Chapter 8
What are male germline stem cells?
Factors controlling germ cell production

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A man produces sperm at the rate of ~1,000 per heartbeat, equivalent to 72,000 sperm in a minute, over 100 million in a day, and 3 billion in a month. Furthermore, male gametogenesis (spermatogenesis) can continue for almost the entirety of a man's life. Unlike women, therefore, men can father genetic children at an advanced age even beyond 70 yrs. The foundation of this remarkable functionality of the male reproductive system is spermatogonial stem cells (SSCs), the stem cell population of male germ line. These cells reproduce themselves (self-renew) while continuously generating committed germ cells that are destined to become spermatozoa (commitment/differentiation). This chapter discusses the basic biology of SSCs and their potential for clinical applications.

How are SSCs defined and how many of them are there?

SSCs represent a small fraction of diploid male germ cells, spermatogonia. In general, stem cells are defined functionally by their ability to regenerate and maintain an adult cell lineage. SSCs are thus recognized by their ability to reconstitute spermatogenesis and support continuous sperm production. Such a function is unequivocally detected when cells of interest are transplanted into the seminiferous tubules devoid of spermatogenesis. This technique, called spermatogonial transplantation, was first established in the mouse model. A single-cell suspension of donor cells is prepared by enzymatic digestion of testes and injected into the seminiferous tubules of recipient mice; recipients lack spermatogenesis due to a genetic mutation or a pretreatment with an alkylating agent, such as busulfan (Fig. 1). SSCs included in the donor cell preparation then engraft and regenerate spermatogenesis, and in successful cases, recipients become able to sire offspring through natural mating. Spermatogonial transplantation is thus an unequivocal SSC detection assay based on the functional definition of stem cells. After

transplantation, only stem cells can survive and regenerate spermatogenesis. Non-stem germ cells do not have long-term self-renewal ability and are thus lost through differentiation even if they survive after transplantation. Notably, SSCs have never been purified or made visible under a microscope, emphasizing the importance of spermatogonial transplantation approach to detect SSCs unequivocally. In addition, since a colony of donor-derived spermatogenesis (blue segments seen in Fig. 1) arises from a single SSC, the number of SSCs that engrafted can be determined simply by counting the number of colonies after transplantation. Two unique properties of spermatogonial transplantation are noted. One is that this is an assay that allows for the detection of SSCs 'indirectly' by observing terminally differentiated cells, spermatozoa. The other is that spermatogonial transplantation detects SSCs only retrospectively; we would not know if there are SSCs in a cell sample or how many of them exist in it before transplantation. Using this technique, SSCs have been estimated to represent 0.01–0.02% of total cells in the seminiferous epithelium in mice. SSCs are expected generally to

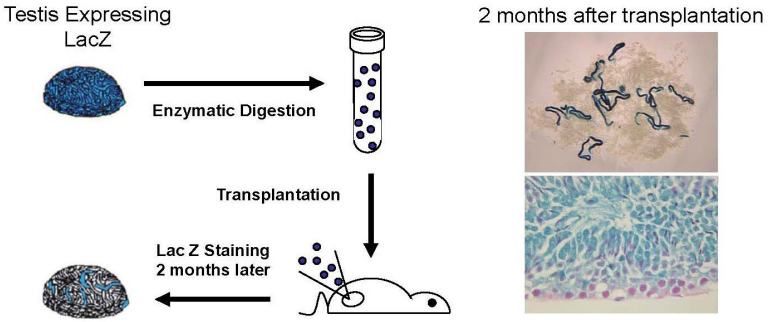

Figure 1. Spermatogonial transplantation in mice. The donor testis is derived from a transgenic mouse carrying the bacterial LacZ gene. The donor testis is digested into single cells using enzymes. These cells are injected into the seminiferous tubules of a recipient mouse that is infertile. Two months later, recipient testes are harvested and reacted with LacZ substrate, which makes donor-derived cells blue. As show in the right panels, SSCs derived from the donor testis regenerate spermatogenesis in the form of blue segments along the recipient seminiferous tubules, which is visualized by the LacZ reaction (blue). Each segment is made of fully regenerated spermatogenesis that the donor SSCs produced.

represent a minute subpopulation of spermatogonia in mammalian species.

What is the morphology of a SSC?

Histological analyses of spermatogenesis have contributed greatly to our understanding of male germ cell biology since the 1800's. One unique aspect of male germ cells is that their cytokinesis is incomplete. After each division, male germ cells remain connected by cytoplasmic bridge, like threaded beads, and this morphology of cell chain is maintained until a cohort of spermatozoa are released from the seminiferous epithelium all at once. This also implies that the cells of origin are isolated and not connected with sister cells. These cells are called Type A-single spermatogonia and had long been believed to be stem cells of the male germ line. We now know that A-single spermatogonia are heterogeneous functionally as well as in terms of other characteristics (e.g., genes and proteins expressed); thus, a majority of A-single spermatogonia do not have the regenerative capacity. On the other hand, some studies have also reported that primitive spermatogonia, which are connected with sister cells, may still retain the regenerative activity and act as SSCs; the regenerative capacity has not yet been lost in these seemingly committed cells. The answer to this question about SSC morphology, therefore, must await the purification and reliable visualization of SSCs in the future.

What is the "fate" of SSCs?

After a division of a SSC, two daughter cells emerge, and for these cells, we can consider three potential paths or fate trajectories. If one of the two daughter cells maintains its "stemness" while the other loses the stem cell activity and decides to become spermatozoa, then, SSC self-renewal and commitment to differentiation are balanced, leading to steady-state spermatogenesis (Fig. 2A). If, however, both daughter cells remain as SSCs, this leads to the proliferation of SSCs and an eventual loss of spermatozoa, disrupting spermatogenesis and male fertility (Fig. 2B). SSC proliferation may cause an abnormal expansion of primitive cells that could develop into cancer. On the other hand, a commitment of both daughter cells to sperm formation might lead to a temporal overproduction of spermatozoa and should cause a loss of SSCs, leading to an eventual loss of spermatogenesis (Fig. 2C). This balance of self-renewal vs. commitment needs not to occur at the level

of individual cells; i.e., not every single SSC needs to take the path of balanced cell division. The normal steady-state spermatogenesis can be achieved when the fate is balanced at the population level. For example, a combination of the fate trajectories shown in Figs. 2B and 2C can lead to an optimal balance of inputs and outputs, at least in theory. This thought experiment also tells us that SSCs do self-renew but must not proliferate to maintain normal spermatogenesis and male fertility.

How is SSC fate controlled?

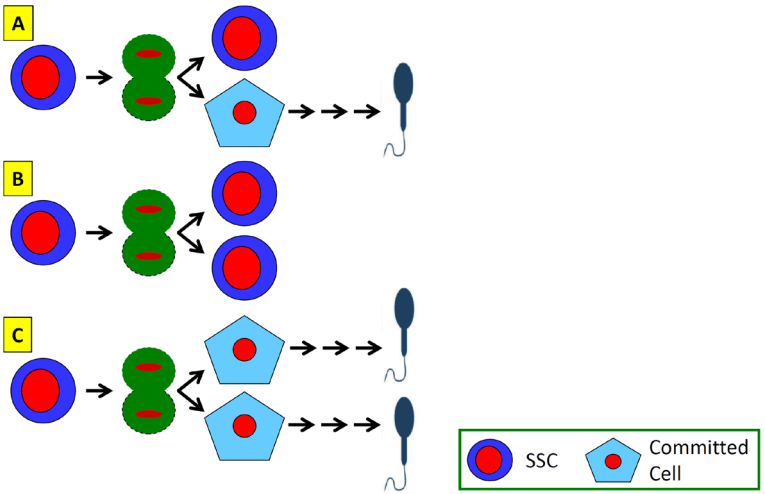

Figure 2. Self-renewal and commitment of SSCs for balanced, steady-state spermatogenesis. SSCs self-renew to maintain a stem cell pool. They can also commit to differentiation to produce sperm. (A) SSC self-renewal and commitment needs to be balanced to maintain normal spermatogenesis. (B) SSCs should not proliferate as a population during normal spermatogenesis. (C) Loss of stemness leads to a temporal overproduction of sperm but eventual loss of spermatogenesis.

This is an area of intensive research and we do not yet know the precise mechanism that controls self-renewal and differentiation of SSCs. However, there are growth factors and hormones that are known to contribute to SSC fate control. Glial-cell-line-derived neurotrophic factor (GDNF) is the critical growth factor that promotes SSC self-renewal. In addition, fibroblast growth factor (FGF) 2 plays

important supportive roles for SSC maintenance. Other growth factors that have been reported to exhibit positive effects on SSC self-renewal include colony-stimulating factor (CSF)-1 and Wnt molecules, whereas retinoic acid is a known differentiation inducer of SSCs and spermatogonia. These factors are secreted by surrounding somatic cells in the environment. The microenvironment that houses stem cells is called a stem cell niche. SSC niches in the seminiferous epithelium are composed of somatic cells, such as Sertoli and myoid cells, acellular elements (extracellular matrices of the basal membrane), and committed germ cells. In addition to soluble factors, SSC niches may influence SSC fate decision through direct cell-cell contact. More studies are required to understand the control mechanism of SSC fate.

Can SSCs be cultured?

Pluripotent stem cells, such as embryonic stem cells (ESCs) and induced pluripotent stem cells (iPSCs), are stem cells generated in vitro and can be cultured for a long time, leading to exponential propagation. Such a property gives these cells practical advantages in biological research as well as clinical applications since they generate an abundant resource of materials to work with. Despite a far lower efficiency of propagation compared to PSCs, mouse SSCs can also be cultured for a long time and propagated exponentially. Mouse SSC culture requires GDNF and FGF2 as well as a feeder cell layer of embryonic fibroblasts that are replication defective. Another essential component for a successful SSC culture is the use of serum-free and lipid-rich medium. Under these conditions and through serial passaging over time, mouse SSCs can be propagated in vitro. Of note, however, is that a majority of cultured spermatogonia are not SSCs, even though they respond to GDNF and FGF2; it is estimated that only ~1–3% of cultured spermatogonia have regenerative capacity. Studies have reported that mouse SSCs are stable in their karyotype and epigenetic marks (e.g., DNA methylation) during long-term culture, in contrast to ESCs, which readily acquire epigenetic and karyotypic abnormalities in vitro. Rat SSCs can also be cultured similarly as mouse SSCs but are far more difficult to maintain in vitro. In vitro culture and amplification of SSCs in other animal species have been attempted and reported but consistent success has been hard to be achieved.

What are the potential practical/clinical applications of SSCs?

Being the foundation of spermatogenesis, SSCs are expected to become a critical resource for male fertility preservation and restoration. For example, anti-cancer therapies can induce male infertility. While sperm cryopreservation is an important option for adult men, it is not applicable for prepubertal boys due to their developmental immaturity. However, since SSCs exist in the testis from the time of birth, these cells can be harvested before the therapy, cryopreserved, and transplanted afterwards to regenerate spermatogenesis. Fertility may be restored spontaneously or via assisted reproductive technologies. Similarly, SSCs of non-human animal species can be used for preservation of species or favored agricultural traits. SSCs can also be used for germline gene modifications. In fact, gene editing in SSCs and even transchromosomal transgenesis via SSCs have been reported in mice to modify the phenotype of offspring. It is important to note that every single step of the SSC-based strategy of male fertility preservation and restoration (harvest testis cells including SSCs, enrich the cells for SSCs, cryopreserve SSCs, propagate SSC in vitro, transplantation, mating that results in offspring production) has been realized in the mouse model. The efforts to translate this achievement in clinical settings are currently underway world-wide.

What is the male mutation bias for transmission of genetic diseases and its linkage to SSCs?

There are inherited diseases and familial syndromes caused by genetic mutations that are transmitted to children solely by fathers, the phenomena called the male mutation bias. Interestingly, the frequency of such diseases increases as fathers become older. A particularly strong male mutation bias has been reported in point mutations of Ret, which is a signal-transducing component of the GDNF receptor, as well as FGF receptor (FGFR) 1 to 3. These mutations result in constitutive activation of these receptors and their down-stream signaling cascades. Ret mutations lead to multiple endocrine neoplasia 2A and 2B and familial medullary thyroid carcinoma while FGFR mutations cause Apert, Crouzon, and Pfeiffer syndromes. Interestingly, GDNF and FGF are two essential growth factors required for long-term amplification of mouse SSCs in vitro (see above). It has thus been proposed that the constitutive

activation of signaling pathways that are beneficial for propagation of SSCs and primitive spermatogonia gradually give these cells proliferative advantage over time, eventually taking over a significant proportion of the sperm production, which results in male mutation biases that become more prevalent as a father ages.

What are SSC markers?

Similar to most bioassays, the functional detection of SSCs provides the information only retrospectively, which makes SSC research difficult and data interpretation confusing. Prospective detection of SSCs requires "SSC markers". A number of marker molecules have been identified at the transcript and protein levels for SSCs as well as their descendants. If such markers are cell-surface proteins, cells expressing and not expressing a marker or markers can be sorted and harvested using immunological methods, such as fluorescent-activated cell sorting or immunomagnetic cell separation. When transplanted, if marker-expressing cells generate more colonies of donor-derived spermatogenesis than unsorted control cells, then, the marker protein is called a positive SSC marker. If colony numbers decline, such a marker is considered to be a negative SSC marker. Immunological cell sorting using these proteins can increase the concentration of SSCs (SSC enrichment), but SSCs have not been purified even in the mouse model and cannot be visualized unequivocally. Considering multiple markers are required to identify hematopoietic stem cells (or a cell population that is highly enriched for these stem cells) in mice and humans, it is logical to assume that SSC identification may also require multiple markers. Thus, it is necessary to be cautious in interpreting data when SSCs are defined or analyzed only by a single gene or protein. Probably, the most important issue regarding SSC markers, which calls for particular attention and care, is the fact that gene X or protein Y may be expressed by SSCs, but not all the cells that express such a gene or a protein are SSCs. For example, GFRα1 is a receptor of GDNF and is a SSC maker, but it is also expressed by primitive spermatogonia that have lost regenerative activity; thus, not 100% of GFRα1-expressing cells are SSCs.

Suggested reading

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