4 months, followed by transplantation into testes of infertile mice in order to examine whether SSCs existed in the culture (Nagano et al. 1998). Donor cell-derived spermatogenesis was generated in the recipient testes, indicating that SSCs existed in the long-term culture. This result clearly demonstrated that SSCs could be maintained in culture for several months, although the number of SSCs was reduced (Nagano et al. 1998). Several cytokines such as FGF2, leukemia inhibitory factor (LIF) and SCF were thought to be candidate mitogens for SSCs. FGF2 and SCF are potent mitogens for PGCs (Matsui et al. 1992; Resnick et al. 1992), while LIF is an essential self-renewing factor for ES cells (Smith et al. 1988; Williams et al. 1988). Using a short-term culture experiment, none of these three cytokines improved survival of SSCs (Nagano et al. 2003). A critical finding came from a study using GDNF-overexpressing and -deficient mice (Meng et al. 2000). Although GDNF was originally discovered as a survival factor for midbrain dopaminergic neurons (Lin et al. 1993), the factor is secreted from Sertoli cells in the testis (Viglietto et al. 2000). In the GDNF-overexpressing mice, dysregulated proliferation of spermatogonia was observed, whereas hemizygous GDNF-targeted mice gradually lost their spermatogonial population, indicating that GDNF regulates spermatogonial proliferation (Meng et al. 2000). Furthermore, gene transfer of a GDNF-expression plasmid into Sertoli cells resulted in an increase of SSC number (Yomogida et al. 2003). In addition to these in vivo studies, an in vitro experiment also indicated a beneficial effect of GDNF on cultured SSCs using serum-supplemented medium in a short-term culture experiment; however, an increase in SSC number was not observed under these culture conditions (Nagano et al. 2003).

In 2003, it was reported that gonocytes from newborn mice of genetic background ICR or BDF1 (C57BL/6 × DBA/2), could form grape-like colonies and proliferate on mouse embryonic fibroblasts (MEF) feeders in a serum-supplemented proprietary StemPro-34® (Life technology)-based medium, which contained the original StemPro-34® supplement plus 16 individual compounds and fetal bovine serum (FBS) with a cytokine mixture of GDNF, FGF2, epidermal growth factor (EGF), and LIF (Kanatsu-Shinohara et al. 2003). Proliferating cells on MEF express several spermatogonial markers such as ITGA6, ITGB1, and EPCAM, and

generated spermatogenic colonies following transplantation into infertile mouse testes, indicating they had SSC potential. In this culture condition, cell proliferation was restricted to the genetic background indicated above, and gonocytes from C57BL/6 or 129/Sv never formed colonies. The grape-like proliferating cells were named GS (germline stem) cells, because they possessed SSC activity, which was equivalent to that of freshly-isolated gonocytes (~12 colonies/10^5 cells transplanted (Kanatsu-Shinohara et al. 2003)). Using the transplantation assay, gonocytes purified by FACS generated ~17 colonies per 10^5 cells transplanted, while purified undifferentiated spermatogonia enriched for SSCs generate 160 ~ 350 colonies per 10^5 cells transplanted (Kubota et al. 2003, 2004a). Under the culture condition, GS cells that arose from gonocytes in culture spontaneously transformed to pluripotent stem cells, which were designated multipotent GS (mGS) cells, with characteristics similar to ES cells (Kanatsu-Shinohara et al. 2004a). Because spontaneous transformation of SSCs in normal mouse testes is extremely rare, this suggests that GS cells might not be identical to normal SSCs. In addition, GS cells could proliferate in an anchorage independent condition (Kanatsu-Shinohara et al. 2006b), which is one criterion of transformed cells. These unique characteristics of GS cells might be acquired during the process of in vitro cultivation. GS-like cell lines, which possess SSC activity with spontaneous dedifferentiation to pluripotent cells, have also been reported to arise from adult testes using similar culture conditions (Guan et al. 2006; Ko et al. 2009; Seandel et al. 2007). Therefore, although SSCs are unipotent stem cells in the testis, the transformation capability of GS cells into pluripotent stem cells makes them a valuable tool to investigate and understand pluripotent characteristics of the germline. Furthermore, a recent study reported that FGF2-dependent GS cells were established in the absence of GDNF (Takashima et al. 2015). Spermatogonial proliferation does not occur in the testes of GDNF-knockout mice (Naughton et al. 2006); therefore, it is not clear whether a population equivalent to FGF2-dependent GS cells exists in normal testes. Because FGF2 is a potent mitogen for PGCs, and the FGF2-dependent GS cells express the KIT receptor, it would be valuable to investigate whether they are capable of transforming into pluripotent stem cells as seen in PGCs or GS cells cultured with GDNF.

To establish long-term culture conditions for SSCs, identification of essential extrinsic factors for self-renewal of SSCs is crucial (Kubota and Brinster 2008). For this purpose, serum-free culture conditions are useful. In addition to considerable batch variation, serum contains complex undefined substances including biologically active compounds, such as hormones and growth factors. In addition, serum contains growth factors that stimulate proliferation of fibroblasts, including FGFs or platelet-derived growth factors (PDGFs); therefore, serum-containing medium supports proliferation of fibroblasts in a selective manner (Sato et al. 1960). Such fibroblast-dominant culture conditions are detrimental for many types of cells, including germ cells. In the 1970s, Gordon Sato's group developed serum-free hormonally defined media, which supported many different types of cells, by adding appropriate hormones or growth factors (Barnes and Sato 1980). Common requirements for all cells in serum-free conditions were insulin, albumin, transferrin, selenium, and fatty acids (Barnes and Sato 1980). Further studies demonstrated that

extracellular matrices that provide anchorage for adherent cells are important components of serum (Enat et al. 1984). Thus, in serum-free conditions, anchorage materials, which can be extracellular matrices or inactive feeder cells, should be added to the culture. For stem cell cultures, feeder cells are preferable, because the initial stem cell number placed in culture is generally low, and feeder cells are beneficial in this situation (Kubota and Brinster 2008). Based on these principles, a culture system for SSCs was developed consisting of a serum-free defined medium containing minimum components and STO feeder cells, which was originally developed for hepatic stem/progenitor cells (Kubota and Reid 2000). The serum-free medium consisted of alpha MEM basal medium containing insulin, transferrin, selenium, putrescine, 2-mercaptoethanol, free fatty acids, HEPES, and antibiotics. This defined culture system was used to screen various growth factors for their effect on SSC proliferation (Kubota et al. 2004a), and it was found that SSCs from DBA background mice continuously proliferated in the presence of GDNF (Kubota et al. 2004b). Although the STO cell line produces LIF and SCF constitutively (Schmitt et al. 1991), these factors did not show any beneficial effect on SSC proliferation (Kubota et al. 2004a). Additionally, EGF did not support proliferation of SSCs (Kubota et al. 2004a). Although GDNF alone supports proliferation of SSCs isolated from DBA/2 background mice, GDNF alone was not sufficient to support continuous proliferation of SSCs from other mouse strains, such as C57BL/6. In the GDNF-containing medium, they initially formed clumps, but ceased proliferation and disappeared within a few weeks (Kubota et al. 2004b). Interestingly, a relatively small amount of FGF2 (~1 ng/mL) was very effective in enhancing proliferation of SSCs from non-DBA/2 mouse strains. Furthermore, addition of soluble GFRA1 enhanced the effect of GDNF on SSCs (Kubota et al. 2004b). The GDNF receptor complex consists of RET receptor tyrosine kinase and GFRA1 that is a glycosil phosphatidylinositol-anchored ligand binding subunit. Soluble GFRA1 has been shown to potentiate RET activation by direct binding with GDNF-GFRA1 complexes (Paratcha et al. 2001). Combining both factors with GDNF, SSCs from all mouse strains examined including 129/Sv, which is one of nonpermissive strains to generate GS cells (Inoue and Ogura 2015), could self-renew and proliferate indefinitely in culture (Kubota et al. 2004b). Under these culture condition, undifferentiated spermatogonia that were freshly isolated and highly enriched for SSCs form morula-like, tightly packed cellular clumps and continuously proliferate (Fig. 11.2A). The proliferating cells were $THY1^{+}$ $ITGA6^{+}$ $ITGAV^{lo}$ and their SSC activity as determined by the functional assay was 300 ~ 400 colonies per 10^{5} cell transplanted, which is essentially equivalent to freshly isolated undifferentiated spermatogonia from postnatal testes (Kubota et al. 2004b).

A serum-free culture system containing key extrinsic factors has allowed development of a simple culture condition to support self-renewal and proliferation of SSCs. Once a simple culture condition for ex vivo expansion of SSCs was determined, several minor factors appeared to be important for successful establishment of long-term cultures. One important factor is the catalog number (type) and lot number of bovine serum albumin (BSA). Although the basis is not clear, BSA purified by a heat-shock process rather than the Cohn cold ethanol precipitation process

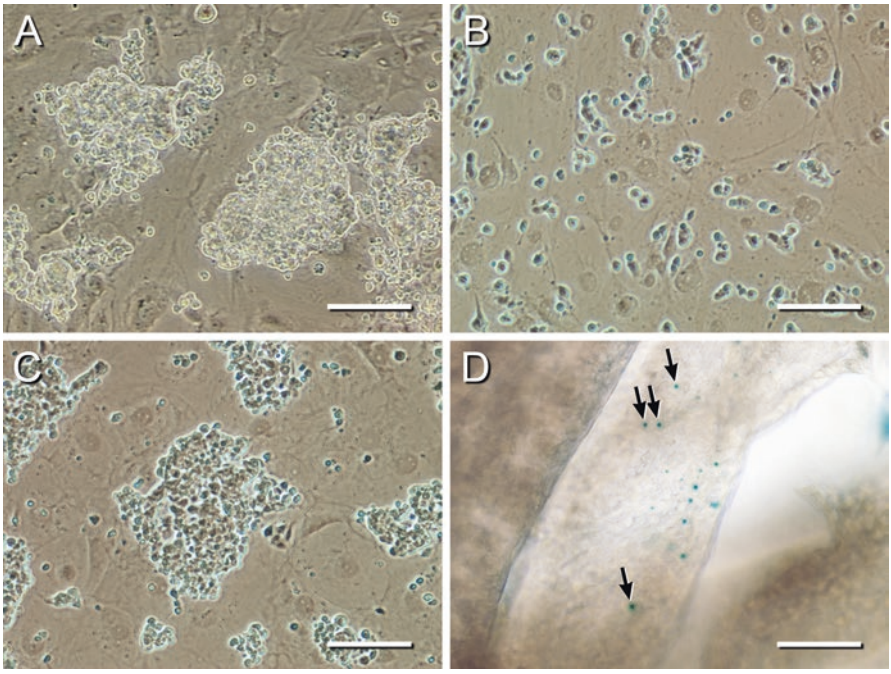

Fig. 11.2 Comparative analysis of mouse SSCs and rabbit SSCs. (**A**–**C**) A phase-culture images of proliferating mouse SSCs clumps on STO feeder cells (**A**), rabbit SSCs cultured on STO feeders (**B**), and rabbit SSCs on C166 mouse endothelial cell feeders (**C**). Although no cellular clumps of rabbit SSCs were formed on STO feeder cells, they formed clumps on C166 feeder layers and continuously proliferated. (**D**) Seminiferous tubules of recipient testes transplanted with the β-galactosidase-expressing rabbit SSCs. Recipient testes were analyzed with X-gal staining at 23 weeks after transplantation. Donor rabbit cells were stained blue (*arrows*). Blue cells and clusters were found throughout the recipient seminiferous tubules. Scale bars = 100 μm

appeared more suitable for SSC culture. The catalog number and lot number of BSA that allows SSC survival and proliferation in culture must be determined empirically. In addition, a culture gas atmosphere of 10% O_2 was significantly better than 21% O_2 (Kubota et al. 2009). In particular, long-term cultures of SSCs from W^v/W^v mice could be established only in a 10% O_2 atmosphere (Kubota et al. 2009). Because the SSC number is extremely low in W^v/W^v testes, it was impossible to enrich SSCs from W^v/W^v mouse testes by antibody-based enrichment such as FACS using markers for SSC. However, even without an enrichment step, a 10% O_2 condition could support proliferation of W^v/W^v SSCs and establish a long-term culture. Furthermore, a 10% O_2 atmosphere made it possible to establish a long-term culture from an individual colony generated from a single SSC colonization event in a seminiferous tubule of a recipient testis (Schmidt et al. 2011). In addition, an increased proliferation rate of SSCs from wild-type C57Bl/6 mice was found in a 10% O_2 atmosphere (Kubota et al. 2009). Significantly, the number of SSCs in a germ cell clump and the cell surface phenotype was not different between 10% O_2 and 21%

O_2 cultures, indicating that O_2 concentration improved the proliferation rate of cultured SSCs.

11.3.2 Rat

Using mouse SSC culture conditions as a foundation, two rat SSC culture systems have been developed (Hamra et al. 2005; Ryu et al. 2005). One of these conditions is essentially the same as the mouse SSC culture system consisting of serum-free medium, STO feeders, and growth factors including, GDNF and FGF2 (Ryu et al. 2005). GFRA1 also has a supportive effect on the GDNF dependent proliferation (Ryu et al. 2005). Under these culture conditions, rat spermatogonia indefinitely proliferated as clumps, and cultured cells generated spermatogenic colonies in infertile recipients following transplantation. When they were transplanted into Busulfan-treated recipient rat testes, offspring were successfully produced, indicating that cultured cells were indeed rat SSCs (Ryu et al. 2005).

However, some modifications of the mouse SSC culture method were beneficial for long-term culture of rat SSCs (Kubota and Brinster 2008). First, although the components (insulin, transferrin, selenium, putrescine, 2-mercaptoethanol, and free fatty acids) of the serum-free medium for rat SSCs are the same as those of the medium for mouse SSCs, the concentration of several components was increased. Second, the medium osmolality was reduced by addition of water to the serum-free medium (Brinster 1965). Third, a reduced concentration of trypsin with a short exposure time during subculture facilitated recovery of cells. Lastly, a low atmospheric concentration of oxygen (5 ~ 10%) was beneficial. In addition, overgrowth of testicular somatic cells was even more of a problem for rat SSC proliferation then previously observed for mouse SSC (Kubota et al. 2004a). Therefore, it is critical that somatic cells be removed from the cultures. Thus, when fibroblasts or other somatic cells were present in the culture, rat germ cell clumps were collected by gentle pipetting of medium across the surface of the feeder layers because regular trypsin-treatment resulted in recovering all somatic cells in the culture. Before placing collected germ cells on fresh STO feeders, they could be digested with a low concentration of trypsin (e.g. 0.01%). All together, these minor modifications are important for establishment of long-term cultures of rat SSCs (Ryu et al. 2005). An important finding arising from rat SSC cultures is that self-renewal of rat SSCs is also dependent on GDNF, suggesting that it may be essential in other species. Furthermore, these studies using defined culture conditions for mouse and rat SSCs have identified the main essential exogenous factors for their unlimited proliferation and made possible generation of large numbers of SSCs in vitro, which is a prerequisite for biochemical and molecular investigation of SSCs (Oatley et al. 2006; Schmidt et al. 2009).

In a second long-term culture system for rat SSCs, a serum-free medium containing proprietary B27 supplement® minus vitamin A (Life technology), with GDNF, FGF2, and MEF feeder cells was used (Hamra et al. 2005). Initially in this system, when serum-supplemented medium was used, the number of rat germ cells

decreased after each subculture (Hamra et al. 2005). Serum was removed from the medium because the decrease in germ cell numbers in the culture seemed to be caused by a combination of serum and testicular somatic cells, which had been demonstrated in mouse germ cell cultures (Kubota et al. 2004a). The basic culture medium was StemPro-34-based GS cell medium, but serum was replaced to the proprietary B27 supplement, a serum-free supplement developed for supporting neural cell cultures (Brewer et al. 1993). Although the original B27 supplement contains vitamin A, the B27 minus vitamin A was used for rat SSC culture because vitamin A is a potent differentiation factor for spermatogonia. When the B27 supplement minus vitamin A replaced serum in the StemPro-34 based GS cell medium, rat SSCs continuously proliferated (Hamra et al. 2005). A subsequent study showed that a medium without StemPro-34 supplement seemed better than medium containing StemPro-34 supplement (Wu et al. 2009b). Interestingly, like rat SSCs, removal of FBS and B27 supplementation in the GS medium improved cell proliferation speed of mouse GS cells, although FBS appeared to be necessary for initial derivation of mouse GS cell lines on primary MEF feeder cells (Kanatsu-Shinohara et al. 2005b). Rat pluripotent stem cell lines have not been developed from cultured rat SSCs in either culture system.

11.3.3 Rabbit

The basic requirements for self-renewal of murine and rat SSCs appear to be identical. Although rodent SSCs have been investigated intensively using the in vitro culture techniques in addition to the functional transplantation assay (Brinster 2002; Kubota and Brinster 2006; Oatley and Brinster 2012), knowledge about non-rodent SSCs is limited. Rabbits diverged phylogenetically from rodents about 60 million years ago (McKenna and Bell 1997), while the time of divergence between mice and rats is considered to be approximately 11 million years ago (Catzeflis et al. 1993). Thus, cultivation of rabbit germ cells could provide an excellent foundation for development of SSC cultures from other species that diverged from the mouse 60 million or more years ago, and this possibility served as one objective for studying rabbit SSCs. When rabbit germ cells were transplanted into immunodeficient mouse testes, putative rabbit SSCs colonized the basement membrane of seminiferous tubules and proliferated for several months; however, no spermatogonial differentiation occurred (Dobrinski et al. 1999a). This indicates that exogenous factors to promote rabbit SSCs exist in mouse seminiferous tubules. GDNF and FGF2 are strong candidates for those factors. However, the rodent SSC culture conditions that consist of STO feeders and serum-free medium containing GDNF and FGF2 could not support proliferation of rabbit germ cells (Kubota et al. 2011). In the serum-free rodent SSC culture system, involvement of mitotically inactive feeder layers for expansion of mouse SSCs is important, because other feeder cells such as Sertoli cell feeders could not support initiation of derivation or maintenance of mouse SSCs (Kubota and Brinster 2008). In particular, testicular fibroblast feeders were detrimental for maintenance of murine SSCs (Kubota et al. 2004a). Similar feeder

effects were observed in the culture system for rat SSCs (Hamra et al. 2005). Besides embryonic fibroblasts, endothelial cells are often used as feeder cells for several types of stem cells (Shen et al. 2004), and we found endothelial cells critical for rabbit SSC culture. Although rabbit spermatogonia could not form clumps and proliferate on STO feeder cells on which rodent SSCs replicate continuously (Fig. 11.2A, B), rabbit germ cells could form clumps on C166 feeders, a yolk sac-derived endothelial cell line, and proliferated continuously in the presence of GDNF (Fig. 11.2C). The proliferation of rabbit germ cell clumps was dependent on GDNF, but FGF2 was not required for continuous proliferation. In addition to expression of DDX4, a definitive germ cell marker, they expressed several SSC marker proteins including ZBTB16, POU5F1, GFRA1, and THY1, which were found in rodent SSCs (Kubota et al. 2011).

To demonstrate that the clump-forming germ cells on C166 feeder cells are rabbit SSCs, functional characterization is necessary. However, an assay to evaluate rabbit SSC activity that includes both self-renewal and differentiation capability to produce functional sperm has not been established. For non-rodent SSCs, colony formation following transplantation into the seminiferous tubules of immunodeficient mouse testes represents the most reliable identification of SSC potential and has been used in several other xenogenetic transplantation assays (Dobrinski et al. 1999a, 2000; Hermann et al. 2007; Nagano et al. 2001b, 2002; Oatley et al. 2004). In the transplantation assay, there are two issues to be carefully addressed. One is identification of donor cells in recipient testes. They should be unequivocally distinguished from recipient cells. The other is characterization of colonized cells. If the phenotype of colonized cells is not undifferentiated spermatogonia, transplanted cells might not be SSCs. In addition, some types of somatic cells could be colonizing (Dobrinski et al. 2000; Shinohara et al. 2003). To avoid any misinterpretation, labeling transplanted cells by introducing a reporter gene, β-galactosidase or GFP, is one of the best techniques. In our experiments, clump-forming rabbit germ cells on C166 feeders were labeled with the β-galactosidase or GFP gene using lentivirus vectors and transplanted into seminiferous tubules of Busulfan-treated nude mice (Kubota et al. 2011). For as long as 23 weeks after transplantation, the β-galactosidase-labeled donor cells were identified by stereomicroscopic analyses (Fig. 11.2D). In addition, flow cytometry readily identified the GFP-labeled donor cells in the testis cell suspension from recipient seminiferous tubules. More importantly, rabbit donor cells in recipient mouse testes retained the undifferentiated spermatogonial phenotype for 6 months, although they did not produce spermatogenesis (Kubota et al. 2011). Thus, it is very likely that the transplanted clump-forming cells cultured on C166 feeders contained rabbit SSCs. To unequivocally demonstrate that the clump-forming germ cells are genuine rabbit SSCs, evaluation by transplantation experiments using allogeneic or ideally syngeneic rabbits as recipients is required, because a transplantation assay is the gold standard of identification of stem cells in any type of tissue. At present there is no report demonstrating a successful long-term cultivation of SSCs derived from non-rodent species. Nonetheless, the rabbit culture system serves as a critical foundation for efforts to culture the SSCs of other non-rodent species, including human SSCs.

11.3.4 Human

Propagation of human SSCs in vitro is of great clinical value. Since the first report of long-term culture of human SSCs in 2009 (Sadri-Ardekani et al. 2009), several culture conditions have been reported (Conrad et al. 2014; Goharbakhsh et al. 2013; Guo et al. 2015; Kokkinaki et al. 2011; Koruji et al. 2012; Lim et al. 2010). Most of these are modified methods of rodent SSC cultures using StemPro34-based media supplemented with GDNF, FGF2, EGF, LIF, and FBS. In these conditions, two types of colonies appeared after about 2–4-weeks in culture. One type consisted of individually visible cells, while the second type consisted of tightly packed colonies, which appeared similar to ES cell colonies (Sadri-Ardekani et al. 2009). The former colonies were designated SSCs, because they expressed several spermatogonial markers, such as ZBTB16, GFRA1, UCHL1, and GPR125 (Sadri-Ardekani et al. 2009, 2011). On the other hand, ES cell-like colonies were considered to be pluripotent stem cells, because they expressed pluripotent markers, such as POU5F1 and NANOG. Culturing human testicular cells in ES cell culture conditions also produced ES-like colonies (Conrad et al. 2008; Golestaneh et al. 2009; Kossack et al. 2009; Mizrak et al. 2010). In order to investigate the biological activity of proliferating cells in cultures, the putative human SSCs were transplanted into infertile immunodeficient mouse testes and the recipient testes analyzed 10 weeks after transplantation. In the recipient testes, a small number of donor-derived cells were identified (Sadri-Ardekani et al. 2009, 2011). Although other research groups also reported culture of human SSCs using similar culture conditions, they were not transplanted (Conrad et al. 2014; Goharbakhsh et al. 2013; Guo et al. 2015; Kokkinaki et al. 2011; Koruji et al. 2012; Lim et al. 2010), and in none of these studies were the transplanted donor human cells recovered from the recipient mouse testes and analyzed for surface markers, as done for the rabbit, or characteristic transcription factors.

The absence of a functional assay system to identify unequivocally human SSCs has resulted in considerable controversy regarding interpretation of results in these and other human SSC culture experiments (Kossack et al. 2013; Langenstroth et al. 2014; Zheng et al. 2014). Although the cellular identity of human SSCs in the cultures was demonstrated by expression of SSC markers previously identified in the SSCs of rodents, recent studies also have clearly demonstrated that many putative markers used for identifying human SSCs are not reliable, due to their expression in non-germ cells of primary and cultured human testis cells (Kossack et al. 2013; Zheng et al. 2014). Importantly, GPR125, ZBTB16, UCHL1, ITGA6, and GFRA1, which were used to identify human SSCs in previous studies, were expressed in testicular somatic cells. Furthermore, in the transplantation experiments, donor germ cells cannot differentiate in the xenotransplantation system, and non-germ cells can colonize recipient seminiferous tubules as well; therefore, characterization of colonized cells would require demonstrating that the colonized cells were human undifferentiated spermatogonia. Information describing molecular signatures and transcriptome analysis of human undifferentiated spermatogonia in recent studies will be useful to identify donor cells in recipient testes (Valli et al. 2014; Wu et al.

2009a). Possible approaches to avoid the problem would be elimination of non-germ cells prior to transplantation or definitive characterization of colonized cells as donor-derived germ cells, both of which represent formidable challenges.

Collectively, while several reports proposed that human SSCs could be continuously cultured over months using StemPro-34-based culture medium containing a growth factor cocktail with FBS, these published reports of long-term human SSC culture are not universally accepted (Medrano et al. 2016). Development of a functional assay to evaluate the self-renewal and differentiation capability for human SSCs and confirm their identity will be essential to the widespread acceptance of any published techniques.

11.4 Potential Applications of Spermatogonial Stem Cells and Future Directions

The transplantation technique has made it possible to generate functional spermatozoa from SSCs isolated from a donor cell population introduced into the seminiferous tubules of a recipient animal. Furthermore, it has been shown that SSCs can be cryopreserved for at least 14 years and will regenerate spermatogenesis and functional spermatozoa after thawing and transplantation (Avarbock et al. 1996; Wu et al. 2012). Thus, cryopreservation of SSCs or SSC containing cell populations provides an effective mechanism by which to preserve the germline of individual males for long periods providing a potentially immortal lifespan for male germlines. Although semen cryopreservation is commonly used to preserve the germline of certain economically, biologically, or scientifically valuable males, including livestock breeds or endangered animal species, semen cryopreservation methods must be developed for each species. Cryopreservation of SSCs is more suitable for this purpose, because simple cryopreservation procedures for somatic cells are applicable to SSCs (Avarbock et al. 1996). Although semen preservation techniques have only been developed for a limited number of species (Barbas and Mascarenhas 2009), SSCs of many species are readily cryopreserved by regular procedures for somatic cells (Avarbock et al. 1996; Dobrinski et al. 1999a, 2000; Hermann et al. 2007; Nagano et al. 2001b, 2002; Oatley et al. 2004). In addition, the potential genetic recombination possibilities of a germline are only partially conserved with spermatozoa since variability is limited by the number of spermatozoa present in the sample, whereas, the stem cell preserves all the potential recombination possibilities of any germline. Moreover, SSCs have the potential to be expanded in culture. Although long-term culture techniques are available for a few species at present, techniques for many of the valuable species, including human, livestock, or endangered animals are certain to be worked out in the future (Kubota and Brinster 2006).

Functional spermatozoa can be obtained by transplantation of SSCs from immature males before puberty or even from fetuses. Thus, an important and potential clinical application for human SSCs is in prepubertal boys undergoing chemotherapy or radiation treatment (Brinster 2007; Kubota and Brinster 2006) (Fig. 11.3). Germ cells including SSCs are extremely sensitive to chemotherapeutic agents and

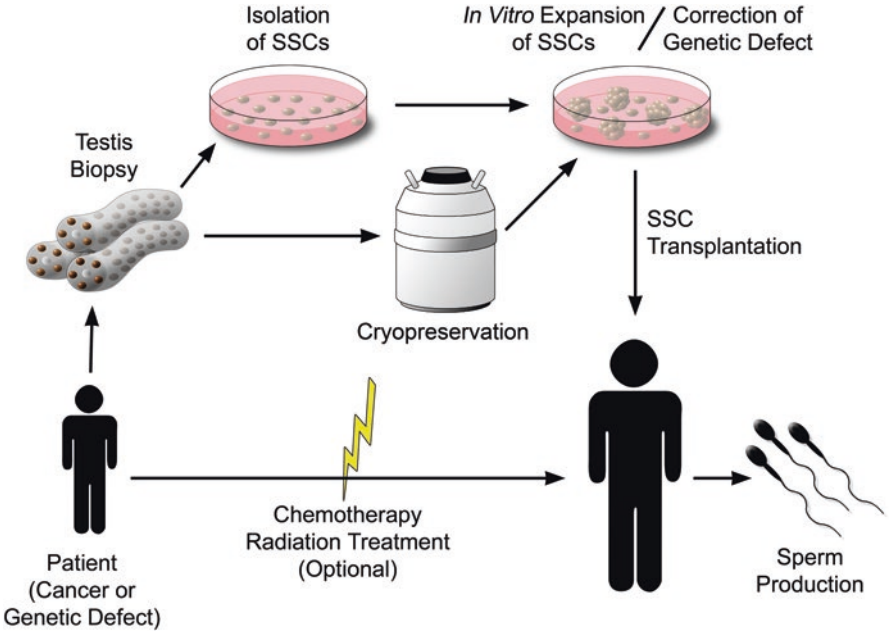

Fig. 11.3 A proposed clinical application of human SSCs. Before treatment for cancer by chemotherapy or irradiation, a prepubertal patient could undergo a testicular biopsy to recover SSCs. The SSCs could be cryopreserved and/or cultured to expand in vitro. After treatment, the SSCs would be transplanted to the patient's testes for the production of spermatozoa. A possible step for genetic correction to rescue a genetic disorder is indicated prior to transplantation. Modified from Kubota and Brinster (2006)

radiation, and it is estimated that approximately 1 in 5000 male cancer survivors of reproductive age are infertile or extremely sub-fertile as a result of treatments for childhood cancer (Ginsberg et al. 2010). While adults can cryopreserve semen before germ cell destroying therapies for future use in artificial insemination or in vitro fertilization, this option is not available for prepubertal boys, because complete spermatogenesis has not been established. For prepubertal boys, cryopreservation of a testicular biopsy can be used for future autologous transplantation into the seminiferous tubules following successful cancer treatments (Ginsberg et al. 2014). The biopsy contains SSCs, which have the potential to colonize and restore spermatogenesis following transplantation. In addition, when efficient culture methods to allow ex vivo expansion of human SSCs become available, the number of SSCs can be greatly increase before cryopreservation or transplantation, which maximizes recovery of spermatogenesis in recipient testes (Fig. 11.3).

An important concern is potential contamination of malignant cells in donor cell suspensions. To avoid this, it is important to determine the unique surface phenotype of human SSCs, which will allow both enrichment of human SSCs and elimination of cancer cells before transplantation. The antigenic profile of mouse SSCs is highly conserved in putative human SSCs. In particular, THY1 is a useful cell

surface marker to enrich putative SSCs in human, and the characteristic of MHC-I negative is also valuable to eliminate malignant cells, because MHC-I is strongly expressed on almost all somatic cells, including tumorigenic cells but not on SSCs (Hermann et al. 2011). Recent studies suggest that contamination of tumorigenic cells in donor cell suspension is avoidable by FACS with combinations of several surface markers (Dovey et al. 2013).

An enormously valuable application of SSCs is for germline modification (Brinster 2002). The first transgenic animal using SSCs was created by transduction of a retrovirus vector containing the β-galactosidase gene into mouse SSCs (Nagano et al. 2001a). Although retroviral transduction was used in the initial approach, subsequent development of a long-term culture system now allows a variety of techniques to select successful modifications, resulting in generation of not only knock-out mice by homologous recombination (Kanatsu-Shinohara et al. 2006a), but also gene-editing mice using the TALEN or, in particular, the CRISPR/Cas9 system (Sato et al. 2015; Wu et al. 2015). In rats, similar to the mouse system, the first SSC-based transgenic rats were generated using a lentiviral vector (Hamra et al. 2002), and the CRISPR/Cas9 system in rat SSCs has been developed (Chapman et al. 2015; Sato et al. 2015; Wu et al. 2015). In addition, recent studies demonstrated the possibility of germline gene therapy using the CRISPR/Cas9 system in mice (Wu et al. 2015). These gene-editing approaches will eventually be applicable to a variety of animals, for instance, valuable breeds of companion animals and farm animals (Tan et al. 2013).

A number of genetic mutations causing human disease have been identified, and at some future date germline gene-editing may be considered for therapeutic correction. However, a recent report of gene-editing in human preimplantation embryos using CRISPR/Cas9 is highly controversial (Kang et al. 2016; Liang et al. 2015), because it raises serious ethical concerns (Bosley et al. 2015). The scientific community has agreed that this technology is not sufficiently developed and should not be used for human clinical reproductive purpose (Baltimore et al. 2015). In 2016, only a few countries have approved the use of gene-editing techniques on human embryos for research purposes. Once cultivation and differentiation techniques of human SSCs become available, controversy will inevitably arise regarding the ethics of research designed to generate spermatozoa from gene-edited human SSCs.

It can be seen from the above discussion that SSC transplantation, culture and cryopreservation have revolutionized the study of the male germline of not only research species, but of all mammals, including companion animals, farm animals, primates, and endangered species. The possibility to increase knowledge about SSCs and spermatogenesis regarding biological regulation and for practical purposes is now limited only by future development and understanding of male germline stem cells and their differentiated daughters, which is a rapidly emerging field as seen in the contents of other chapters in this volume.

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