Chapter 12
Heterogeneity of Spermatogonial Stem Cells

Hiroshi Kubota

Abstract Germ cells transfer genetic materials from one generation to the next, which ensures the continuation of the species. Spermatogenesis, the process of male germ cell production, is one of the most productive systems in adult tissues. This high productivity depends on the well-coordinated differentiation cascade in spermatogonia, occurring via their synchronized cell division and proliferation. Spermatogonial stem cells (SSCs) are responsible for maintaining the spermatogonial population via self-renewal and the continuous generation of committed progenitor cells that differentiate into spermatozoa. Like other stem cells in the body, SSCs are defined by their self-renewal and differentiation abilities. A functional transplantation assay, in which these biological properties of SSCs can be quantitatively evaluated, was developed using mice, and the cell surface characteristics and intracellular marker gene expression of murine SSCs were successfully determined. Another approach to elucidate SSC identity is a cell lineage-tracing experiment using transgenic mice, which can track the SSC behavior in the testes. Recent studies using both these experimental approaches have revealed that the SSC identity changed depending upon the developmental, homeostatic, and regenerative circumstances. In addition, single-cell transcriptomic analyses have further indicated the instability of marker gene expression in SSCs. More studies are needed to unify the results of the determination of SSC identity based on the functional properties and accumulating transcriptomic data of SSCs, to elucidate the functional interaction between SSC behavior and gene products and illustrate the conserved features of SSCs amidst their heterogeneity. Furthermore, the deterministic roles of distinct SSC niches under different physiological conditions in the SSC heterogeneity and its causal regulators must also be clarified in future studies.

Keywords Spermatogonial stem cells · Germline stem cells · Functional assay · Stem cell transplantation · Cell lineage-tracing · Genetic-labeling · Stem cell

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identity · Stem cell markers · Stem cell niche · Self-renewal · Differentiation · Regeneration · Spermatogonia · Spermatogenesis · Testis

Introduction

Spermatogenesis occurs in the seminiferous tubules in the testes, and it is presumed to be one of the most productive cell-renewing systems in the adult tissues. In males, millions of spermatozoa are produced daily since the onset of puberty until old age [1]. The high productivity of spermatogenesis is conserved among most animals; therefore, this process is advantageous for the continuation of the species. This high productivity depends on the well-coordinated differentiation cascade in spermatogonia, which occurs via their synchronized cell division and proliferation. Because one spermatocyte produces only four spermatids by reductive cell division, a continuous supply of large numbers of spermatocytes requires at least a quarter of the population of spermatogonia, which are the precursors of spermatocytes and represent mitotic cell populations in spermatogenesis [2].

In the testes, the basic structure of seminiferous tubules is formed by the epithelia of Sertoli cells and the surrounding peritubular myoid cells (Fig. 12.1). The tight junctions of Sertoli cells form the blood-testis barrier and separate the basal and adluminal compartments of the seminiferous tubules. The basal compartment contains spermatogonia, which are located on the basement membrane, and preleptotene spermatocytes. The adluminal compartment contains pachytene spermatocytes and subsequent haploid germ cells, including spermatids and spermatozoa, which are never exposed to the blood and lymph constituents. This is important to keep them separate from the immune system in order to avoid unwanted immune reactions against the haploid germ cells after meiotic recombination [3].

Spermatogonial stem cells (SSCs) are responsible for maintaining the spermatogonial population during reproductive life via self-renewal and the generation of daughter cells that commit to differentiation [4, 5]. Among various mammalian species, mouse spermatogenesis is the most intensely investigated, and thus, mice are the most established model for studying spermatogenesis in several mammals [6]. Although spermatogenesis in non-primate mammals is similar to mouse spermatogenesis, primate spermatogenesis, including that in humans, displays notable differences in the classification of spermatogonial cell populations and their differentiation process, compared to those in non-primates [7]. The biology of human SSCs is tremendously important in order to understand the unique human reproductive system and to develop new therapies for male infertility; however, the identity of human SSCs has not yet been unequivocally determined and still raises marked controversy among researchers [8]. Currently, our knowledge about mammalian SSCs has stemmed mainly from mouse studies. In addition, several critical experimental methods and approaches to elucidate stem cell behavior are only available in mouse-based systems. Therefore, this review focuses on mouse SSCs and discusses their heterogeneity.

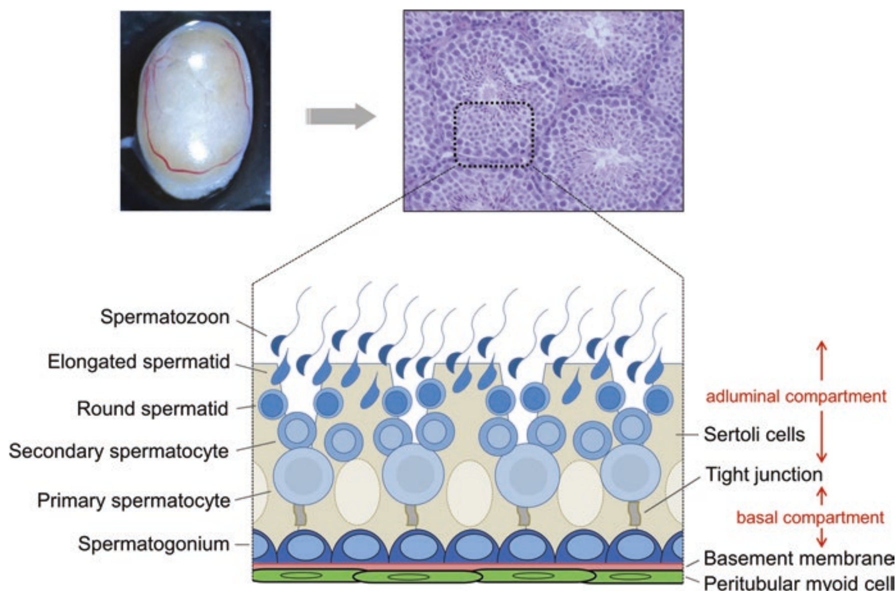

Fig. 12.1 Schematic representation of mouse seminiferous tubules. (Upper left) Adult mouse testis. (Upper right) Cross-section of adult mouse testis stained with hematoxylin–eosin. (Bottom) Schematic magnified view of the indicated square region of a seminiferous tubule in the histological section. The seminiferous tubules consist of the epithelia of Sertoli cells, the surrounding peritubular myoid cells, and the germ cells at various stages of their development. The tight junctions between Sertoli cells form the blood-testis barrier separate the basal and adluminal compartments of the seminiferous tubules. The basal compartment contains spermatogonia, which are located on the basement membrane, and early primary spermatocytes (preleptotene spermatocytes), which are not shown in this diagram. The adluminal compartment contains late primary spermatocytes (pachytene spermatocytes–), secondary spermatocytes, spermatids, and spermatozoa

Classical Spermatogonial Stem Cell Definition Based on Morphology

Spermatogonia are located in the basal compartment and on the basement membrane in the periphery of the seminiferous tubules (Fig. 12.1). They are classified into several subpopulations. At first, they were subdivided into type A and type B. Type A spermatogonia present no heterochromatin in the nuclei, whereas type B spermatogonia display heterochromatin. In mice, subsequently, spermatogonia of an intermediate (In) type were found; the nuclei of In spermatogonia contain a moderate amount of heterochromatin [2]. Type A spermatogonia form the initial population of cells that undergo spermatogenesis, followed by In and type B spermatogonia, which give rise to spermatocytes (Fig. 12.2). Type A spermatogonia are further subdivided into undifferentiated, A1, A2, A3, and A4 spermatogonia (Fig. 12.2). The six types of spermatogonia from A1 to type B are generated by one cell division

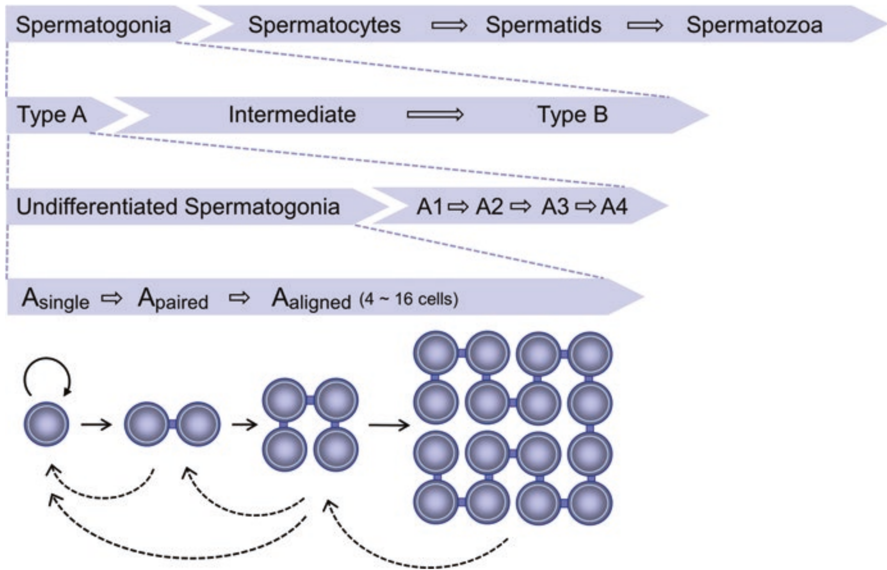

Fig. 12.2 Scheme of male germ cell lineage in mice. Murine spermatogonia are classified into type A, intermediate type (In), and type B spermatogonia. Type A spermatogonia are further subdivided into undifferentiated, A1, A2, A3, and A4 spermatogonia. Undifferentiated spermatogonia, in turn, are further subdivided to A_s, A_{pr}, and A_{al}. A_{al} have 2^n (n = 2–4, rarely 5) cells due to their incomplete cytokinesis. The A_s model assumes that only the A_s undifferentiated spermatogonia are SSCs that can self-renew (circle arrow). The dotted arrows indicate fragmentation from longer A_{al} to shorter A_{al}, A_{pr}, or A_s. This suggests that all undifferentiated spermatogonia have stem-cell potential. Refer to the text for details

each [9], and a single type B spermatogonium divides into two preleptotene spermatocytes. Thus, one A1 spermatogonium can generate 64 preleptotene spermatocytes, which eventually produce 256 spermatozoa. In each cell division, the two daughter cells do not separate completely and are connected by an intercellular bridge [10]. Because subsequent cell divisions are synchronized, their differentiation process proceeds in a well-coordinated manner.

The most immature type A spermatogonia are named undifferentiated spermatogonia, which are further subdivided into A_{single} (A_s), A_{paired} (A_{pr}), and $A_{aligned}$ (A_{al}), based on their morphological characteristics (Fig. 12.2). An A_s cell represents a single or an isolated undifferentiated spermatogonium, while A_{pr} cells are two interconnected undifferentiated spermatogonia with an intercellular bridge. $A_{al\text{-}4}$, $A_{al\text{-}8}$, $A_{al\text{-}16}$, and the rare $A_{al\text{-}32}$ comprise 4, 8, 16, and 32 undifferentiated spermatogonia, respectively, with intercellular bridges. These A_{al} spermatogonia differentiate into A1 spermatogonia without cell division; thus, theoretically, a single A_s spermatogonium can generate a maximum of 4096 or 8192 spermatozoa. However, because a significant number of germ cells undergo apoptosis during differentiation [11], this high yield of spermatozoa does not occur naturally.

The morphological analyses, in combination with cell kinetic studies via radioisotope labeling, whole mount analysis, and differentiation-arrest models, including models fed with vitamin A-deficient diets and those with cryptorchid testes, support the hypothesis that A_s spermatogonia are SSCs (A_s model) [4, 9]. When A_s spermatogonia divide, they have two options: one cell can either generate two A_s spermatogonia or two A_{pr} spermatogonia. The former represents cell division for self-renewal, and the latter represents differentiation, which results in the generation of $A_{al\text{-}4}$, $A_{al\text{-}8}$, $A_{al\text{-}16}$, and $A_{al\text{-}32}$ following subsequent synchronized cell divisions. In adult testes, the number of SSCs is consistent; therefore, after a self-renewal cell division, only one A_s is maintained as an A_s spermatogonium, whereas the other undergoes differentiation and generates A_{pr} by the subsequent cell division. In contrast, during the developmental phase after birth or regeneration phases after injury such as irradiation or chemotherapy, A_s spermatogonia in the testes must increase in number and repeat self-renewal cell divisions. In the A_s model, the generation of A_{pr} is the first step of differentiation. Furthermore, the model suggests that A_s cells are assumed to only be derived from self-renewing cell divisions of A_s cells; therefore, A_s cells are the most primitive spermatogonia and have been exclusively considered as SSCs in the testes [12, 13]. It is not clear, however, whether all A_s have the ability to maintain and regenerate long-term spermatogenesis and whether non-A_s subpopulations such as A_{pr}, A_{al}, or other spermatogonia have such a potential or occasionally behave as SSCs. Although the A_s model assumes that both A_{pr} and A_{al} are irreversibly committed for differentiation, recent reports have observed fragmentation from long A_{al} to short A_{al}, A_{pr}, and A_s, which accompanied dedifferentiation [14, 15]. The differentiation order of mouse spermatogonia is indicated in Fig. 12.2.

Spermatogonial Stem Cell Definition Based on Functional Transplantation

Stem cells are defined by their biological activity [16]; however, until 1994, a method to assess the stem cell activity was only available for murine hematopoietic stem cells. When testicular germ cells were introduced into germ cell-depleted seminiferous tubules, a particular cell population migrated and colonized the basement membrane and regenerated, resulting in donor-derived spermatogenesis [17] (Fig. 12.3). The reconstituted spermatogenesis continued throughout the lifespan of the host and successfully generated a progeny upon mating with females [18]. One spermatogenic colony in the recipient testes could be generated from a single colonized cell [19, 20]. These results demonstrated that the spermatogenic colony-forming cells had the ability to self-renew and differentiate into functional gametes, and the transplantation system served as a quantitative functional assay for SSCs [8]. This SSC transplantation system, in which both the self-renewal and differentiation activities of SSCs can be evaluated, is the second functional assay for stem cells, the first transplantation assay being bone marrow transplantation into

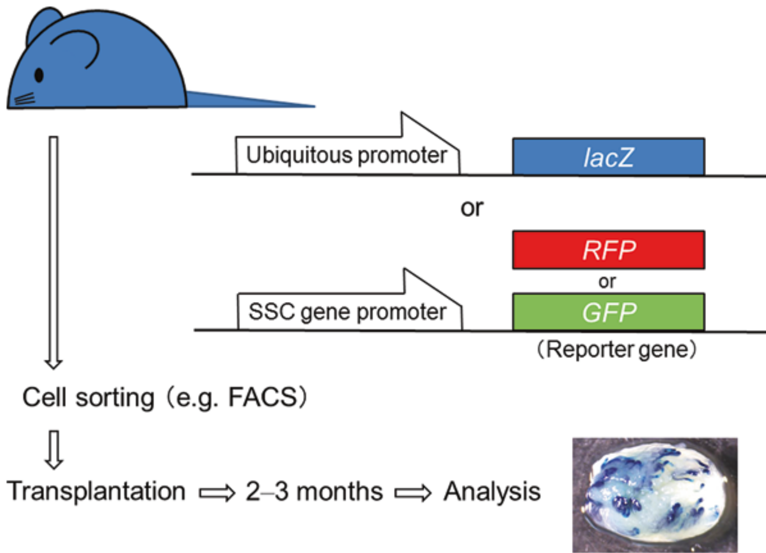

Fig. 12.3 Schematic representation of the transplantation assay for SSCs to determine the SSC identity. To explore the antigenic profile of SSCs, donor cells are prepared from transgenic mice that constitutively express a reporter gene (e.g., *lacZ* encoding β-galactosidase) under the control of a ubiquitous promoter (e.g., *Rosa26* locus), isolated into their candidate fractions by FACS or MACS, and transplanted into the seminiferous tubules of infertile recipient mice. About 2–3 months after transplantation, the recipient testes were analyzed to identify donor-derived spermatogenic colonies (β-galactosidase-expressing colonies can be identified as blue colonies by staining with X-gal, 5-bromo-4-chloro-3-indoyl-β-D-galactopyranoside). To explore the intracellular molecules expressed in SSCs, donor cells are prepared from transgenic mice that express a fluorescent reporter gene (e.g., GFP or RFP) under the control of an endogenous gene promoter, which possibly drives SSC-specific gene expression (SSC gene promoter). The fluorescent signal-positive cells are isolated by FACS, followed by the transplantation assay. About 2–3 months after transplantation, the recipient testes are analyzed to identify donor-derived fluorescent spermatogenic colonies (not shown in this figure)

recipients with hematopoietic destruction, for hematopoietic stem cells, which was established in 1961 [21].

Using the transplantation assay for SSCs in combination with fluorescence-activated cell sorting (FACS), the antigenic profile of SSCs, which is a phenotypic identity of SSCs, can be determined (Fig. 12.3). A series of studies using antibodies against various cell surface molecules have identified the ITGA6, ITGB1, THY1, EPCAM, MCAM, CD9, CD24, CDH1, and GFRA1 expressed on SSCs [22–28]. Because these molecules are also expressed on non-stem spermatogonia, no SSC-specific surface molecules have been identified. During the course of studies, to determine the surface phenotype of SSCs, negative results (no expression) have been found to be important. For example, SSC activity was concentrated in the major histocompatibility complex-1 (MHC-1)$^{-}$- and KIT^{-}-cell fractions [25]. MHC-1 is expressed in most somatic cells, whereas KIT is expressed in spermatogonia, differentiating from A1 to type B [29]. A combination of the positive and

aforementioned negative markers has facilitated the identification of a specific subpopulation of testicular cells enriched for SSCs. In diploid testicular cells, MHC-1$^-$ KIT$^-$ cells represent undifferentiated spermatogonia, in which ITGA6, CD9, CDH1, MCAM1, and THY1 are expressed. THY1 was strongly expressed in a few somatic cells, including fibroblasts and T lymphocytes; however, its expression in a germ cell fraction was detected at a low level [25, 30]. A THY1$^+$ ITGA6$^+$ KIT$^-$ MHC-1$^-$ subpopulation comprises SSC activity-containing undifferentiated spermatogonia and is one of the most SSC-enriched subpopulations, in which 1 in 15–30 cells is assumed to be an SSC [25]. THY1 appeared to be heterogeneously expressed in ITGA6$^+$ spermatogonia [30]. High SSC activity was detected in THY1$^+$, whereas low SSC activity was detected in THY1$^-$ spermatogonia, indicating the heterogeneity of the cell surface phenotype of SSCs. In addition, THY1 expression on SSCs decreased gradually from neonates to adults, suggesting that the cell-surface phenotype of SSCs in infants and adults or in a proliferation phase and stable phase is different [30]. The regulatory mechanism of THY1 expression in undifferentiated spermatogonia needs to be determined.

The transplantation assay, in conjunction with FACS, can be utilized for investigating intracellular molecules expressed in transplantable SSCs (Fig. 12.3). To identify the SSCs via intracellular molecules, transgenic mice expressing noninvasive reporter genes, such as GFP, YFP, or RFP, under the regulatory elements of the genes of interest, are required. In most cases, a reporter gene is knocked-in to the original genomic locus or inserted into a large genomic fragment or bacterial artificial chromosome (BAC) clone containing regulatory sequences of the gene. Based on the reporter gene expression, subpopulations of spermatogonia can be isolated by FACS, and the SSC activity of these spermatogonia can be determined via the transplantation assay. Using this approach, it has been reported that *Pou5f1 (Oct3/4)*, *Sox2*, *Tert*, and *Id4* are expressed in SSCs [31–34]. *Pou5f1* and *Sox2* are transcription factors essential for pluripotency, and *Tert* is telomerase reverse transcriptase, which is expressed in self-renewing cells to ensure unlimited proliferation. *Id4*, a transcriptional repressor, was identified in SSC-enriched THY1$^+$ undifferentiated spermatogonia from juvenile males [35]. The biological function of ID4 in the SSCs has not been elucidated. Nonetheless, determination of the cell surface phenotype of transplantable SSCs has made it possible to obtain SSC-enriched populations by FACS or magnetic-activated cell sorting (MACS), which could be used for the identification of genes expressed in SSCs, such as *Id4*, by transcriptomic analyses. The transplantation assay for SSCs using reporter mice is a valuable approach to validate the functionality of the gene products with regard to the self-renewal and differentiation potential of SSCs.

These reporter gene-expressing transgenic mice can be used to assess the topographical distribution and cell identity of the reporter gene$^+$ cells in seminiferous tubules. Histological analyses of adult *Id4-GFP* transgenic mice injected with an *Id4*-BAC clone revealed that the GFP signal in the testes was detected in a small subset of *Zbtb16* (*Plzf*)$^+$ undifferentiated spermatogonia (~2%), which were primarily A_s and a few A_{pr}, and pachytene spermatocytes, indicating that *Id4* could be an A_s marker [34]. *Zbtb16* is a transcription repressor that is required for the maintenance

of continuous spermatogenesis in adult males and is expressed in all undifferentiated spermatogonia [36–38]. Intriguingly, the histological and flow cytometric analyses of adult testes of *Id4-GFP* knock-in mice indicated a more widespread GFP expression, including that from ~30% of *Zbtb16*$^+$ spermatogonia, spermatocytes, and spermatids [38, 39]. Although these results of the *Id4-GFP* knock-in mice were contrasting to those obtained for the *Id4*-BAC clone transgenic mice, which claimed that the major *Id4-GFP*$^+$ cells in the testes were A_s, the expression of *Id4-GFP* in a minor subset of undifferentiated spermatogonia was commonly observed in both the *Id4-GFP* transgenic mouse lines. However, it should be noted that flow cytometric analysis of the *Id4*-BAC clone transgenic mice was performed in the developing testes (8 days postpartum; 8 dpp), which did not contain differentiated and haploid germ cells [40]; therefore, it is important to compare the flow cytometric data of the *Id4-GFP*$^+$ cells in the adult testes of the *Id4*-BAC clone transgenic mice with those of the *Id4-GFP*$^+$ cells in the adult testes of the *Id4-GFP* knock-in mice, to clarify the proportion of *Id4*$^+$ cells in mouse testes.

Because it has been reported that SSCs and gonocytes, the precursor cells of SSCs, in postnatal testes could transform the pluripotent stem cells in culture [41–44], the expression of *Pou5f1* and *Sox2*, which are critical transcription factors for pluripotency, in SSCs, is of great interest. Although several *Pou5f1* transgenic mice have been developed, two *Pou5f1-GFP* transgenic mouse lines developed by introducing an 18-kb genomic fragment containing the minimal promoter and proximal and distal enhancers (GOF18) or an 18-kb genomic fragment lacking the proximal enhancer sequences (GOF18ΔPE) were used for the SSC transplantation assay [31, 45]. Either construct is sufficient for reproducing the endogenous gene expression pattern in the germline [46]. Almost all gonocytes in the neonatal testes of the two *Pou5f1-GFP* transgenic mice were GFP$^+$; however, the GFP expression of the undifferentiated spermatogonia in juvenile testes (5–8 dpp) was heterogeneous [31, 38, 47]. While the *Pou5f1-GFP*$^+$ cells from the developing testes contained transplantable SSCs, *Pou5f1-GFP*$^-$ cells also generated spermatogenic colonies, albeit at low numbers [31, 47]. Intriguingly, *Pou5f1-GFP*$^+$ cells in the adult testes (GOF18ΔPE) comprised A_{al} undifferentiated spermatogonia, KIT$^+$ spermatogonia, and spermatids [38]. A_s and A_{pr} undifferentiated spermatogonia in the mice were *Pou5f1-GFP*$^-$ cells. As expected, the *Pou5f1-GFP*$^-$ cells, which were basically *Gfra1*$^+$, generated spermatogenic colonies following transplantation; however, the *Pou5f1-GFP*$^+$ cells in adult testes, which had no A_s spermatogonia, also generated spermatogenic colonies [38]. The SSC activity in the *Pou5f1-GFP*$^-$ undifferentiated spermatogonia of the adult testes was threefold higher than that of the *Pou5f1-GFP*$^+$ cells. Collectively, the expression of *Pou5f1* in SSCs changes developmentally and does not associate faithfully with SSC activity.

To investigate *Sox2* expression in SSCs, *Sox2-GFP* knock-in mice were generated [32]. In the adult testes of the *Sox2-GFP* mice, rare GFP$^+$ cells were detected on the basement membrane of the seminiferous tubules of adult testes. However, the flow cytometric analysis of testicular cells from young *Sox2-GFP* mice (2-week-old) revealed twice as many KIT$^+$ *Sox2-GFP*$^+$ cells as KIT$^-$ Sox2-GFP$^+$ cells, indicating that the differentiating spermatogonia expressed *Sox2*. In the transplantation

assay, the KIT^- *Sox2-GFP*$^+$ cells isolated by FACS displayed SSC activity; however, the KIT^+ *Sox2-GFP*$^+$ cells did not generate any colonies, indicating that no transplantable SSCs existed in the KIT^+ *Sox2-GFP*$^+$ spermatogonia. The discrepancy in the results of the flow cytometric analysis and immunohistochemistry of GFP expression in the *Sox2-GFP* mouse testes remains unexplained; however, *Sox2*, a crucial transcription factor for pluripotency, is expressed in transplantable SSCs. Furthermore, these studies indicate that the expression patterns of *Pou5f1* and *Sox2* in transplantable SSCs are not correlated with each other.

Telomere maintenance is critical for self-renewal in stem cells [48]. In testicular germ cells of adult *Tert-RFP* mice, the *Tert*^{high} population comprised undifferentiated spermatogonia and KIT^+-differentiating spermatogonia, whereas the *Tert*^{low} comprised KIT^+ differentiated spermatogonia [33, 49]. Transplantation assay demonstrated that SSC activity was detected only in the *Tert*^{high} KIT^- cell population. Although the *Tert*^{high} KIT^- cells expressed homogenous ZBTB16, GFRA1 was heterogeneously expressed, and the whole-mount immunocytochemistry indicated that *Tert*^{high} $ZBTB16^+$ $GFRA1^-$ cell population included the long and short chains of A_{al} undifferentiated spermatogonia. To address the SSC activity in the $GFRA1^+$ and $GFRA1^-$ cells, the *Tert*^{high} KIT^- cells were subdivided into the $GFRA1^+$ and $GFRA1^-$ cells, and the transplantation assay was carried out [49]. The *Tert*^{high} KIT^- $GFRA1^+$ population produced threefold more spermatogenic colonies than the *Tert*^{high} KIT^- $GFRA1^-$ cells, but the important point is that the *Tert*^{high} KIT^- $ZBTB16^+$ $GFRA1^-$ cells contained transplantable SSCs. Furthermore, the number of *Tert*^{high} KIT^- $GFRA1^-$ cells was thrice the number of the *Tert*^{high} KIT^- $GFRA1^+$ cells; therefore, the total numbers of transplantable SSCs of the $GFRA1^+$ and $GFRA1^-$ populations are comparable. Intriguingly, the $GFRA1^-$ cells converted to $GFRA1^+$ following transplantation. These results indicate that transplantable SSCs are not limited in the A_s population, and they suggest that SSC niche factors dictate the conversion from A_{al} to A_s after transplantation.

Although the number of A_s spermatogonia in adult mice is estimated to be about 35,000 [50], the number of transplantable SSCs was estimated to be about 3000 [51]. The discrepancy of the numbers of A_s and transplantable SSCs suggest that not all A_s spermatogonia are transplantable SSCs; in other words, a small subset of A_s represent transplantable SSCs. Furthermore, considering the fact that some A_{pr} and A_{al} possessed SSC activity, by which they could regenerate donor-derived spermatogenesis in the transplantation assay, the proportion of A_s among the 3000 transplantable SSCs would further decline.

Spermatogonial Stem Cell Definition Based on Genetic Lineage Tracing

Spermatogonial transplantation is a powerful approach to unequivocally identify SSCs existing in any donor cell population by assessing their self-renewal and differentiation activities. In the transplantation assay, however, SSCs are colonized in

the infertile recipient testes, in which the germ cells were depleted by Busulfan, an alkylating agent, or absent due to a congenital genetic defect, such as *Kit* mutation. Because donor-derived spermatogenesis is regenerated in the germ cell-depleted microenvironments, the SSCs identified by the transplantation assay are likely to represent those under a regeneration condition. It is important to understand the SSC identity and behavior under homeostatic conditions such as those in normal adult testes. To address this, a cell lineage-labeling system using transgenic mice has been developed (Fig. 12.4). For labeling candidate SSCs, a transgenic mouse line expressing a tamoxifen-inducible Cre recombinase (CreER^{T2}) under the control of the regulatory sequence of a gene of interest is crossed with another transgenic mouse line with a floxed-stop *GFP* or *lacZ* reporter gene under the control of a

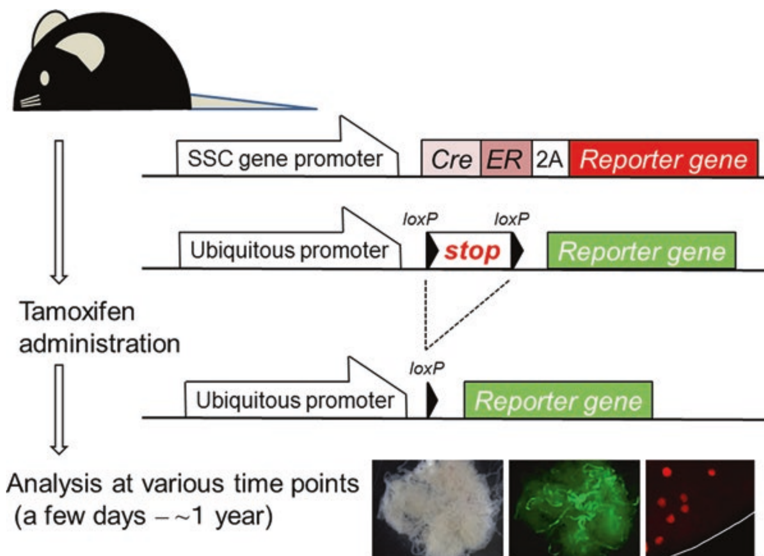

Fig. 12.4 Schematic representation of a cell lineage-tracing experiment to investigate the SSC identity and behavior. Genetic lineage tracing requires a transgenic mouse line with two constructs, Cre recombinase under the control of the regulatory sequence of a gene of interest (SSC gene promoter) and an inducible reporter. In the Cre recombinase construct, Cre is fused to a tamoxifen-inducible mutated estrogen receptor (CreER^{T2}), and a fluorescent reporter (e.g., RFP) is inserted downstream of the SSC gene promoter. Additionally, the two genes are connected by a small 2A peptide sequence that mediates a co-translational cleavage, producing CreER^{T2} and RFP. Alternatively, an internal ribosome entry site (IRES) sequence can be introduced between the CreER^{T2} and RFP sequences to allow the co-expression of the two genes. The RFP enables the identification of SSC candidates, as well as the visualization of the Cre-expressing cells. For a reporter construct, a reporter gene (e.g., GFP) under the control of a ubiquitous promoter is flanked by a *loxP-stop-loxP* (floxed-stop) sequence. When tamoxifen is transiently administered to the mice, CreER^{T2} is translocated to the nucleus and the floxed-stop sequence is removed. The Cre-expressing cells are irreversibly labeled with the reporter gene under the control of the ubiquitous promoter. At various time points after tamoxifen administration, the testes are dissected and analyzed to identify fluorescent spermatogenic colonies. This genetic cell-labeling system can be used in normal and regenerative circumstances

ubiquitous promoter [52]. Tamoxifen administration transiently induces the translocation of CreER^{T2} to the nucleus, followed by the removal of the floxed-stop sequence [53]. Consequently, the Cre recombinase-expressing cells, presumably the cell population expressing the gene of interest, are irreversibly labeled with the introduced reporter gene. The progenies of the cell population are permanently labeled with the reporter gene under the control of the ubiquitous promoter. At various time points after tamoxifen administration, the testes from mice administered with tamoxifen are analyzed to identify the reporter$^+$ spermatogenic colonies. If the labeled cells are SSCs, they replenish the existing differentiated germ cells by forming newly generated spermatogonia; thus, patches of spermatogenic colonies expressing the reporter gene are formed. If the labeled cells are non-SSCs, such as transit-amplifying cells, the reporter gene-expressing spermatogenic colonies are transient and will eventually disappear. Additionally, when a second reporter gene is connected to CreER^{T2} by a small 2A peptide sequence or an internal ribosome entry site (IRES) sequence to allow the co-expression of the two genes, identification of Cre recombinase-expressing cells is possible by detecting the second reporter gene$^+$ cells (RFP in Fig. 12.4), and their topographical distribution in seminiferous tubules can be investigated.

This CreER^{T2}-mediated cell lineage-tracing system demonstrated that *Gfra1*-, *Id4*-, *Sox2*-, *Zbtb16*-, *Bmi1*-, *Pax7*-, *Nanos2*-, and *Axin2*-expressing cells could give rise to reporter gene-labeled long-term spermatogenesis under homeostatic conditions in adult testes, indicating that the cells expressing these molecules are SSCs in undisturbed testes [14, 15, 32, 38, 54–58]. In addition, *Id4*-, *Bmi1*-, and *Pax7*-expressing cells have been shown to be able to regenerate long-term spermatogenesis under regenerative conditions after irradiation or chemotherapy [55–57]. Transgenic mouse studies or whole-mount immunocytochemistry confirmed that these genes were expressed in A_s spermatogonia. As mentioned earlier, while it was shown that most of the GFP$^+$ cells in the testes of *Id4*-BAC clone transgenic mice were A_s [34], the GFP$^+$ spermatogonia in the *Id4-GFP* knock-in mice were not limited to A_s; instead, GFP was broadly expressed in undifferentiated spermatogonia, including A_{pr} and A_{al} [38]. In the third type of *Id4* transgenic mice, that is, *Id4-2A-CreER^{T2}-2A-RFP* knock-in mice, the major population of RFP$^+$ cells comprised A_s spermatogonia, but the age of the mice analyzed was not described, and again, flow cytometric analysis was carried out only in juvenile testes (8 dpp) [55]. Nonetheless, the study clearly demonstrated that *Id4*$^+$ undifferentiated spermatogonia are SSCs in normal and regenerative conditions.

The drawback of the Cre-mediated cell-labeling system is that the quantification of the colony-forming cells is difficult. The efficiency of the tamoxifen-induced removal of the floxed-stop sequence is not 100%. The threshold of intracellular tamoxifen concentration to complete the genetic recombination might be affected by the cell types or cell conditions. In addition, the efficiency of successful recombination will be affected by several factors including the promoter activity of the gene of interest, turnover and stability of Cre recombinase, and gene repair machinery available in the target cells. If the target gene is weakly expressed due to the high stability of the gene product, the target cells might not be efficiently labeled with the Cre recombinase.

Heterogeneity of Spermatogonial Stem Cells

Currently, SSCs are identified by the transplantation assay or cell lineage-tracing experiments. These two methods have revealed that SSCs exhibit heterogeneous phenotypes and that no universal feature covering all SSCs was observed. At the least, almost all SSC activity was detected in undifferentiated spermatogonia, comprising phenotypically and biologically heterogeneous cells. As originally described, undifferentiated spermatogonia display single (isolated), paired, and aligned morphological features. Furthermore, while the expression of ZBTB16 and CDH1 is relatively constant in the A_s, A_{pr}, and A_{al} spermatogonia, and has been used to identify all undifferentiated spermatogonia, the A_s, A_{pr}, and A_{al} spermatogonia display a trend of differential gene expression patterns [14]. *GFRA1*, *Id4*, *Bmi1*, *Pax7*, *Nanos2*, *Lhx1*, *Bcl6b*, *Etv5*, *T*, *Sall4*, *Sox2*, *Eomes*, and *Pdx1* are shown to be preferentially expressed in A_s, whereas *Pou5f1*, *Ngn3*, *Lin28A*, *Sohlh1*, *Sox3*, and *Rarg* are preferentially expressed in long A_{al} in adult testes [14, 15, 32, 38, 54–57]. It should be noted that the use of different transgenic mouse lines or detection methods including flow cytometry, whole-mount immunocytochemistry, and immunohistochemistry, occasionally resulted in a different conclusion (for the *Id4-GFP* mouse, *Id4-RFP* mouse, *Sox2-GFP* mouse, etc.). In general, undifferentiated spermatogonia expressing A_s-oriented genes present high SSC activity in either the transplantation assay or cell lineage-tracing experiments; however, the SSC activity is not limited to the cells expressing A_s-oriented genes. The A_s-oriented gene$^-$ or A_{al}-oriented gene$^+$ undifferentiated spermatogonia from adult testes presented a low level of SSC activity, indicating that SSCs with different gene expression profiles exist. Undoubtedly, the undifferentiated spermatogonia with high SSC activity in developing testes, normal adult testes, and regenerative adult testes appeared to be different. Undifferentiated spermatogonia expressing *Pou5f1* or *Ngn3*, both of which are A_{al}-oriented genes in adult testes, exhibit high SSC activity in the transplantation assay using juvenile testis cells or cell lineage-tracing experiments under a regenerative condition, respectively [14, 15, 31, 38, 47]. The possible key factor for generating heterogeneous SSCs is the surrounding microenvironment or the SSC niche. In developing testes, the SSC niche produces abundant growth factors for self-renewing proliferation and less inhibitory factors, which are supposed to be produced from differentiated germ cells, for self-renewal [59]. On the other hand, the SSC niche in homeostatic adult testes produces a moderate amount of mitogenic factors to maintain a stable SSC number. Accordingly, the majority of the SSCs in developing testes are in a mitotic state, while the SSCs in homeostatic conditions are likely to be quiescent or in a slow cycling state. In regenerating testes, the SSC niche again stimulates growth factor production for SSC expansion.

The dynamic exchange of undifferentiated spermatogonia can occur in regenerative conditions. In the normal adult testes, under homeostatic conditions, undifferentiated spermatogonia expressing A_s-oriented genes (e.g., *Ngn3*$^-$ *Gfra1*$^+$ cells) self-renew and maintain spermatogenesis; however, under regenerative conditions,

spermatogonia expressing A_{al}-oriented genes (e.g., *Ngn3*$^+$ *Gfra1*$^-$ cells) re-express the A_s-oriented genes and acquire SSC activity [14, 38]. In addition, the transplantation assay, which mimics a regenerative condition, detected SSC activity in *Ngn3*$^+$ *Gfra1*$^-$ cells. *Ngn3*$^+$ *Miwi2* (*Piwil4*)$^+$ *Gfra1*$^-$ *Kit*$^-$ spermatogonia, which are mostly A_{pr} and A_{al} and possess characteristics of transit-amplifying cells, are not responsible for homeostatic spermatogenesis. However, this cell population is crucial for regeneration after injury in the adult testes. The transplantation assay demonstrated that the *Ngn3*$^+$ *Miwi2*$^+$ *Gfra1*$^-$ cells indeed have a robust reconstitution activity [60].

Collectively, several lines of evidence indicate that SSC activity is not limited to A_s; the A_{pr} and A_{al} retain or regain SSC activity. Furthermore, live cell imaging experiments have indicated that the differentiation process from A_s to A_{al} spermatogonia is not unidirectional, because A_s or A_{pr} spermatogonia could be occasionally generated from A_{al} by fragmentation [15]. Another intriguing research has reported that KIT$^+$ A1 spermatogonia became transplantable SSCs after culturing in vitro, indicating a possible dedifferentiation from the differentiating spermatogonia to SSCs [61]. These studies should be confirmed using different experimental settings, and the molecular mechanisms underlying these findings should be elucidated.

Concluding Remarks and Future Directions

In the past two decades, several SSC marker genes or A_s-oriented genes expressed in SSCs have been identified by the transplantation assay and cell lineage-tracing experiments (Table 12.1). Unfortunately, the functional roles of the majority of these genes, including *Id4*, *Bmi1*, *Pax7*, *Sox2*, *Eomes*, and *Pdx1*, in SSCs, have not been determined. However, *Gfra1*, which is the receptor of GDNF, is an exception. GDNF is an essential growth factor for SSC self-renewal, and directly drives the self-renewal of SSCs, followed by binding to GFRA1 [62, 63]. *Bcl6b*, *Lhx1*, *Etv5*, *T*, and *Nanos2* have been suggested to be involved in GDNF-signaling in cultured SSCs [64–66]. The list of genes expressed in undifferentiated spermatogonia is increasing, but the functional interaction between these genes and their identified gene products is poorly understood. Recent studies using single-cell transcriptomic analysis of fresh testicular cells and undifferentiated spermatogonia enriched for SSCs have revealed that the expression patterns of all A_s-oriented genes are not correlated with each other [38, 67–70]. Presumably, the gene expression patterns in SSCs are more dynamic and unstable than previously thought. Future studies should address and elucidate the biological significance of the complex omics data, including not only the transcriptome but also methylome, proteome, and metabolome data obtained from undifferentiated spermatogonia. Furthermore, the deterministic roles of different SSC niches in developing, homeostatic, and regenerating testes in SSC heterogeneity should be clarified. Elucidating the functional roles and interactions of characteristic gene products and finding their causal regulators from the SSC

Table 12.1 Cell surface and intracellular molecules directly demonstrated to be expressed in spermatogonial stem cells via two experimental approaches

	Transplantation assay		Cell lineage-tracing experiment	
	Juvenile	Adult	Homeostasis	Regeneration
GFRA1/*Gfra1*	[28]	[49]	[15]	[15]
THY1	[30]	[25, 30]		
CDH1		[23]		
MCAM		[27]		
ITGA6		[22, 30]		
CD9		[26]		
Pou5f1[a]	[31, 47]	[38][b]		
Miwi2[a]		[60]		
Id4	[40]		[55]	[55]
Sox2		[32]	[32]	
Zbtb16			[38]	
Bmi1			[56]	[56]
Pax7			[57]	[57]
Nanos2			[54]	
Axin2			[58]	
Ngn3[a]				[14]

[a]Expressed in A_{al} spermatogonia, transit-amplifying cells, in adult testes
[b]Higher SSC activity in *Pou5f1*$^{-}$ cells than *Pou5f1*$^{+}$ cells

niches will provide new insights into SSC biology and clarify the universal identity of SSCs, that is, characters that could be conserved among various mammalian species including humans, amidst their heterogeneity.

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