
2 Germ Line Stem Cells

Makoto C. Nagano

CONTENTS

INTRODUCTION
DEVELOPMENT OF GERM CELLS AND THEIR RELATIONS TO EMBRYONIC STEM CELLS
SPERMATOGONIAL TRANSPLANTATION—FUNCTIONAL ASSAY TO DETECT STEM CELL ACTIVITY
THE FREQUENCY OF SSCs
KINETICS AND REGULATION OF SSC PROLIFERATION
THE STEM CELL NICHE
SSC IDENTIFICATION MARKERS
SSCs, MALE MUTATION BIAS, AND GENETIC DISEASES
SUMMARY
REFERENCES

1. INTRODUCTION

Stem cells are a unique cell population in both biological and clinical contexts. Stem cells are defined by their function to reproduce themselves (self-renewal) and concurrently generate daughter cells that are committed to differentiation (1). The differentiation of stem cells leads to the production of all cell types in a given cell lineage. Furthermore, stem cells conduct this dual function for a lifetime, thereby continuously providing specialized cells that perform normal functions of self-renewing tissues/organs (e.g., bone marrow, intestinal epithelium; *see ref. 2*). Clinically, these characteristics of stem cells allow them to be a potentially powerful resource for various applications, such as regenerative medicine and gene therapy (3). Our ability to understand stem cell biology is therefore crucial for such practical applications.

In general, stem cells can be categorized into three types. One type is a stem cell population of embryonic origin, and the other two are postnatal stem cells in

From: *Contemporary Endocrinology: Stem Cells in Endocrinology*

Edited by: L. B. Lester © Humana Press Inc., Totowa, NJ

somatic and germ cell lineages. Each of these three stem cell types has a unique property. Whereas somatic stem cells (e.g., hematopoietic stem cells) are indispensable for the survival of an individual, germ line stem cells are essential for the survival of a species. Thus germ line stem cells are the foundation for the manifestation of evolutionary processes, whereas somatic stem cells function as receptors of the selection pressure exerted by their environment. Stem cells derived from embryos have the broadest differentiation potential among the three and can produce all cell types in the body, including somatic and germ cell lineages. On the other hand, postnatal stem cells are essentially tissue-specific and produce only cells of the corresponding cell lineage (4).

Among these three types of stem cells, somatic and embryonic stem cells have been more intensively investigated than germ line stem cells. However, because all embryonic and somatic cells originate from gametes, studies of germ line stem cells may facilitate understanding the biology of other two stem cell types. Alternatively, because the development of germ cells is initiated in embryos separately from that of somatic cells (*see* the following section), germ line stem cells might possess unique properties that distinguish them from other types of stem cells.

In this chapter, the current state of germ line stem cell research and knowledge accumulated during the past decade almost exclusively using a mouse model is discussed. Although some clinical perspectives are discussed in the last section of this chapter, readers are advised to refer elsewhere (Chapter 11 and ref. 5) for more detailed discussions on applications of germ line stem cells. After brief descriptions on fetal germ cells, the main focus of this chapter will be stem cells of the postnatal male germ line, because using the strictest definition of stem cells, no stem cells exist in the fetal and female germ lines in mammals. In addition, the discussion in this chapter will focus on studies in which stem cells were analyzed by functional assays, even though extensive morphologic studies have historically provided important knowledge on germ line stem cells (6). This is because stem cells are defined essentially by their functions and not by their morphology.

2. DEVELOPMENT OF GERM CELLS AND THEIR RELATIONS TO EMBRYONIC STEM CELLS

During embryonic development, germ cells first emerge at a specific location segregated from somatic cell development in both vertebrates and invertebrates (7–9). This physical segregation of germ cells has been hypothesized to allow germ line specification to occur with a minimal influence from somatic cell development (8). In mammals, germ cell development has been best studied in mice. Mouse germ cells are first recognized at the base of allantois in the

extraembryonic mesoderm at approximately 7 days postcoitum (dpc) as a cluster of approximately 50 cells that exhibit the alkaline phosphatase activity (7–9). These fetal germ cells are called primordial germ cells (PGCs). PGCs are then transferred from the extraembryonic tissue to the embryo *per se* at approximately 8.5 dpc and migrate through the hindgut while rapidly proliferating. These cells further migrate through the dorsal mesentery into the genital ridges (fetal gonads) at approximately 10.5 dpc. PGCs continue to proliferate in the genital ridge until approximately 12.5 dpc when the sex differentiation becomes morphologically evident. On 13.5 dpc, approximately 25,000 PGCs can be found in the genital ridge (10); thus, PGCs increase 500-fold in number from the time of their emergence. At the initiation of sex differentiation, female and male germ cells take different developmental pathways (Fig. 1). In females, PGCs enter meiosis and then become arrested at meiotic prophase. In males, PGCs encapsulated in the testicular cords (fetal seminiferous tubules) become mitotically arrested. The arrested stage continues until birth in both sexes. After birth, female germ cells are periodically recruited for meiotic maturation, whereas male germ cells initiate mitosis. In the male, these diploid postnatal germ cells are called *spermatogonia*. Spermatogonia undergo mitosis and sequentially differentiate before committing to meiosis (11). In mice, first meiotic male germ cells (spermatocytes) appear around 10 days of age and first spermatozoa, approximately 35 days of age (12,13). The male gametogenesis (spermatogenesis) continues throughout life.

The mechanism of germ cell specification, migration, proliferation/survival, and sex-dependent differentiation during embryonic development remains elusive and is beyond the scope of this chapter (*see refs. 8,9,14–17 for detail*). However, the process of germ cell development depicts a unique characteristic of germ line stem cells. Because all female germ cells enter meiosis on sex differentiation, they lose self-renewal potential before birth, resulting in the loss of stem cells in the postnatal female germ line. In contrast, a population of self-renewing cells (stem cells) remains in the male germ line throughout life. This is the foundation of continuous spermatogenesis and the regeneration of spermatogenesis following testicular insults, including sterilizing cancer therapy (5,18). Consequently, the number of gametes during the reproductively active periods in males far exceeds that in females. Such a sex-dependent difference in the existence of stem cells cannot be seen in other types of stem cells.

PGCs are a transient cell type and are not true stem cells under a strict stem cell definition. These cells are rather “precursors” of germ line stem cells, because the cells that have characteristics identical to PGCs do not exist in normal postnatal mammals, reflecting the lack of extended self-renewal activity of PGCs. In fact, many embryonic cells are precursors. For example, although the cells in the inner cell mass of blastocysts are the origin of embryonic stem cells

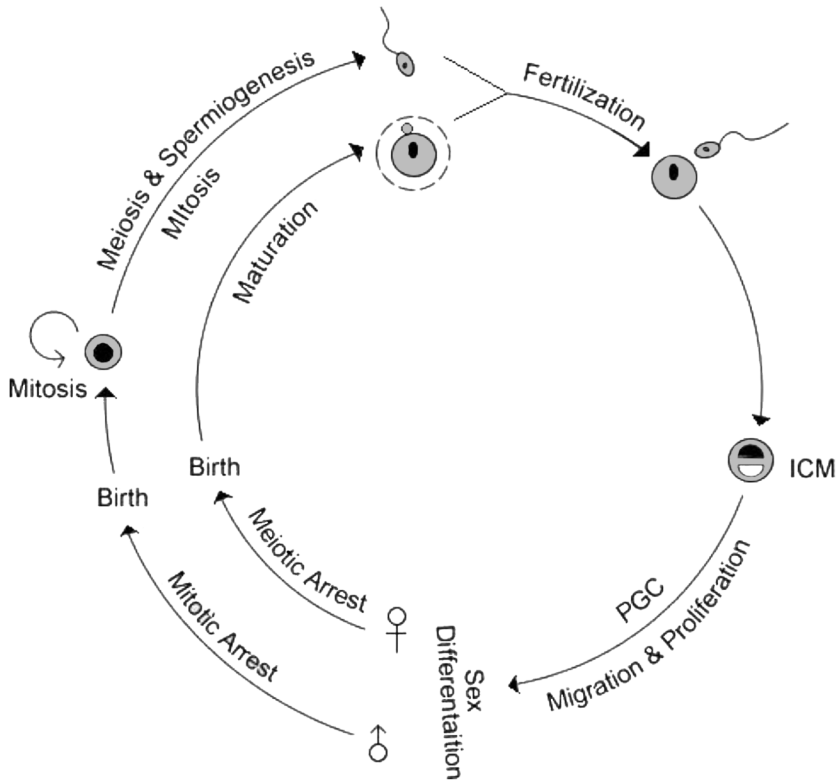


Fig. 1. The life cycle of germ cells. Fertilization of an egg by a sperm triggers the embryonic development. The cells in the ICM (shown as a dark part) of the blastocyst are the origin of all cell types in the body as well as ES cells. PGCs are first found in the extraembryonic mesoderm and translocate into the embryo *per se*. Then, they migrate toward the embryonic gonads while actively proliferating. These proliferating PGCs are capable of transforming to totipotent EG cells. After the migration into the gonads, male germ cells enter mitotic arrest, whereas all female germ cells enter meiosis, followed by the arrest at the meiotic prophase. After birth, male germ cells reinitiate mitosis and undergo meiosis and spermiogenesis, which is a complex morphologic transformation of haploid spermatids to sperm. Mitosis, meiosis, and spermiogenesis continue throughout life in the male germ line (spermatogenesis). It is important to note that a population of stem cells exists in the male germ line, whereas it is absent in the female germ line. (Modified from ref. 95 with permission.)

(ES cells; Chapter 1), they do not self-renew but rather disappear during normal embryonic development.

Although PGCs are a transient cell population, these cells have significant potential to be a source of stem cells, not only for male germ line stem cells but also for pluripotent embryonal carcinoma (EC) cells and totipotent embryonic

germ (EG) cells (*see* Chapter 1). From the 1950s to the 1970s (reviewed in ref. 19), Leroy Stevens at the Jackson Laboratory intensively studied teratomas/teratocarcinomas (hereafter, only the term “teratocarcinomas” will be used for simplicity). Using mice, he showed that the implantation of blastocysts or of the genital ridge (10.5–12.5 dpc) into adult testes resulted in the formation of teratocarcinomas, in which a wide range of differentiated tissues can be observed (*see* Chapter 1). He identified PGCs as the origin of teratocarcinomas that arise after implanting genital ridges into adult testes (20). Teratocarcinomas were found to contain the stem cells that were later isolated and called EC cells (*see* Chapter 1). The self-renewal potential and pluripotency (potential to differentiate into multiple, *but not all*, cell types) of EC cells was proven by Barry Pierce, thus confirming the stem cell properties of these cells (21). Ralph Brinster later demonstrated by injecting EC cells into blastocysts that these tumor stem cells could be integrated into the normal developmental process to produce chimeric mice without causing tumorigenesis (22), although EC cells do not enter the germ line. Therefore, PGCs are the source of pluripotent EC cells that can contribute to the generation of chimeric mice.

In 1992, Matsui et al. (23) and Resnick et al. (24) reported that mouse PGCs transformed to cells that morphologically resembled ES cells when cultured in vitro with a cocktail of growth factors. These cells, called EG cells, were derived by culturing proliferating PGCs (8.5–12.5 dpc) with leukemia inhibitory factor (LIF), Steel factor (also called c-Kit ligand), and basic fibroblast growth factor (bFGF). Using similar culture techniques, EG cells have also been obtained from pigs and humans (25,26). The studies using mice have shown that EG and ES cells are similar not only in their morphology, but also in their function. As with ES cells, EG cells can be maintained in undifferentiated states indefinitely in vitro and will generate teratocarcinomas in vivo (23). When injected into blastocysts, both ES and EG cells are integrated into the normal developmental process of host mice and contribute to both somatic and germ cell lineages (27). Therefore, ES and EG cells are both totipotent (differentiate into *all* cell types), although they are apparently not identical (28). Another characteristic shared by both of these tumor stem cells is that they are derived from transient cell types that express Oct-3/4 transcription factor: ES cells from the inner cell mass/epiblast and EG cells from PGCs (8,9,29,30) (*see* Chapter 1).

Although mouse PGCs have the potential to transform into totipotent stem cells, ironically these cells have never been successfully used for cloning by nuclear transplantation (31,32). Mice can be successfully cloned using the nuclei of many somatic cell types (33). However, no reports have shown live birth of offspring cloned from PGC nuclei (31,32). Yamazaki et al. have shown that embryos cloned using nuclei of 10.5-dpc PGCs can develop normally until mid-gestation but die shortly thereafter (32). Furthermore, embryos cloned from

later-stage PGCs never develop normally (32). These results might indicate that the genomes of germ cells may be programmed in a specific manner. For example, it is well known that the patterns of DNA methylation and imprinting in the germ line are distinct from those of somatic cell lineages (34,35). Therefore, it is possible that genomic modifications specific to the germ line render the reprogramming of the germ cell genome difficult in cloning by nuclear transplantation.

3. SPERMATOGONIAL TRANSPLANTATION—FUNCTIONAL ASSAY TO DETECT STEM CELL ACTIVITY

Postnatal male germ line stem cells, also called spermatogonial stem cells (SSCs), are a small fraction of spermatogonia (6,18). SSCs and spermatogonia reside at the periphery of the seminiferous tubules on the basement membrane surrounded by Sertoli cells, which are somatic regulatory and supportive cells for spermatogenesis (Fig. 2).

Because stem cells are defined by their dual functions, studies of stem cells require a functional assay to detect stem cell *activity* (long-term self-renewal and differentiation). The unequivocal detection system for stem cells is a transplantation assay, in which the presence of stem cells is retrospectively confirmed by their ability to replenish a complete cell lineage in a damaged tissue. For example, the presence of hematopoietic stem cells (HSCs) in the bone marrow can be inferred when transplantation of marrow cells into lethally irradiated recipients results in a complete regeneration and long-term maintenance of hematopoiesis of donor origin (1). Likewise, SSCs can be detected by a transplantation assay (spermatogonial transplantation). In fact, HSCs and SSCs are the only stem cell populations for which a transplantation assay has been established. The detection of other stem cells relies on more equivocal assay systems, such as an in vitro differentiation assay for neural stem cells (36).

The spermatogonial transplantation assay was developed in 1994 using the mouse as a model species (Fig. 3; refs. 37,38). In this assay system, a single cell suspension is first prepared from the testes of donor mice (39,40). These cells are injected into the lumen of recipient mouse seminiferous tubules using a micro-injection needle. The recipient mice are pretreated with an alkylating reagent, busulfan, to ablate endogenous spermatogenesis, thereby providing access and space for donor stem cells to colonize on the basement membrane and to develop spermatogenesis (39–41). On transplantation, SSCs migrate from the tubular lumen to the basement membrane, settle in the microenvironment surrounded by recipient Sertoli cells, and regenerate spermatogenesis. The donor origin of regenerated spermatogenesis is readily confirmed by using transgenic mice as donors and wild-type mice as recipients. In an experiment shown in Fig. 3, donors were ROSA26 transgenic mice that ubiquitously express β -galactosi-

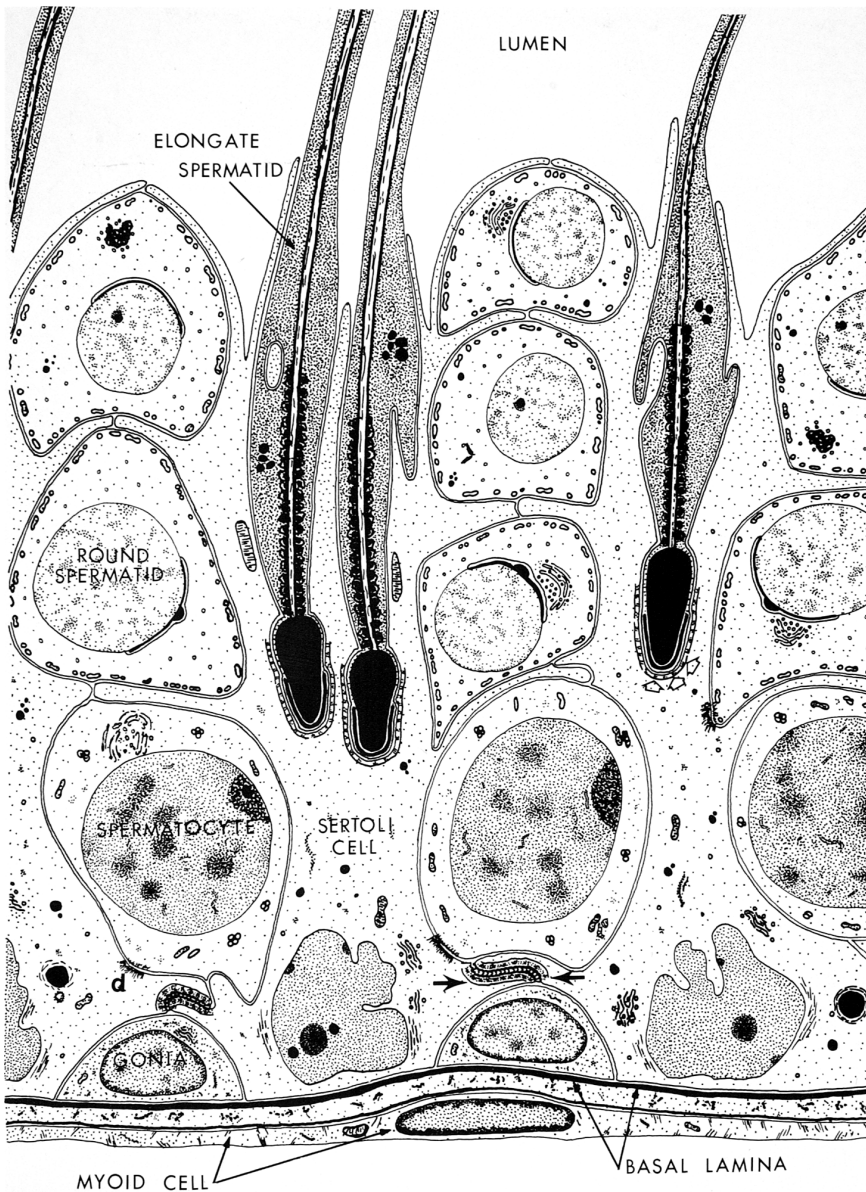


Fig. 2. A schematic representation of seminiferous epithelium. Diploid spermatogonia reside on the basement membrane at the periphery of seminiferous tubules, surrounded by Sertoli and peritubular myoid cells. Meiotic and haploid germ cells are located in the adluminal compartment and migrate toward the lumen during their development. The final product of spermatogenesis, spermatozoa, is released into the lumen. Spermatogonial stem cells are a small fraction of spermatogonia. (From ref. 11 with permission.)

dase, and recipients were immunocompatible wild-type mice pretreated with busulfan. Thus donor-derived spermatogenesis can be detected as a blue segment (colony) in seminiferous tubules by staining recipient testes with X-gal. After transplantation, recipient mice can regain fertility and produce offspring carrying the donor genotype (*see* Fig. 3). Because such a complete recovery and long-term maintenance of spermatogenesis can be achieved only by stem cell activity, spermatogonial transplantation is the unequivocal assay for SSCs.

Spermatogonial transplantation has provided a unique opportunity to study the role of hormones in spermatogenesis. Although testosterone is essential to spermatogenesis, the target of its action (germ cells or somatic cells) was elusive for decades. This question was addressed using transplantation of testis cells obtained from the *tfm* (i.e., testicular feminization) mutant mice, which lacked functional testosterone receptors (42). Transplantation of these cells into the testes of immunocompatible wild-type mice resulted in the complete regeneration of spermatogenesis in recipient testes. The result thus demonstrated that SSCs exist in the *tfm* mice and that the testosterone receptor is not required in germ cells but is essential in their environment (42). Similarly, a transplantation study has demonstrated that the estrogen receptor α is not required in germ cells for complete spermatogenesis (43).

Spermatogonial transplantation is not only a qualitative but also a quantitative assay for SSCs. Recent transplantation studies have shown that each colony of donor-derived spermatogonia arises from a single SSC (44,45). Therefore, the number of colonies observed in recipient testes directly correlates with the number of functional SSCs that have successfully colonized the recipient environment and regenerated spermatogenesis.

Although spermatogonial transplantation is a time-consuming assay that detects SSCs only retrospectively, it is currently the only definitive assay for SSCs, and studies of SSCs have heavily relied on the development of this assay system.

4. THE FREQUENCY OF SSCs

A common characteristic observed in virtually all stem cell systems is that stem cells are a rare cell population in a cell lineage. For example, definitive HSCs represent only 0.007% of nucleated bone marrow cells in mice (1). Using spermatogonial transplantation as a SSC bioassay, a recent study has demonstrated that adult mouse SSCs represent 0.01% of total testis cells (approximately 3000 SSCs/testis, ref. 46). Thus 1 in 10,000 cells in an adult testis is a stem cell, a similar frequency to that observed with mouse HSCs in the bone marrow.

However, the number of SSCs appears to vary during postnatal development and between species. It has been shown that the total number of functional SSCs in a testis (those capable of regenerating spermatogenesis in recipient testes after

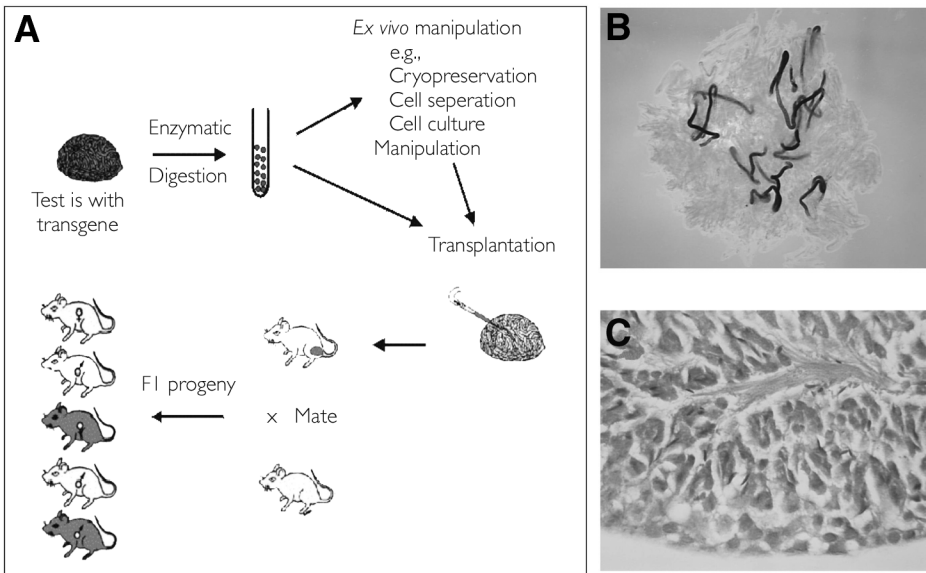


Fig. 3. (A) A flow chart of the spermatogonial transplantation procedure. Donor testes are enzymatically digested to a single cell suspension. These cells can be used for ex vivo manipulations, such as cryopreservation, cell selection, cell culture, and genetic modification. Donor cells are introduced into the lumen of seminiferous tubules. Spermatogonial stem cells included in the donor cell preparation colonize recipient testes and develop donor-derived spermatogenesis. Recipient males can become fertile and produce offspring carrying the donor genotype. Because such a complete and long-term regeneration of spermatogenesis cannot be accomplished without stem cells, spermatogonial transplantation is the unequivocal assay to detect stem cell activity. **(B)** When donor cells are derived from the testes of transgenic mice that express a marker gene (*lacZ* in this example) in germ cells, donor-derived spermatogenesis can be readily observed as blue-stained segments in seminiferous tubules (colonies; dark segments in this panel). The formation of morphologically distinct colonies allows quantitative analyses of stem cells by counting the number of colonies. **(C)** At 2 months posttransplantation, complete spermatogenesis is established in a colony. (From ref. 96 with permission.)

transplantation) increases up to 40-fold from the day of birth to adulthood in mice (47). In the rat, the total number of functional SSCs also dramatically increases (70-fold) during postnatal development (41,48). However, compared with mouse SSCs, rat SSCs are 2- to 10-fold higher in their concentration (frequency) and 30- to 120-fold higher in their total number per testis, depending on the stage of postnatal development (41,47,48). Thus the frequency and the number of SSCs appear to differ greatly with age and between species.

5. KINETICS AND REGULATION OF SSC PROLIFERATION

Although proliferation kinetics of SSCs in the steady state (i.e., in intact testes) are unknown, some data are available on the SSC proliferation kinetics under experimental conditions *in vivo* and *in vitro*.

5.1 *In Vivo Studies*

SSC proliferation activity after transplantation has recently been characterized. On transplantation, adult mouse SSCs migrate to the basement membrane and settle in the recipient seminiferous epithelium in the first week (46). During this period, the proliferation of SSCs is undetectable. From 1 to 4 weeks posttransplantation, SSCs rapidly proliferate (approximately eightfold), but thereafter, their proliferation activity significantly diminishes (46,49). SSCs divide slowly from 1 month to more than 1 year after transplantation, and their population doubling time has been estimated to be approximately 80 days during this period (49). Although SSCs slowly divide after 1 month posttransplantation, colonies of donor-derived spermatogonia continue to expand in a linear fashion. Interestingly, during this linear growth period, the concentration of SSCs in a colony appears to stay constant at approximately 8 SSCs per 2 mm of colony (46,49). This consistent stem cell concentration during the colony growth phase suggests that the proliferation of SSCs is tightly regulated in coordination with the progress of spermatogenic regeneration and the number of differentiating germ cells produced by SSCs.

It is well known that spermatogenesis takes place in a species-specific cycle duration (11). For example, one cycle of spermatogenesis takes 35 days in the mouse and 52 days in the rat. Such a species-specific cycle of spermatogenesis could be related to regulation of balance between the activity of SSCs and the progress of spermatogenesis described previously. A long-standing question was whether the spermatogenic cycle length is determined by germ cells, supporting Sertoli cells, or by both. This question was addressed by using xenogeneic spermatogonial transplantation, in which SSCs derived from transgenic rats were transplanted into the testes of immunodeficient mice (50). The results showed that regenerated rat spermatogenesis exhibited spermatogenic cycle length characteristics of the rat (52 days), even though rat SSCs and spermatogenesis were supported by mouse Sertoli cells. Therefore, it was concluded that the cycle length of spermatogenesis is predominantly regulated by germ cells, rather than by Sertoli cells (50). Because the progress of spermatogenesis apparently depends on the activity of SSCs to self-renew and supply differentiating germ cells (46,49), the difference in the spermatogenic cycle length observed in various species might correlate with a species-specific SSC activity.

The proliferation of SSCs must also be regulated in concert with their differentiation and death (6). For example, if one cell division of a SSC results in the production of one daughter stem cell and one differentiating spermatogonium (50% probability each for self-renewal and differentiation), the size of SSC population should be maintained at constant. If the probability is distorted to 100% self-renewal or 100% differentiation, it should result in the expansion of SSCs and the loss of differentiating cells or vice versa. Likewise, the death of daughter cells should result in a decrease in stem and/or differentiating cells. The pattern and mechanism of such stem cell regulation are unknown for SSCs (6). However, observations of spermatogenesis in mutant mice suggest that the differentiation of spermatogonia/SSCs is less stable than their proliferation.

Mutant mice that lack functional Steel factor are devoid of spermatogenesis and infertile because Sertoli cells cannot support spermatogenesis. However, SSCs are retained in their testes and are capable of regenerating complete spermatogenesis when transplanted into the testes of wild-type mice (51). A detailed study of spermatogenesis in Steel factor mutant mice has demonstrated that the loss of spermatogenesis results from defective differentiation of spermatogonia (52). Provided with no functional Steel factor, SSCs can still proliferate and produce daughter spermatogonia, but these spermatogonia cannot proceed through further differentiation events and die by apoptosis. Similarly, adult *jsd* (juvenile spermatogonial deficiency) mice lack spermatogenesis and are infertile because of the intrinsic inability of spermatogonia produced by SSCs to complete differentiation, which results in spermatogonial death (52,53). In another mutant mouse strain that overexpresses glial cell line-derived neurotrophic factor (GDNF), primitive spermatogonia accumulate in the seminiferous tubules, probably because of the blockade in spermatogonial differentiation while spermatogonial proliferation continues (54). A similar pattern of spermatogonial accumulation caused by the differentiation blockade has also been observed in mutant mice that misexpress the anti-apoptotic *bcl-2* gene in germ cells (55).

In contrast, despite the production of numerous mutant strains of infertile mice to date (56), specific defects in the proliferation of spermatogonia or SSCs have not clearly been demonstrated. These studies thus suggest that the proliferation of spermatogonia and possibly SSCs is a rather autonomous function of these cells; whereas their differentiation is the vulnerable and potentially manipulative process during early spermatogenesis.

5.2 In Vitro Studies

Proliferation characteristics of SSCs have also been studied in vitro using retroviral vectors. These viral vectors can deliver and integrate a target gene into the genome of host cells. Because stem cells are retrospectively detected by their

function, retroviruses are particularly powerful vectors to place a marker in stem cells before they exhibit their regeneration activity. In addition, retroviruses require host cell division for a successful gene delivery; thus, stem cell proliferation can be detected using these vectors. To evaluate the activity of SSC proliferation in vitro, a marker gene (*lacZ*) was delivered into SSCs using retroviral vectors in culture, followed by transplantation of cultured cells (57). After 7 days of culture, the marker gene was found in 0.3% of total stem cells cultured. Therefore, at least 1 of 300 SSCs entered self-renewal during 1 week of culture. It has also been shown that the self-renewal activity of prepubertal SSCs derived from 1-week-old mice is approximately 10-fold higher than that of adult SSCs (58), corresponding well with the behavior of prepubertal SSCs observed in vivo. Although adult SSCs are believed to be mostly quiescent, prepubertal spermatogonia and SSCs proliferate more actively during the first postnatal week in mice (6).

Although the studies using retroviruses demonstrated that SSCs could divide in vitro, it has also been shown that the total number of mouse SSCs continuously decreases during 1 week of culture (59). About half of SSCs placed in culture are lost within the first 2 days and only 12% of SSCs remain for 1 week. However, this continuous decrease in SSC numbers should not be a simple loss of SSCs during culture because SSCs undergo self-renewal in vitro. Because the number of SSCs detected by spermatogonial transplantation reflects a cumulative result of stem cell division, differentiation, and death, the observed decrease in the number of SSCs should imply that the differentiation or the death of SSCs exceeded their self-renewal under the culture conditions employed.

Using the same culture system, effects of soluble factors on the in vitro SSC maintenance have been examined (59). Activin A is a member of transforming growth factor- β (TGF- β) family and has been shown to stimulate the proliferation of spermatogonia in vitro (60). However, transplantation experiments using testis cells cultured with activin A have demonstrated a marked decrease in SSC numbers to 30% of those in control culture with no factors. The results thus suggest that activin A may act on differentiating spermatogonia, rather than SSCs (59). Because spermatogenesis is a one-way differentiation cascade from stem cells to spermatozoa (Fig. 4), it may be reasonable to speculate that the proliferation of advanced spermatogonia stimulated by activin A might induce the recruitment of SSCs into differentiation at a rate that exceeds the self-renewal of SSCs to support expanding nonstem spermatogonia, thereby diminishing stem cell population in vitro.

In contrast to the effect of activin A, GDNF, which is another member of the TGF- β family, stimulates the maintenance of SSCs in vitro (59). It has been shown that GDNF blocks the differentiation of spermatogonia in transgenic mice that constitutively express the factor, and undifferentiated spermatogonia accu-

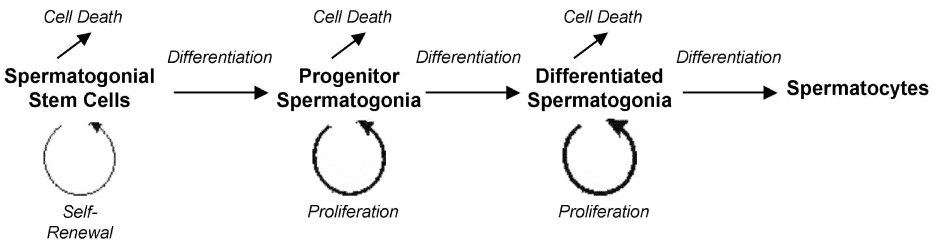


Fig. 4. A schematic representation of the cascade of spermatogonial differentiation. Spermatogenesis is a one-way differentiation event from stem cells to sperm. Therefore, a blockade in one step of differentiation may result in the accumulation of cells at prior differentiation stages, and ultimately stem cells. The amplification of stem cells can be achieved also by encouraging stem cell self-renewal and inhibiting stem cell death.

mulate in their testes (54). In addition, transfection of GDNF in Sertoli cells leads to the expansion of the SSC population in vivo (61). Likewise, GDNF may exert a similar function to block spermatogonial differentiation in vitro. A blockade of a differentiation process in unidirectional spermatogenesis could ultimately result in the accumulation of SSCs, thereby supporting the maintenance of SSCs in culture (Fig. 4).

The same study also suggested a similarity in regulatory mechanisms of SSCs and HSCs (59). Although bone morphogenetic protein 4 (BMP4) is known to play a critical role in germ cell specification during embryonic development in mice (14), BMP4 was found to decrease SSC numbers to 40% of control levels in vitro (59). In this context, it has recently been shown that the number of HSCs significantly increases in transgenic mice that lack functional BMP signaling machinery (62). Thus the BMP signaling might be involved in negatively regulating the number of stem cells in both the male germ line and the hematopoietic lineages.

The interpretation of these results described previously is based on the hypothesis that the regulation of spermatogonial/SSC differentiation alters the maintenance of SSCs in vitro. As illustrated in Fig. 4, however, the number of SSCs can also differ depending on the status of their death or proliferation. If the death of SSCs is inhibited or their proliferation stimulated, more SSCs should remain in culture. Thus further studies are required to better understand the mechanism of SSC fate decision (self-renewal, differentiation, or death), and the in vitro culture of SSCs is expected to provide an effective experimental system for such studies.

Recently, a culture system has been reported by which SSCs are dramatically amplified (63). In this study, when testis cells derived from newborn mice were cultured with GDNF, bFGF, LIF, and epidermal growth factor, groups of aggregated germ cells emerged. Importantly, when digested using trypsin and replated

into culture, these aggregated cells emerged again, indicating that the cells can be passaged. Repeated passaging of these cells resulted not only in a long-term maintenance of SSCs over 5 months, but also resulted in their remarkable amplification, estimated to be more than 10^{14} -fold. Such an extremely high degree of stem cell amplification has not been achieved with any other stem cell system, which may be related to intrinsic proliferation potential of different types of stem cells. For example, whereas mouse HSCs regenerate complete hematopoiesis after transplantation, their proliferation activity could cease even when the stem cell number reaches at only 5% of normal value (64,65). Such an inhibition of repopulation activity has not been observed for mouse SSCs (49). Thus SSCs may have a highly robust proliferation potential that can be induced in vitro using growth factors.

However, it should be noted that this remarkable proliferation was observed with neonatal SSCs and it remains to be addressed if adult SSCs retain a similar degree of proliferation potential. Furthermore, the robust proliferation activity of mouse SSCs was found to be strain-dependent (63). Although neonatal SSCs derived from the DBA, DBA \times C57BL/6 (B6) F₁, or ICR strain actively proliferate in vitro, those derived from the B6 or 129 strain do not. Therefore, there appear to be genetic mechanisms specific to mouse strains that predispose the proliferation potential of SSCs.

6. THE STEM CELL NICHE

The activity of stem cells and their fate decision are believed to be regulated intrinsically by stem cells and also extrinsically by supporting cells in the microenvironment where stem cells reside (66). This microenvironment is called the stem cell niche. Although the mechanism of stem cell regulation by their niches is largely unknown for most of mammalian stem cell systems (66), some information has begun to emerge recently. For HSCs, osteoblasts are now known to be a crucial element of their niches (62,67). When osteoblasts are stimulated in vivo by parathyroid hormone/parathyroid hormone-related protein, they produce high levels of jagged 1, a ligand to Notch, and thereby stimulate HSC self-renewal, resulting in approximately twofold increase in the HSC population (67). In addition, a twofold increase in the number of HSCs has been observed in mutant mice depleted of a BMP receptor, coinciding the doubling of osteoblast population in the HSC niches (62). Similarly, with SSCs, it is known that when more vacant niches are available, more SSCs colonize recipient testes after transplantation (41,68). Thus the availability of niches determines the number of stem cells in hematopoietic and germ cell lineages in vivo.

It is believed that SSC niches are composed of Sertoli cells, the basement membrane, and peritubular myoid cells, because all spermatogonia, including

SSCs, are contained in a space formed by these cellular and extracellular elements (*see* Fig. 2). However, how these niche components cross-talk with SSCs and regulate the SSC activity remains elusive. A study using a gene trap strategy in Sertoli cells has shown that these cells rapidly change their gene expression profiles in response to the contact with germ cells in vitro (69). Such an in vitro approach may shed some light on the mechanism of intercellular communications involved in the niche functions.

7. SSC IDENTIFICATION MARKERS

Probably the most significant impedance in the study of stem cells is that the detection of stem cells relies on their function, rendering their assay retrospective and time-consuming with a low sensitivity. To prospectively study stem cells, identification markers need to be determined. A general strategy to determine stem cell markers is as follows. First, a molecule that could be a stem cell marker is chosen. Second, a population of cells is selected that express this molecule. Third, these selected cells are assayed for stem cell activity. If the molecule is expressed selectively or preferentially by stem cells, the selected cells should contain more stem cells and exhibit a higher level of stem cell activity than unselected cells.

Consequently, stem cell markers need to be cell-surface molecules, because the selected cells must be alive and functionally intact. Although an intracellular molecule, such as a transcription factor, could be expressed exclusively in stem cells, such a molecule cannot be used as a stem cell marker because it does not allow the selection of target cells without damaging or killing them. (An intracellular molecule can be used as a stem cell marker if donor animals are transgenic or donor cells are transfected with a marker gene (such as green fluorescent protein) and its expression is driven by the promoter of the molecule. *See* ref. 70.) More specifically, in the search of SSC markers, testis cells are first selected based on the expression of a particular cell-surface molecule and then transplanted into recipient testes. If transplantation of selected cells gives rise to more spermatogenic colonies than that of unselected cells, the molecule is identified as a positive marker for SSCs. Conversely, if selected cells show a reduced stem cell activity, the molecule is identified as a negative marker for SSCs.

Based on the fact that SSCs reside on the basement membrane, Shinohara et al. investigated the enrichment efficiency of adult mouse SSCs by panning testis cells using tissue culture dishes that were coated with extracellular matrix (71). Among the major components of seminiferous epithelium basement membrane, namely, laminin, collagen IV, and fibronectin, only the cells panned out using laminin showed a higher SSC activity (fourfold) than unselected cells, indicating that SSCs preferentially attach laminin in the basement membrane. Next, when

testis cells were selected using antibodies against integrin (Int)- β 1, a laminin receptor subunit, by magnet-assisted cell sorting, SSCs were enriched approximately fourfold (71). Another laminin receptor subunit, Int- α 6, was found to be more effective, allowing an eightfold enrichment for SSCs (71). Although c-kit (Kit) has been known to be a marker for PGCs, HSCs, and ES cells, it was not expressed on SSCs, confirming previous results that Kit is expressed in advanced spermatogonia (72).

An even higher SSC enrichment was achieved using fluorescent-activated cell sorting (FACS) technique (73). It should be noted, however, that SSCs cannot be enriched by FACS from *intact* adult testis cells, probably because FACS is so sensitive that its cell separation ability is obscured by a high noise level resulting from the rare presence of SSCs in highly heterogeneous testis cells (73). To circumvent this problem, adult testis cells were enriched for SSCs *in vivo* before FACS by suturing the testes of donor mice on the abdominal wall. A high body core temperature destroys heat-sensitive spermatogenesis, eliminating differentiating germ cells. Because 95% of testis cells are germ cells, this procedure (experimental cryptorchidism) results in an approximately 25-fold enrichment for SSCs (74). The cells derived from cryptorchid testes were found to be more amenable for FACS. When transplanted into recipient testes, the Int- α 6^{hi} Int- α v⁻ Side Scatter^{lo} (which reflects cell complexity) fraction of FACS-selected cells exhibited up to 7.2-fold higher stem cell activity than unselected cryptorchid testis cells (73). Therefore, this two-step enrichment strategy (cryptorchidism + FACS) resulted in a total of 166-fold enrichment of intact adult testis cells for SSCs [(23 by cryptorchidism) \times (7.2 by FACS)] (73).

Recent studies have identified more SSC markers and demonstrated a higher SSC enrichment efficiency. Kubota et al. have shown that SSCs do not express major histocompatibility complex (MHC)-I molecules but do express Thy-1, which is a known antigen for lymphocytes and HSCs (75). After cryptorchidism and FACS, SSCs can be enriched approximately 700-fold in the Thy-1⁺ MHC-I⁻ Kit⁻ fraction. The cells in the Thy-1⁺ MHC-I⁻ Kit⁻ fraction also show an antigenic phenotype of Int- α 6⁺ CD24⁺ Int- α v⁻ Sca-1⁻ CD34⁻. Kanatsu-Shinohara et al. have shown that SSCs also express CD9, which is associated with integrins and other cell-surface receptors (76). Taken together, five molecules each are known to be expressed on SSCs (positive markers) and not expressed on SSCs (negative markers; Table 1).

Male germ line stem cells can also be selected based on their morphology (77). Gonocytes (neonatal spermatogonia) derived from rat testes can readily be identified in a single cell suspension because of their large size. Some of the large cells have a round shape, but others have a more irregular shape with pseudopods. When rat gonocytes with or without pseudopods were individually picked up, pooled, and transplanted, stem cell activity was almost exclusively found in the

Table 1
Cell-Surface Marker Molecules for SSCs

<i>Positive markers^a</i>	<i>Negative markers^a</i>
Integrin $\alpha 6$	Integrin αv
Integrin $\beta 1$	MHC-I
Thy-1	Sca-1
CD24	CD34
CD9	c-Kit

^aPositive markers are the molecules expressed SSCs and negative markers are those not expressed by SSCs. (Based on refs. 71,74–76.)

pseudopod cell population, demonstrating that rat neonatal stem cells can be morphologically selected (77). However, this procedure is labor-intensive, and spermatogonia with pseudopods are not readily found in a single-cell suspension derived from adult rat testes or from mouse testes.

These studies show that the determination of SSC markers is in a rapid progress. Notably, however, even the highest enrichment of SSCs achieved to date (700-fold; ref. 75) does not allow the purification of stem cells. Because SSCs represent only 0.01% of total testis cell population (46), the 700-fold enrichment gives only 7% of stem cells in selected cells (700×0.01 , 1 of 14 testis cells). Therefore, further investigations are required to determine SSC markers and prospectively identify stem cells.

Among different types of stem cells, the highest enrichment has been achieved with HSCs. Mouse HSCs have been enriched up to 2000-fold in the Lineage⁻ Kit⁺ Sca-1⁺ Thy-1⁺ fraction of bone marrow cells (1). Because definitive HSCs represent 0.007% of bone marrow cells, 14% of selected marrow cells are stem cells (one of seven marrow cells). These studies of SSCs and HSCs raise three characteristics of stem cell markers. First, both SSCs and HSCs cannot be identified by the expression of a single molecule. In this context, it should be noted that none of the individual marker molecules are specific to stem cells. For instance, Thy-1 is also expressed in lymphocytes. Second, some, but not all, marker molecules are expressed in both stem cell populations (75). Third, although a high degree of enrichment for HSCs and SSCs is possible by a multiparameter cell selection, purification and definitive identification of these stem cells have not been achieved. In addition, another difficulty in the determination of stem cell markers is that they could be species-specific. For example, HSC markers are different in mice and humans (3). Thus, although SSC markers have been investigated only in mice, the markers determined in this species could be inappropriate to select/identify SSCs in another.

It has been an intensive focus of investigations for some time whether or not a set of molecules are expressed by all types of stem cells. Such molecules that could define “stemness” have been investigated using the DNA microarray technology (78–80). Two studies explored the stemness genes expressed commonly in HSCs, ES cells, and neural stem cells and identified approximately 300 genes that were expressed in the three stem cell populations (78,79). However, although these studies used virtually identical cell populations for microarray analyses, only six genes were commonly detected in both studies (1.2% overlap, ref. 81). A similar study using microarrays identified nearly 400 genes shared by ES cells, neural stem cells, and retinal stem cells (80). The comparison of these data demonstrated that there was only one gene detected in all the three studies (thus in HSCs, ES cells, neural stem cells, and retinal stem cells), namely, integrin- α 6 (80). Although this molecule is also expressed in SSCs (*see* Table 1), it can be found in a number of nonstem cells and its function on stem cell populations is unknown.

Several causes of this discrepancy can be raised. Technical difficulties could be one of them. As described previously, no stem cells have been purified. Thus the power of microarray technology could be clouded by the impurity of cell sources (82,83). In addition, it is possible that stemness genes are differentially expressed depending on *in vivo* status of the cells (e.g., cell cycle, development) or differentially affected by experimental procedures (e.g., physical stresses during cell separation or culture, exposure to enzymes and growth factors, temperature). It is also possible that stemness genes are not present on microarrays currently available (82,83). On the other hand, the simplest explanation could be that there are no universal stemness genes. Furthermore, even if such genes exist, they may not be regulated at the transcription level. The microarray-based studies of stem cell gene profiling collectively suggest at least that the stemness genes may not be expressed at a high level, which is detectable and distinguishable in microarray analyses (82,83).

9. SSCs, MALE MUTATION BIAS, AND GENETIC DISEASES

SSCs are the foundation for the life-long production of sperm that transmit genes to the next generation. Therefore, any abnormality that occurs in the SSC genome can result in germ line mutations and cause inherited diseases in offspring. In this regard, recent clinical studies, including those of endocrine cancers, have shown that a strong sex-dependent bias exists in germ line mutations (84), which, as described in the following sections, suggests the involvement of the regulation of stem cell fate decision in pathogenesis of inherited diseases.

It has been described that point mutations (base substitutions) occur primarily in the male germ line and are age-dependent, whereas small chromosomal

changes (mainly intragenic deletions) occur more frequently in the female germ line and are not age-dependent (84). A particularly strong male mutation bias has been demonstrated in point mutations of FGF receptor 2 (FGFR2), FGFR3, and Ret, which have been suggested to occur in spermatogonia and possibly in SSCs (85–88).

Point mutations in FGFR1–3 are the cause of Apert, Crouzon, and Pfeiffer syndromes (86–89). Apert syndrome (achondroplasia) is associated with short-limbed dwarfism, Crouzon syndrome with craniosynostosis and dysmorphic facial features, and Pfeiffer syndrome with premature fusion and deformity of the sutures of the skull. Ret is a signal-transducing component of GDNF receptor. Point mutations in Ret cause three familial cancer syndromes (90–92): multiple endocrine neoplasia 2A (MEN2), which is associated with medullary thyroid carcinoma, pheochromocytoma, and parathyroid hyperplasia; MEN2B, medullary thyroid carcinoma associated with pheochromocytoma, enteric ganglioneuroma, and skeletal and ocular abnormalities; and familial medullary thyroid carcinoma. In all of these cases, point mutations that occur in corresponding genes (i.e., FGFRs and Ret) are gain-of-function mutations (84,85). Furthermore, virtually all cases of these diseases are of paternal origin. In other words, mutations occur nearly exclusively in the male germ line and are transmitted to offspring through sperm (84,85).

The incidence of these diseases has long been known to increase with paternal age (84,85). The widely accepted cause of this age-dependent male mutation bias was the difference in the number of chromosomal replications that take place during male and female gametogenesis in humans. In the female, there are a total of 23 chromosomal replications regardless of age. In the male, the number of total chromosomal replications increases with age: 161 at age 20 and 610 at age 40 (84). Thus it was believed that the male germ line has a greater chance for mutations.

However, an increase in mutation rate was not sufficient to explain the near-exclusive paternal origin of the diseases (86–88). Only a slight increase in mutant sperm with paternal age was observed, which was much less than predicted by the clinical data (84). In a recent study of Apert syndrome (caused by point mutations in FGFR2), Goriely et al. have suggested that the FGFR2 mutations are enriched with age because spermatogonia/SSCs with the mutant FGFR2 have a selective advantage that leads to their clonal expansion over time, in much the same way as the clonal expansion of some tumors (86). Furthermore, a similar observation has also been reported with FGFR3 (87), and it has been proposed that this may also be true for mutations in Ret, a GDNF receptor (84,85).

It is intriguing to compare the observations made in these genetic diseases with those made in animal studies described earlier. Primitive spermatogonia accumulate in the testes of mutant mice that overexpress GDNF, which should

result in the activation of its receptor (54,61). In addition, GDNF augments the maintenance of SSCs *in vitro* (59), and the combination of GDNF and bFGF highly stimulates the proliferation of cultured SSCs (63). Based on the results of clinical and animal studies, therefore, it is likely that the stimulation of signaling pathways involving FGF and GDNF confers to spermatogonia/SSCs the selective advantage that results in their accumulation. This would result in a clonal expansion of mutant SSCs or their descendants over time, leading to age-dependent male mutation bias observed in human genetic diseases. Further investigations into the roles of FGFs and GDNF on the actions of SSCs and spermatogonia may be important to better understand the origin of male mutation bias and the mechanism of SSC fate decision.

10. SUMMARY

The development of spermatogonial transplantation in 1994 allowed, for the first time, investigations into SSCs based on their function. Since then, studies of SSCs have progressed rapidly and these cells can even be used for transgenesis (93,94). However, some critical SSC properties still remain to be addressed and will be an important focus for future studies. Further investigations into the SSC identification markers are essential to better understand the biology of SSCs and to carry out prospective studies of SSCs. The mechanism of SSC fate decision is another important issue in SSC biology. The fate of SSCs should be regulated by the intrinsic potential of SSCs and by the extrinsic stimuli exerted by their niches. Investigations into the function of SSC niches could be facilitated by *in vitro* experiments, whereas those into the intrinsic potential of SSCs may require the determination of SSC markers or genetic manipulations of SSCs. Recent progress in clinical studies of sex-dependent mutation bias has provided important insights into the SSC biology. Because SSCs may contribute to the pathogenesis of paternally transmitted genetic diseases, our ability to understand the biology of SSCs will be critical for the development of potential therapeutic strategies for those diseases.

ACKNOWLEDGMENTS

The author thanks Kyle Orwig and Frances Clerk for their critical reading of this manuscript. The studies conducted at author's laboratory were supported by the Canadian Institutes of Health Research (MOP49444) and the Canada Foundation for Innovation (4177).

REFERENCES

1. Lagasse E, Shizuru JA, Uchida N, Tsukamoto A, Weissman IL Toward regenerative medicine. *Immunity* 2001;14:425–436.

2. Loeffler M, Potten CS. Stem cells and cellular pedigrees—a conceptual introduction. In: Potten CS, ed. *Stem Cells*. San Diego, Academic Press, 1997, pp. 1–27.
3. Weissman IL. Translating stem and progenitor cell biology to the clinic: barriers and opportunities. *Science* 2000;287:1442–1446.
4. Rudnicki MA. Marrow to muscle, fission versus fusion. *Nature Med* 2003;12:1461–1462.
5. Nagano MC. A surgical strategy using spermatogonial stem cells for restoring male fertility. In: Gosden RG, Tulandi T, eds. *Preservation of Fertility*. Lancaster, UK, The Parthenon Publishing Group, 2004, pp. 125–139.
6. Meistrich ML, Van Beek MEAB Spermatogonial stem cells. In: Desjardins C, Ewing LL, eds. *Cell and Molecular Biology of the Testis*. New York, Oxford University Press, 1993, pp. 266–295.
7. Kiger AA, Fuller MT. Male germ-line stem cells. In: Marshak DR, Gardner RL, Gottlieb D, eds. *Stem Cell Biology*. Cold Spring Harbor, Cold Spring Harbor Laboratory Press, 2001, pp. 149–187.
8. Hogan B. Primordial germ cells as stem cells. In: Marshak DR, Gardner RL, Gottlieb D, eds. *Stem Cell Biology*. Cold Spring Harbor, Cold Spring Harbor Laboratory Press, 2001, pp. 189–204.
9. Lawson KA, Hage WJ. Clonal analysis of the origin of primordial germ cells in the mouse. *Ciba Found Symp* 1994;182:68–91.
10. Tam PPL, Snow MHL. Proliferation and migration of primordial germ cells during compensatory growth in the mouse embryo. *J Embryol Exp Morph* 1981;64:133–147.
11. Russell LD, Ertlin RA, Shih H, Clegg ED, eds. *Mammalian spermatogenesis*. In: *Histological and Histopathological Evaluation of the Testis*. Clearwater, FL, Cache River Press, 1990, pp. 1–40.
12. Bellve AR, Cavicchia JC, Millette CF, O'Brien DA, Bhatnagar YM, Dym M. Spermatogenic cells of the prepubertal mouse. Isolation and morphological characterization. *J Cell Biol* 1977;74:1:68–85.
13. McCarrey JR. Development of the germ cell. In: Desjardins C, Ewing LL, eds. *Cell and Molecular Biology of the Testis*. New York, Oxford University Press, 1993, pp. 58–89.
14. Lawson KA, Dunn NR, Roelen BA, et al. Bmp4 is required for the generation of primordial germ cells in the mouse embryo. *Genes Dev* 1999;13:424–436.
15. Saitou M, Barton SC, Surani MA. A molecular programme for the specification of germ cell fate in mice. *Nature* 2002;418:293–300.
16. Godin I, Wylie CC. TGF beta 1 inhibits proliferation and has a chemotropic effect on mouse primordial germ cells in culture. *Development* 1991;113:1451–1457.
17. Zhao G-Q, Garbers DL. Male Germ cell specification and differentiation. *Dev Cell* 2002;2:537–547.
18. Brinster RL. Germline stem cell transplantation and transgenesis. *Science* 2002;296:2174–2176.
19. Illmensee K, Stevens LC. Teratomas and chimeras. *Sci Am* 1979;240:120–133.
20. Stevens LC. Origin of testicular teratomas from primordial germ cells in mice. *J Natl Cancer Inst* 1967;38:549–552.
21. Pierce GB. Teratocarcinoma: model for a developmental concept of cancer. *Curr Top Dev Biol* 1967;2:223–246.
22. Brinster RL. The effect of cells transferred into the mouse blastocyst on subsequent development. *J Exp Med* 1974;104:1049–1056.
23. Matsui JY, Zsebo K, Hogan BLM. Derivation of pluripotential embryonic stem cells from murine primordial germ cells in culture. *Cell* 1992;70:841–847.
24. Resnick JL, Bixler LS, Cheng L, Donovan PJ. Long-term proliferation of mouse primordial germ cells in culture. *Nature* 1992;359:550–551.

25. Shim H, Gutierrez-Adan A, Chen LR, BonDurant RH, Behboodi E, Anderson GB. Isolation of pluripotent stem cells from cultured porcine primordial germ cells. *Biol Reprod* 1997;57:1089–1095.
26. Shamblott MJ, Axelman J, Wang S, et al. Derivation of pluripotent stem cells from cultured human primordial germ cells. *Proc Natl Acad Sci USA* 1998;95:13726–13731.
27. Stewart CL, Gadi I, Bhatt H. Stem cells from primordial germ cells can reenter the germ line. *Dev Biol* 1994;161:626–628.
28. Labosky PA, Barlow DP, Hogan BL. Mouse embryonic germ (EG) cell lines: transmission through the germline and differences in the methylation imprint of insulin-like growth factor 2 receptor (Igf2r) gene compared with embryonic stem (ES) cell lines. *Development* 1994;120:3197–3204.
29. Smith A 2001 Embryonic stem cells. In: Marshak DR, Gardner RL, Gottlieb D, eds. *Stem Cell Biology*. Cold Spring Harbor, Cold Spring Harbor Laboratory Press, 2001, pp. 205–230.
30. Brook FA, Gardner RL. The origin and efficient derivation of embryonic stem cells in the mouse. *Proc Natl Acad Sci USA* 1997;94:5709–5712.
31. Tsunoda Y, Tokunaga T, Imai H, Uchida T. Nuclear transplantation of male primordial germ cells in the mouse. *Development* 1989;107:407–411.
32. Yamazaki, Y, Mann RW, Lee SS, et al. 2003 Reprogramming of primordial germ cells begins before migration into the genital ridge, making these cells inadequate donors for reproductive cloning. *Proc Natl Acad Sci USA* 2003;100:12207–12212.
33. Wakayama T, Yanagimachi R. Mouse cloning with nucleus donor cells of different age and type. *Mol Reprod Dev* 2001;58:376–383.
34. Constancia M, Pickard B, Kelsey G, Reik W. Imprinting mechanisms. *Genome Res* 1998;8:881–900.
35. Monk M. Epigenetic programming of differential gene expression in development and evolution. *Dev Genet* 1995;17:188–197.
36. Gage FH. Mammalian neural stem cells. *Science* 2000;287:1433–1438.
37. Brinster RL, Zimmermann JW. Spermatogenesis following male germ-cell transplantation. *Proc Natl Acad Sci USA* 1994;91:11298–11302.
38. Brinster RL, Avarbock MR. Germline transmission of donor haplotype following spermatogonial transplantation. *Proc Natl Acad Sci USA* 1994;91:11303–11307.
39. Ogawa T, Arechaga JM, Avarbock MR, Brinster RL. Transplantation of testis germinal cells into mouse seminiferous tubules. *Int J Dev Biol* 1997;41:111–122.
40. Nagano MC. Spermatogonial transplantation. In: Gardner DK, Lane M, Watson A, eds. *A Laboratory Guide to the Mammalian Embryo*. Oxford, UK, Oxford University Press, 2004, pp. 334–351.
41. Ryu BY, Orwig KE, Avarbock MR, Brinster RL. Stem cell and niche development in the postnatal rat testis. *Dev Biol* 2003;263:253–263.
42. Johnston DS, Russell LD, Friel PJ, Griswold MD. Murine germ cells do not require functional androgen receptors to complete spermatogenesis following spermatogonial stem cell transplantation. *Endocrinology* 2001;142:2405–2408.
43. Mahato D, Goulding EH, Korach KS, Eddy EM. Spermatogenic cells do not require estrogen receptor- α for development or function. *Endocrinology* 2000;141:1273–1276.
44. Zhang X, Ebata KT, Nagano MC. Genetic analysis of the clonal origin of regenerating mouse spermatogenesis following transplantation. *Biol Reprod* 2003;69:1872–1878.
45. Dobrinski I, Ogawa T, Avarbock MR, Brinster RL. Computer assisted image analysis to assess colonization of recipient seminiferous tubules by spermatogonial stem cell from transgenic donor mice. *Mol Reprod Dev* 1999;53:142–148.

46. Nagano MC. Homing efficiency and proliferation kinetics of male germ line stem cells following transplantation in mice. *Biol Reprod* 2003;69:701–707.
47. Shinohara T, Orwig KE, Avarbock MR, Brinster RL. Remodeling of the postnatal mouse testis is accompanied by dramatic changes in stem cell number and niche accessibility. *Proc Natl Acad Sci USA* 2001;98:6186–6191.
48. Orwig KE, Shinohara T, Avarbock MR, Brinster RL. Functional analysis of stem cells in the adult rat testis. *Biol Reprod* 2002;66:944–949.
49. 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.
50. Franca LR, Ogawa T, Avarbock MR, Brinster RL, Russell LD. Germ cell genotype controls cell cycle during spermatogenesis in the rat. *Biol Reprod* 1998;59:1371–1377.
51. Ogawa T, Dobrinski I, Avarbock MR, Brinster RL. Transplantation of male germ line stem cells restores fertility in infertile mice. *Nat Med* 2000;6:29–34.
52. de Rooij DG, Okabe M, Nishimune Y. Arrest of spermatogonial differentiation in *jsd/jsd*, *Sl17H/Sl17H*, and *cryptorchid* mice. *Biol Reprod* 1999;61:842–847.
53. Boettger-Tong HL, Johnston DS, Russell LD, Griswold MD, Bishop CE. Juvenile spermatogonial depletion (*jsd*) mutant seminiferous tubules are capable of supporting transplanted spermatogenesis. *Biol Reprod* 2000;63:1185–1191.
54. Meng X, Lindahl M, Hyvonen ME, et al. Regulation of cell fate decision of undifferentiated spermatogonia by GDNF. *Science* 2000;287:1489–1493.
55. Furuchi T, Masuko K, Nishimune Y, Obinata M, Matsui Y. Inhibition of testicular germ cell apoptosis and differentiation in mice misexpressing Bcl-2 in spermatogonia. *Development* 1996;122:1703–1709.
56. Matzuk MM, Lamb DJ. Genetic dissection of mammalian fertility pathways. *Nat Cell Biol* 2002;4(Suppl.):s41–s49.
57. Nagano M, Shinohara T, Avarbock MR, Brinster RL. Retrovirus-mediated gene delivery into male germ line stem cells. *FEBS Lett* 2000;475:7–10.
58. Nagano M, Watson DJ, Ryu BY, Wolfe JH, Brinster RL. Lentiviral vector transduction of male germ line stem cells in mice. *FEBS Lett* 2002;524:111–115.
59. Nagano M, Ryu BY, Brinster CJ, Avarbock MR, Brinster RL. Maintenance of mouse male germ line stem cells in vitro. *Biol Reprod* 2003;68:2207–2214.
60. Mather JP, Attie KM, Woodruff TK, Rice GC, Phillips DM. Activin stimulates spermatogonial proliferation in germ-Sertoli cell cocultures from immature rat testis. *Endocrinology* 1990;127:3206–3214.
61. Yomogida K, Yagura Y, Tadokoro Y, Nishimune Y. Dramatic expansion of germinal stem cells by ectopically expressed human glial cell line-derived neurotrophic factor in mouse Sertoli cells. *Biol Reprod* 2003;69:1303–1307.
62. Zhang J, Niu C, Ye L, et al. Identification of the haematopoietic stem cell niche and control of the niche size. *Nature* 2003;425:836–841.
63. Kanatsu-Shinohara M, Ogonuki N, Inoue K, et al. Long-term proliferation in culture and germline transmission of mouse male germline stem cells. *Biol Reprod* 2003;69:612–616.
64. Pawliuk R, Eaves C, Humphries RK. Evidence of both ontogeny and transplant dose-regulated expansion of hematopoietic stem cells in vivo. *Blood* 1996;88:2852–2858.
65. Iscove NN, Nawa K. Hematopoietic stem cells expand during serial transplantation in vivo without apparent exhaustion. *Curr Biol* 1997;7:805–808.
66. Watt FM, Hogan BLM. Out of Eden: stem cells and their niches. *Science* 2000;287:1427–1430.
67. Calvi LM, Adams GB, Weibrecht KW, et al. Osteoblastic cells regulate the haematopoietic stem cell niche. *Nature* 2003;425:841–846.

68. Ohta H, Yomogida K, Dohmae K, Nishimune Y. Regulation of proliferation and differentiation in spermatogonial stem cells: the role of c-kit and its ligand SCF. *Development* 2000;127:2125–2131.
69. Vidal F, Lopez P, Lopez-Fernandez LA, et al. Gene trap analysis of germ cell signaling to Sertoli cells: NGF-TrkA mediated induction of Fra1 and Fos by post-meiotic germ cells. *J Cell Sci* 2001;114:435–443.
70. Giuli G, Tomljenovic A, Labrecque N, Oulad-Abdelghani M, Rassoulzadegan M, Cuzin F. Murine spermatogonial stem cells: targeted transgene expression and purification in an active state. *EMBO Rep* 2002;3:753–759.
71. Shinohara T, Avarbock MR, Brinster RL. β 1- and α 6-integrin are surface markers on mouse spermatogonial stem cells. *Proc Natl Acad Sci USA* 1999;96:5504–5509.
72. Yoshinaga K, Nishikawa S, Ogawa M, et al. Role of c-kit in mouse spermatogenesis: identification of spermatogonia as a specific site of c-kit expression and function. *Development* 1991;113:689–699.
73. 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.
74. Shinohara T, Avarbock MR, Brinster RL. Functional analysis of spermatogonial stem cells in Steel and cryptorchid infertile mouse models. *Dev Biol* 2000;220:401–411.
75. 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.
76. Kanatsu-Shinohara M, Toyokuni S, Shinohara T. CD9 is a surface marker on mouse and rat male germline stem cells. *Biol Reprod* 2004;70:70–75.
77. Orwig KE, Ryu BY, Avarbock MR, Brinster RL. Male germ-line stem cell potential is predicted by morphology of cells in neonatal rat testes. *Proc Natl Acad Sci USA* 2002;99:11706–11711.
78. Ramalho-Santos M, Yoon S, Matsuzaki Y, Mulligan RC, Melton DA. “Stemness:” transcriptional profiling of embryonic and adult stem cells. *Science* 2000;298: 97–600.
79. Ivanova NB, Dimos JT, Schaniel C, Hackney JA, Moore KA, Lemischka IR. A stem cell molecular signature. *Science* 2002;298:601–604.
80. Fortunel NO, Otu HH, Ng HH, et al. Comment on “ ‘Stemness’: transcriptional profiling of embryonic and adult stem cells” and “a stem cell molecular signature.” *Science* 2003;302:393.
81. Evsikov AV, Solter D. Comment on “ ‘Stemness’: transcriptional profiling of embryonic and adult stem cells” and “a stem cell molecular signature.” *Science* 2003;302:393.
82. Vogel G. ‘Stemness’ genes still elusive. *Science* 2003;302:371.
83. Ivanova NB, Dimos JT, Schaniel C, et al. Response to comments on “ ‘stemness’: transcriptional profiling of embryonic and adult stem cells” and “a stem cell molecular signature” *Science* 2003;302:393.
84. Crow JF. The origins, patterns and implications of human spontaneous mutation. *Nat Rev Genet* 2000;1:40–47.
85. Crow JF. There’s something curious about paternal-age effects. *Science* 2003;301:606–607.
86. 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.
87. Tiemann-Boege I, Navidi W, Grewal R, et al. The observed human sperm mutation frequency cannot explain the achondroplasia paternal age effect. *Proc Natl Acad Sci USA* 2002;99: 14952–14957.
88. Oldridge M, Lunt PW, Zackai EH, et al. Genotype-phenotype correlation for nucleotide substitutions in the IgII-IgIII linker of FGFR2. *Hum Mol Genet* 1997;6:137–143.

89. Vajo Z, Francomano CA, Wilkin DJ. The molecular and genetic basis of fibroblast growth factor receptor 3 disorders: the achondroplasia family of skeletal dysplasias, Muenke cranio-synostosis, and Crouzon syndrome with acanthosis nigricans. *Endocr Rev* 2000;21:23–39.
90. Santoro M, Carlomagno F, Romano A, et al. Activation of RET as a dominant transforming gene by germline mutations of MEN2A and MEN2B. *Science* 1995;267:381–383.
91. Santoro M, Melillo RM, Carlomagno F, Fusco A, Vecchio G. Molecular mechanisms of RET activation in human cancer. *Ann N Y Acad Sci* 2002;963:116–121.
92. Takahashi M. The GDNF/RET signaling pathway and human diseases. *Cytokine Growth Factor Rev* 2001;12:361–373.
93. 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* 2001;98:13090–13095.
94. 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.
95. Donovan PJ. Growth factor regulation of mouse primordial germ cell development. *Curr Top Dev Biol* 1994;19:189–225.
96. Nagano M, Avarbock MR, Brinster RL. Pattern and kinetics of mouse donor spermatogonial stem cell colonization in recipient testes. *Biol Reprod* 1999;60:1429–1436.



<http://www.springer.com/978-1-58829-407-4>

Stem Cells in Endocrinology

Lester, L.B. (Ed.)

2005, XIV, 274 p., Hardcover

ISBN: 978-1-58829-407-4

A product of Humana Press