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Regulation of Spermatogonial Stem Cell Self-Renewal in Mammals

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Abstract

Mammalian spermatogenesis is a classic adult stem cell–dependent process, supported by self-renewal and differentiation of spermatogonial stem cells (SSCs). Studying SSCs provides a model to better understand adult stem cell biology, and deciphering the mechanisms that control SSC functions may lead to treatment of male infertility and an understanding of the etiology of testicular germ cell tumor formation. Self-renewal of rodent SSCs is greatly influenced by the niche factor glial cell line–derived neurotrophic factor (GDNF). In mouse SSCs, GDNF activation upregulates expression of the transcription factor–encoding genes *bcl6b*, *etv5*, and *lhx1*, which influence SSC self-renewal. Additionally, the non-GDNF-stimulated transcription factors Plzf and Taf4b have been implicated in regulating SSC functions. Together, these molecules are part of a robust gene network controlling SSC fate decisions that may parallel the regulatory networks in other adult stem cell populations.

Keywords

rodent; stem cell niche; testis; GDNF; transcription factor

INTRODUCTION

Spermatogenesis is the intricate and coordinated process by which thousands of spermatozoa are produced daily within the male gonad or testis (Russell et al. 1990). Morphogenesis of the mammalian testis begins shortly after birth and continues until puberty, at which time the first round of spermatogenesis is completed. This period also establishes the framework for all successive spermatozoa production, which continues until old age in males. In addition to

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DISCLOSURE STATEMENT

The University of Pennsylvania has filed two patent applications on spermatogonial stem cell culture. They are (a) Culture Conditions and Growth Factors Affecting Fate Determination, Self-Renewal, and Expansion of Rat Spermatogonial Stem Cells and (b) Culture Conditions and Growth Factors Affecting Fate Determination, Self-Renewal, and Expansion of Mouse Spermatogonial Stem Cells. R.L.B. is listed as the inventor on each. No patent has been issued.

being the site of spermatozoa production, the testis also serves as an endocrine organ, producing the high levels of testosterone needed for normal spermatogenesis and male phenotypic characteristics. Spermatogenesis is highly coordinated and complex, consisting of three distinct phases (Sharpe 1994). First, in the proliferative phase, spermatogonia undergo a series of mitotic amplifying divisions and differentiate into primary spermatocytes (Clermont 1962) that enter the second phase, or meiotic phase, in which meiosis and genetic recombination occur, resulting in the formation of haploid spermatids (Stern 1993). The third phase, termed spermiogenesis, involves the rearrangement of cytoskeletal structure, transforming round germ cells to specialized spermatozoa (Clermont et al. 1993). Spermatozoa are the vehicle by which male genetic information or germplasm is transmitted to successive generations. Thus, normal spermatogenesis is essential for species preservation and genetic diversity. The entire developmental process from spermatogonia to spermatozoa occurs within seminiferous tubules and is supported by close interaction of the germ cells with somatic Sertoli cells. This interaction forms a complex three-dimensional architecture referred to as the seminiferous epithelium, which is supported by a basement membrane generated from contributions of both Sertoli and outlying peritubular myoid cells (Clermont 1972, Russell 1993). The seminiferous epithelium is divided into a basal compartment, exposed to many lymph- and blood-borne substances, and an adluminal compartment, to which blood-borne substances have limited direct access. These compartments are established by the tight junctions formed between adjacent Sertoli cells, which also provide a major component of the blood-testis barrier. Late-meiotic-stage germ cells, postmeiotic spermatids, and spermatozoa are located in the adluminal compartment, which must, therefore, be an immune-privileged site.

Following puberty, spermatogenesis is one of the most productive cell-producing systems in adult animals, generating approximately 100 million spermatozoa each day in adult men and as many as 1.6 billion sperm per day in male pigs (Sharpe 1994). Approximately 1000 spermatozoa are produced with every heart beat in men. Thus, spermatogenesis is a process in which terminally differentiated cells are continually produced and lost. Like other adult self-renewing tissues whose function relies on the replenishment of differentiated cells at a constant rate or more rapidly after toxic injury, continual spermatogenesis is dependent on an adult tissue-specific stem cell population termed spermatogonial stem cells (SSCs).

SPERMATOGONIAL STEM CELLS

SSCs are the adult stem cell population of the testis, and their biological activities provide a foundation for the high productivity of spermatogenesis. These cells arise postnatally and persist throughout the lifetime of a male. SSCs are rare, with an estimated concentration of 1 in 3000 cells in the adult mouse testis (Tegelenbosch & de Rooij 1993). Thus, little is known of their phenotypic characteristics or mechanisms regulating their functions. Similar to other adult stem cells, SSCs maintain prolonged tissue homeostasis by undergoing both self-renewal and differentiation, which are regulated by extrinsic niche stimuli and intrinsic gene expression.

Origin of SSCs

Postnatally, SSCs arise from more undifferentiated precursors termed gonocytes, which derive from primordial germ cells (PGCs) that migrate from the embryonic ectoderm to the urogenital ridges and take part in formation of the embryonic gonad (Clermont & Perey 1957, Sapsford 1962, McLaren 2003). Upon formation of seminiferous cords during embryogenesis, PGCs become known as gonocytes, which persist until shortly after birth. Transformation of gonocytes into SSCs occurs between 0 and 6 days postpartum (dpp) in male mice (Huckins & Clermont 1968, Bellve et al. 1977, de Rooij & Russell 2000), with the first appearance of biologically active SSCs occurring at approximately 3–4 dpp (McLean et al. 2003). In other species, the transition period of gonocytes into SSCs is largely undefined and may occur over a period of several months in livestock animals or years in humans and other primates. Several studies in mice suggest that two different populations of gonocytes are present in the neonatal mouse testis, in which one subpopulation progresses directly into differentiating spermatogonia and completes the first round of postnatal spermatogenesis without undergoing self-renewal, whereas a second subpopulation transforms into SSCs that then provide the basis for all subsequent rounds of spermatogenesis (de Rooij 1998, de Rooij & Russell 2000, Yoshida et al. 2006). Whether this process is conserved in males of other mammals is currently unknown.

SSC Biological Activities

Similar to other adult stem cell populations, SSCs are capable of undergoing both self-renewal and differentiation (Figure 1*a*). Whether SSC division is a symmetric process or an asymmetric process (Figure 1*b*) in mammals is currently unknown and a topic of debate. Regardless of the symmetry, self-renewal is thought to be an infinite process that results in maintenance of a stem cell pool, allowing for continual spermatogenesis throughout the majority of a male's life span. There are up to nine different spermatogonia populations in mouse and rat, of which there are three major subclasses: type A, intermediate, and type B spermatogonia (Huckins 1978). The type A spermatogonia population consists of A_{single} (A_s), A_{paired} (A_{pr}), A_{aligned} (A_{al}), A_1 , A_2 , A_3 , and A_4 spermatogonia. SSCs are often considered the A_s spermatogonia; this type is the most primitive and does not contain intercellular bridges. As depicted in Figure 1*c*, initiation of spermatogenesis occurs when SSC differentiation results in the production of daughter progeny, the A_{pr} spermatogonia, which are committed to further development into spermatozoa rather than self-renewal (Huckins 1971, Oakberg 1971, de Rooij & Russell 2000). The A_{pr} spermatogonia then undergo a series of mitotic cell divisions to become $A_{\text{al}(4)}$, $A_{\text{al}(8)}$, and $A_{\text{al}(16)}$ spermatogonia, which transform into A_1 spermatogonia, a process that does not include a mitotic division. A series of proliferative divisions then results in the formation of A_2 , A_3 , and A_4 spermatogonia. At this point A_4 spermatogonia mature into intermediate and type B spermatogonia that subsequently enter meiosis to become primary and secondary spermatocytes, leading eventually to the production of haploid spermatids, which undergo a transformation into spermatozoa (Russell et al. 1990). In this model, all spermatogonia more advanced than SSCs (A_s) are considered differentiating spermatogonia (Russell et al. 1990, de Rooij & Russell 2000).

The balance between SSC self-renewal and differentiation is regulated by both extrinsic environmental stimuli and specific intrinsic gene expression. Recent studies suggest heterogeneity of the SSC population in mouse testes, which includes a transiently amplifying population that behaves as SSCs in specific experimental situations and a second, less mitotically active SSC population that is present during normal *in vivo* spermatogenesis (Nakagawa et al. 2007). Direct evidence regarding the origin of these transiently amplifying potential SSCs has not been reported; this population may originate from a subpopulation of the actual SSCs or their early proliferating progeny (Yoshida et al. 2008).

SSC Niche

The function of most, if not all, adult stem cell populations is supported within specialized microenvironments referred to as niches, which provide the extrinsic stimuli to regulate self-renewal and differentiation through both architectural support and growth factor stimulation (Spradling et al. 2001, Scadden 2006). Stem cell niches are formed by contributions of surrounding support cells. In mammalian testes, Sertoli cells are the major contributor to the SSC niche, but contributions by other testicular somatic cells, including peritubular myoid and Leydig cells, are also likely (Figure 1*d*). In recent studies, Yoshida et al. (2007) observed the accumulation of A_{pr} and A_{al} spermatogonia (differentiating daughter progeny of SSCs) in regions of seminiferous tubules adjacent to Leydig cell clusters, suggesting that these cells may contribute to the SSC niche. Additionally, preliminary experiments suggest that Leydig and possibly myoid cell production of the cytokine colony-stimulating factor-1 (CSF-1) influences the self-renewal of SSCs in mice (J.M. Oatley, M.J. Oatley, M.R. Avarbock & R.L. Brinster, unpublished data). Sertoli and Leydig cell function, and likely their niche factor output, is regulated by follicle-stimulating hormone (FSH) and luteinizing hormone (LH) stimulation, respectively. The anterior pituitary gland produces and releases both FSH and LH in response to gonadotropin-releasing hormone (GnRH) stimulation. Studies by Kanatsu-Shinohara et al. (2004b) found that inhibition of GnRH release during postnatal development in mice impairs SSC proliferation, whereas in adult males SSC proliferation is increased when GnRH is suppressed. Other preliminary studies suggest that immunoneutralization of GnRH in mice results in loss of SSC biological activity (J.M. Oatley, L.-Y. Chen, J.J. Reeves & D.J. McLean, unpublished data). These results suggest that gonadotropins play a major role in SSC niche function that may vary depending on the developmental stage of a male.

Currently, a major research focus in adult stem cell biology is the influence that impaired or failed stem cell function may play in the aging process and associated diseases. In principle, stem cells are capable of infinite self-renewal and thus are immune to the normal aging process. However, studies with hematopoietic stem cells (HSCs) suggest that stem cells undergo an aging process and contribute to tissue failure in old age (Siminovitch et al. 1964, Van Zant & Liang 2003, Geiger et al. 2005). Whether SSCs age and contribute to the age-related decline in sperm production experienced by males is currently unknown. Recent studies in mice suggest that SSCs are long-lived and that age-related decreases in fertility are due, at least in part, to impaired function of the niche microenvironment rather than reduced abilities of SSCs to undergo self-renewal and differentiation (Ryu et al. 2006,

Zhang et al. 2006). This result may be due to reduced or modified concentrations of nutrients and hormones in serum of old animals. This hypothesis is supported by work from Conboy et al. (2005) demonstrating that the biological activity of aged liver and muscle progenitor cells in old mice is rejuvenated upon exposure to serum from young parabiotic donors. Clarification of the role that adult stem cells play in aging is likely to be a major research interest in the coming decade, and SSCs with their associated niche may be an effective model system to define principles of adult stem cell aging.

SSC Transplantation

The term stem cell is a biologically functional definition that describes a particular cell type capable of completely reestablishing the functionality of a tissue system from which it is derived. The most direct assay to identify stem cells and examine their biological activities is functional transplantation. In this respect, determination of stem cell identity depends on the ability of a donor cell to reestablish functionality following injection into the stem cell-depleted tissue system of a recipient and to undergo self-renewal and differentiation. Stem cell transplantation assays are available for a multitude of adult stem cell populations, including HSCs (Harrison 1980), neural stem cells (Kelly et al. 2004), epidermal stem cells (Blanpain et al. 2004), and SSCs (Brinster & Avarbock 1994, Brinster & Zimmermann 1994, Nagano et al. 1999, Oatley & Brinster 2006). The SSC transplantation system involves injection of a donor testis cell suspension into the seminiferous tubules of a recipient male in which endogenous germ cells have been depleted by treatment with chemotoxic drugs (e.g., busulfan) or are naturally devoid of germ cells (e.g., W/W^v mutant males). SSCs present in the injected cell suspension are capable of colonizing the recipient seminiferous tubules and reestablishing spermatogenesis (Figure 2). Each colony is clonally derived from a single SSC (Dobrynski et al. 1999, Nagano et al. 1999, Kanatsu-Shinohara et al. 2006). Thus, counting colonies provides a quantifiable measure of SSC number in an injected cell suspension. Currently, this transplantation system is the only unequivocal means to identify SSCs and examine their biological activity. Over the past decade, this transplantation assay system has enabled major advances in elucidating SSC identity and mechanisms that regulate their functions (Brinster 2002, 2007).

SPERMATOGONIAL STEM CELL SURFACE PHENOTYPE

Isolation and identification of SSCs from mammalian testes are essential to examine critically the mechanisms that regulate their functions. Additionally, translation of SSC transplantation techniques from rodents to humans, livestock, or endangered species as an assisted reproductive technology would be greatly benefited by the ability to isolate pure or enriched SSC fractions from total testis cell populations. Currently, there are no known phenotypic or molecular markers to identify mammalian SSCs specifically. All markers described to date are also expressed by other spermatogonia; some markers are even expressed by subpopulations of testicular somatic cells. Although the expression of some markers is restricted to A_s, A_{pr}, and A_{al} spermatogonia sub-types, none described to date can distinguish SSCs (A_s spermatogonia) from their differentiating progeny (A_{pr} and A_{al} spermatogonia). On the basis of the functional definition of a stem cell, SSCs are the only testicular cell type capable of reestablishing spermatogenesis following transplantation,

making the transplantation system the only means to distinguish SSCs from their progeny spermatogonia. Investigators have described several phenotypic cell surface makers that are expressed by SSCs, as well as other spermatogonia, and isolation of testis cell populations on the basis of expression of these markers produces cell populations with varying degrees of SSC enrichment. The expression of some identified phenotypic markers has been legitimately validated by functional transplantation, whereas evidence supporting others has been based mainly on conjecture. In mouse testes, A_{pr} and A_{al} spermatogonia are 2–16 times more abundant than SSCs (de Rooij & Russell 2000). Thus, studies in which analyses are based solely on markers expressed by A_s , A_{pr} , and A_{al} spermatogonia subtypes emphasize differentiating progeny rather than SSCs. Results from those types of studies must be validated by the transplantation technique to distinguish between the different spermatogonial subtypes, or results should be interpreted lightly in regard to advancing the knowledge of SSC biology.

In recent years the expression of several molecules on the surface of SSCs has been reported (Table 1) and has provided an initial understanding of the surface phenotype of mammalian SSCs. There is wide variation in the specificity of these identified phenotypic markers, and no marker described to date is expressed exclusively by SSCs in the testis. Therefore, a pure population of SSCs currently cannot be isolated from any mammalian species. This review focuses on studies that have included transplantation analyses to prove SSC expression of certain markers.

Commonality of Hematopoietic Stem Cell and SSC Surface Phenotypes

Stem cells of many self-renewing tissues are thought to share several characteristics and thus may express similar cell surface molecules. On the basis of this hypothesis, Shinohara et al. (1999) identified expression of α_6 - and β_1 -integrins on the surface of SSCs. In those studies, cell populations expressing these molecules were isolated from testes of adult donor mice by antibody-based magnetic bead isolation and transplanted into testes of infertile adult recipient mice. Results revealed that β_1 - or α_6 -integrin-expressing testis cell subpopulations were enriched approximately four- and eight-fold for SSCs compared with total testis cell populations, respectively. Using a multiparameter fluorescent-activated cell sorting (FACS) approach based on expressions of α_6 -integrin, α_v -integrin, and low side-scatter phenotype (a measure of cellular complexity), Shinohara et al. (2000) isolated, from cryptorchid testes, a testis cell population further enriched for SSCs. Results from those studies revealed that the SSC concentration in the most pure fractions is only approximately 1 in 30–40 cells.

To further increase purity of SSCs in testis cell subpopulations, Kubota et al. (2003) examined cell surface markers known to be expressed by HSCs and identified the expression of the glycosyl phosphatidylinositol (GPI)-anchored glycoprotein molecule Thy1 (CD90) on mouse SSCs. These studies determined that nearly all (~95%) of the SSCs in adult mouse testes are present in the Thy1⁺ cell fraction, which has an SSC concentration of approximately 1 SSC in 15 cells, according to transplantation analyses (Kubota et al. 2003). In adult mouse testes, the Thy1⁺ cell fraction is enriched approximately 30-fold compared with unselected testis cell populations. Additionally, Thy1 expression by SSCs is constant throughout the lifetime of a male mouse (Kubota et al. 2004a). In mouse pups (4–5 dpp), the

Thy1⁺ testis cell population is enriched approximately fivefold compared with the total testis cell population (Kubota et al. 2004a). Together, these studies demonstrated that Thy1 is expressed on mouse SSCs and that the Thy1⁺ cell fraction is highly enriched for SSCs but still does not provide an exclusive identification of SSC phenotype.

Using the same hypothesis that different adult stem cell populations express similar molecules, Kanatsu-Shinohara et al. (2004c) determined that mouse SSCs express CD9, which is also expressed by embryonic stem (ES) cells (Oka et al. 2002), neural stem cells (Klassen et al. 2001), and HSCs (Oritani et al. 1996). However, transplantation analyses revealed that the CD9⁺ testis cell fraction is enriched only 6.9-fold for SSCs compared with the total testis cell population in adult mice (Kanatsu-Shinohara et al. 2004c). This result suggests that CD9 expression is not restricted to SSCs, which was confirmed by further characterization studies revealing CD9 expression in somatic cells and other germ cell types within mouse testes (Kanatsu-Shinohara et al. 2004c). In contrast to conserved expression of Thy1 and CD9, HSCs express high levels of c-kit (Matsui et al. 1990), but SSCs do not share this phenotype (Kubota et al. 2003, Kanatsu-Shinohara et al. 2004c), indicating that the surface phenotypes of all adult stem cells are not identical (Kubota et al. 2003). Unfortunately, the $\alpha_6\beta_1$ -integrin⁺, Thy1⁺, and CD9⁺ testis cell fractions in mice are not composed purely of SSCs. Thus, the SSC phenotype must be further characterized to identify definitive markers with the future applicability of isolating pure SSC populations from the testes of other mammalian species.

The GDNF Receptor Complex as a Specific SSC Phenotype?

The growth factor glial cell line–derived neurotrophic factor (GDNF) is an important niche factor regulating mammalian SSC function (discussed below). GDNF exerts its actions via binding a receptor complex consisting of the transmembrane tyrosine kinase molecule rearranged in transformation (c-Ret) and the GPI-anchored binding molecule GDNF family receptor alpha 1 (Gfra1). Thus, it has been suggested that Gfra1 expression and/or c-Ret expression are restricted to SSCs in mammalian testes. Using transplantation analyses, Ebata et al. (2005) determined that the c-Ret-expressing cell fraction in 6 dpp mice testes is not enriched for SSCs. Characterization studies revealed expression of Gfra1 by multiple spermatogonial subtypes in mouse testes including A_s, A_{pr}, and A_{al} spermatogonia (Ebata et al. 2005, Naughton et al. 2006). Hofmann et al. (2005a, b) isolated Gfra1⁺ cells from 6 dpp mouse testes and determined that this cell fraction expresses several germ cell and spermatogonia makers. These results led to the assumption that Gfra1 is an SSC marker and could be used to isolate SSCs from mouse testes. Unfortunately, functional transplantation experiments did not validate this assumption, and the relative enrichment or purity of SSCs in the Gfra1 cell suspensions isolated by Hofmann et al. (2005a, b) could not be assessed. Additionally, c-kit expression was detected on more than half of these Gfra1⁺ isolated cells (Hofmann et al. 2005b), indicating that Gfra1 is expressed by most spermatogonia, because c-kit expression is first detected on type A₁ spermatogonia in the postnatal mouse testis (Manova et al. 1990, Yoshinaga et al. 1991, Schrans-Stassen et al. 1999). These observations suggest that the majority of Gfra1⁺ cells are not SSCs.

Subsequent studies by Bugeaw et al. (2005) utilizing functional transplantation revealed that the Gfra1⁺ cell fractions of 10 dpp mouse pup testes are less than twofold enriched for SSCs compared with Gfra1-depleted testis cell populations. Thus, the actual SSC content in Gfra1⁺ cell fractions may be less than that in an unselected total testis cell population. This limited SSC content in Gfra1⁺ testis cell fractions was also observed in studies by Ebata et al. (2005), in which SSC enrichment was approximately 2.5-fold higher in Gfra1⁺ cells isolated from 6 dpp mouse pup testes than in the total testis cell population, but a significant difference could not be determined because of experimental variation. Surprisingly, SSCs were reduced approximately 87% in the Gfra1⁺ fraction isolated from adult mouse testes (Ebata et al. 2005), indicating that the majority of Gfra1-expressing cells are non-SSCs at this age. Collectively, these studies strongly demonstrate that the Gfra1⁺ cell fraction, isolated by the procedures described, is at most slightly enriched for SSCs in pup testes. These studies indicate that Gfra1 selection does not result in isolation of SSCs and that use of Gfra1 expression is not an adequate endpoint for analysis of SSCs but likely emphasizes other spermatogonia subtypes that are much more abundant in the testis than are SSCs.

Relation of the Mouse SSC Surface Phenotype to Other Mammalian Species

Translating results describing the SSC surface phenotype in mice to other species has been limited, but the expression of several molecules on the surface of rat SSCs has been identified, and the phenotype of primate SSCs is beginning to be defined. Ryu et al. (2004) used transplantation analyses to reveal expression of Ep-CAM (epithelial cell adhesion molecule) on the surface of rat SSCs. SSC concentration was approximately 1 in 8.5 cells in FACS-isolated Ep-CAM⁺ cell fractions from 8–14 dpp rat pup testes (Ryu et al. 2004). Similar to mouse SSCs, the CD9⁺ cell fraction in rat testes is also enriched for SSCs (Kanatsu-Shinohara et al. 2004c), and Ep-CAM⁺ cell fractions express Thy1 antigen (Ryu et al. 2004). Importantly, recent evidence suggests that nonhuman primate SSCs also express Thy1 (Hermann et al. 2007), and hamster SSCs express α_6 -integrin (Kanatsu-Shinohara et al. 2008). Together, these studies suggest an evolutionarily conserved phenotype of mammalian SSCs, which may be useful for isolating these cells from testes of higher-order mammals, including humans.

EXTRINSIC GROWTH FACTORS INFLUENCING SPERMATOGENIAL STEM CELL SELF-RENEWAL

GDNF Influences Spermatogonial Proliferation and Normal Spermatogenesis in Mice

Currently, knowledge of extrinsic niche factors regulating SSC functions in mammals is limited; only GDNF has been shown to have an essential function. GDNF is a related member of the TGF β superfamily of growth factors and also plays an important role in kidney morphogenesis and the regulation of neuronal progenitor cell function (Sariola & Saarma 2003, Dressler 2006). The first insight that GDNF was an important molecule regulating SSC activity came from studies by Meng et al. (2000), who observed disrupted spermatogenesis in mutant mice carrying one GDNF null allele and accumulation of A_{pr} and A_{al} spermatogonia in testes of male mice that overexpressed GDNF. As discussed above, the GDNF receptor complex consists of c-Ret and Gfra1 (Airaksinen & Saarma 2002). Targeted disruption of GDNF, c-Ret, or Gfra1 results in impaired spermatogenesis in homozygous

null male mice (Naughton et al. 2006). These *in vivo* studies implicated GDNF as a niche factor regulating SSC functions. Importantly, GDNF expression in the mouse testis was localized to Sertoli cells and regulated by the gonadotropin FSH (Tadokoro et al. 2002), which is a major regulator of Sertoli cells' ability to support quantitatively normal spermatogenesis (Griswold 1998, Krishnamurthy et al. 2000). In the course of developing culture systems that support the expansion of mouse and rat SSCs for extended periods of time, GDNF was identified as an essential molecule for self-renewal of SSCs *in vitro* (Kubota et al. 2004b, Kanatsu-Shinohara et al. 2005a, Ryu et al. 2005). Moreover, GDNF enhances the short-term proliferation and survival of bovine (Oatley et al. 2004, Aponte et al. 2005) SSCs and the long-term expansion of hamster (Kanatsu-Shinohara et al. 2008) SSCs *in vitro*. Overall, the importance of GDNF as a niche factor regulating SSC self-renewal both *in vivo* and *in vitro* has been unequivocally demonstrated over the past eight years.

Evolution of Culture Systems that Support Long-Term Self-Renewal of Rodent SSCs

The creation of culture systems that support the self-renewing expansion of SSC numbers for extended periods of time has been achieved over the past five years. Nagano et al. (1998) were the first to demonstrate that SSCs could be maintained *in vitro* for up to four months. Nagano et al. (2003) later suggested that GDNF was important for short-term SSC maintenance *in vitro*, but neither of these studies observed an expansion of stem cell numbers. In 2003, Kanatsu-Shinohara et al. reported the ability for long-term culture of gonocytes, the precursors to SSCs, from 0 dpp mouse pups, which the authors termed germline stem (GS) cells. Although a portion of these cells have stem cell potential, demonstrated via a capability to reinitiate spermatogenesis following transplantation into recipient testes, they likely transform into SSCs following transplantation in a similar fashion as the transformation of gonocytes into SSCs during normal *in vivo* development. Thus, it is possible that *in vitro*, prior to transplantation, GS cells are of a gonocyte or a PGC nature rather than of a true postnatal SSC nature. This reasoning is supported by the detection of Dppa3 (also termed Stella) expression in GS cultures, which is expressed by both PGCs and gonocytes but not spermatogonial subtypes. In addition, expression of Dppa3 is not detected in cultures of self-renewing SSCs established from 6 dpp mouse pups in which gonocytes have converted into SSCs (Oatley et al. 2006). In the original report, Kanatsu-Shinohara et al. (2003) were able to support self-renewing proliferation of mouse GS cells over a five-month period, in which a more-than- 10^{14} -fold increase in cell number was measured. However, the conditions used could support GS cells from only donor mice of a DBA/2 background, and expansion to other donor strains was unsuccessful.

Kubota et al. (2004b) were the first to report long-term culture of postnatal SSCs isolated from 6 dpp or adult mouse testes, ages at which gonocytes have transformed into SSCs. The germ cell clumps that formed in these cultures exponentially expanded for more than five months in serum-free conditions and contained SSCs as shown by functional transplantation. Kanatsu-Shinohara et al. (2005a) subsequently developed a serum-free culture condition for GS cells, in which proliferation of cells from donor mice on a DBA/2 background for more than six months *in vitro* was supported. Hofmann et al. (2005a) reported the generation of immortalized mouse spermatogonial cell lines (e.g., C18-4) and, on the basis of expression

of phenotypic and molecular markers including Gfra1 and Oct3/4, concluded that these cells were SSCs. As discussed above, SSCs are defined by their functional ability to reestablish spermatogenesis following transplantation, and there are currently no known definitive phenotypic or molecular SSC markers. Unfortunately, functional transplantations were not conducted to prove the stem cell nature of cell lines created by Hofmann et al. (2005a), and such cell populations could be other non-SSC spermatogonial subtypes. Homogenous expression of markers by cultured testis cell populations does not qualify them as SSCs. Long-term cultures of GS cells and SSCs homogeneously express specific molecules (including Gfra1 and Oct3/4) but are not all true SSCs capable of colonizing a recipient testis following transplantation (Kanatsu-Shinohara et al. 2003, 2005a, b; Kubota et al. 2004b).

Extension of mouse SSC and GS culture systems to other rodent species has been limited. However, progress has been made for rats. This represents a major technical breakthrough with the potential for creating transgenic or knockout rat lines, techniques that are currently limited owing to difficulties in establishing rat ES cell lines. By optimizing the mouse serum-free condition of Kubota et al. (2004b), Ryu et al. (2005) devised a culture system that supported self-renewing expansion of rat SSCs from several different donor strains for more than seven months. Subsequently, Hamra et al. (2005) demonstrated dramatic expansion of rat SSCs when they were cultured in a complex serum condition similar to that reported by Kanatsu-Shinohara et al. (2003). Recently, Kanatsu-Shinohara et al. (2008) reported long-term culture of hamster SSCs in similar conditions. Extension of serum-free culture conditions that support rodent SSCs to other mammalian species has been slow to evolve but will undoubtedly be a major goal of SSC researchers in the coming years.

GDNF Supplementation Is Essential for Long-Term Self-Renewal of SSCs In Vitro

The development of serum-free culture systems that support SSC expansion has provided major insights into the growth factors important for SSC self-renewal. In a serum-free environment, most cell types require the addition of specific growth factors and hormones to promote their proliferation and survival (Hayashi & Sato 1976, Barnes & Sato 1980). This principle has been especially evident for mouse ES cells, in which maintenance of pluripotency requires supplementation with leukemia inhibitory factor (LIF) (Smith et al. 1988). Over the past five years, the growth factor GDNF has been determined to be an important molecule regulating the proliferation of mouse, rat, hamster, and bull SSCs in vitro (Nagano et al. 2003; Kanatsu-Shinohara et al. 2003, 2008; Kubota et al. 2004a, b; Oatley et al. 2004; Ryu et al. 2005). Using a serum-free, chemically defined condition, Kubota et al. (2004a) demonstrated that GDNF enhances SSC self-renewal over a seven-day period. Kubota et al. (2004b) subsequently reported the definitive evidence that GDNF is essential for SSC self-renewal in vitro, showing that long-term self-renewing expansion of SSCs from several different mouse strains in serum-free conditions is dependent on supplementation of media with GDNF. Recently, Seandel et al. (2007) reported the in vitro expansion of a testis cell population from adult mice, which the authors termed spermatogonia precursor cells (SPCs), for more than one year. Proliferation of SPCs was dependent on GDNF supplementation, and some of the cells were capable of reinitiating spermatogenesis after transplantation, demonstrating the presence of SSCs in the SPC

populations. Additionally, long-term culture of rat (Ryu et al. 2005, Hamra et al. 2005) and hamster (Kanatsu-Shinohara et al. 2008) SSCs relies on the inclusion of GDNF in media, confirming the conservation of GDNF influence on SSC self-renewal in rodent species.

In contrast to all other reports of long-term SSC, GS cell, or SPC cultures, Guan et al. (2006) reported long-term maintenance of SSCs from adult mouse testes in culture conditions without GDNF supplementation and indicated that LIF is the important factor for SSC self-renewal from adult testes. Guan et al. (2006) claimed that the cells could reestablish spermatogenesis following transplantation, but actual evidence was not provided. Thus, it is difficult to assess the SSC content of these GDNF-independent, in vitro-derived testis cell populations on the basis of a single report.

In long-term cultures in which GDNF is the main growth factor supplement, undifferentiated germ cell populations form morula-appearing clumps that are composed of both SSCs and non-SSCs, which are likely A_{pr} and A_{al} spermatogonia produced by differentiation (Kanatsu-Shinohara et al. 2003, 2005b; Kubota et al. 2004b; Ryu et al. 2005; Oatley & Brinster 2006). The relative SSC content of these clumps varies widely at different times during a culture period (Kubota et al. 2004b, Kanatsu-Shinohara et al. 2005b), and in some cases the percentage of true SSCs that can reestablish spermatogenesis following transplantation is low, estimated to be 0.02% in one instance (Kanatsu-Shinohara et al. 2005b). Also, SSC proliferation is extremely limited in serum-free conditions with GDNF as the sole growth factor supplement (Kubota et al. 2004b). These results strongly suggest that other factors besides GDNF are important to fully sustain SSC self-renewal in vitro.

Basic Fibroblast Growth Factor and Epidermal Growth Factor, But Not Leukemia Inhibitory Factor, Supplementation Enhances GDNF-Regulated SSC Self-Renewal In Vitro

Studies to identify additional growth factors that regulate SSC self-renewal have focused on evaluating those that influence the proliferation of other stem cell types. Expansion of PGCs, the embryonic precursors to SSCs, in vitro requires the addition of basic fibroblast growth factor (bFGF) to culture media (Resnick et al. 1992). Kubota et al. (2004b) found that supplementation of bFGF in combination with GDNF enhances long-term self-renewing expansion of SSCs, but bFGF alone is incapable of producing a similar result. Similarly, studies by Kanatsu-Shinohara et al. (2003; 2005a, b; 2006) involving long-term culture of GS cells utilized both serum-containing and serum-free media supplemented with bFGF and GDNF. In feeder-free culture conditions, GS cells proliferated as long as GDNF and either bFGF or epidermal growth factor (EGF) were also included in culture media (Kanatsu-Shinohara et al. 2005a). Similarly, expansion of hamster SSCs in vitro requires supplementation with bFGF in addition to GDNF (Kanatsu-Shinohara et al. 2008). Collectively, these studies demonstrate that bFGF and possibly EGF enhance GDNF-regulation of SSC self-renewal, although the mechanism is undefined. In a quest to identify other factors influencing SSC self-renewal in vitro, several studies have evaluated the effects of supplementing culture media with the pleiotropic cytokine LIF because of its demonstrated importance in maintaining the pluripotency of mouse ES cells (Smith et al. 1988, Williams et al. 1988). The addition of LIF to serum-containing media did not affect the proliferation of mouse SSCs in short-term cultures (Nagano et al. 2003, Kubota et al.

2004a). Moreover, the inclusion of LIF in GDNF-dependent serum-free cultures did not significantly enhance the expansion of mouse SSCs (Kubota et al. 2004b). Cellular response to LIF stimulation involves binding a receptor complex consisting of the promiscuous cytokine receptor gp130 (glycoprotein 130) molecule and a specific LIF receptor (LIFR). Even though weak expression of gp130 on the surface of cultured SSCs was detected by flow cytometry (Kubota et al. 2004b), expression of the transcript was absent in similarly cultured cells (Oatley et al. 2006). Additionally, supplementation of LIF in combination with GDNF had no effect on the proliferation of rat SSCs (Ryu et al. 2005). In contrast, LIF enhances the formation of GS cell clumps in culture but does not affect their self-renewal rate during long-term culture (Kanatsu-Shinohara et al. 2007), suggesting that GS cells may be more PGC-like rather than true postnatal SSCs. Collectively, these studies indicate that, in contrast to its essential role in ES cells, LIF is not a major factor influencing the function of rodent SSCs. Knowledge of other factors that influence SSC self-renewal in vitro is limited. Preliminary studies have revealed that supplementation of GDNF-dependent SSC cultures with CSF-1 enhances mouse SSC self-renewal in vitro (J.M. Oatley, M.J. Oatley, M.R. Avarbock & R.L. Brinster, unpublished data). Because GDNF, bFGF, and CSF-1 are all classified as cytokines, other members of the large cytokine family of factors may also have important roles in regulating SSC functions. Using culture methods to identify growth factors that regulate SSC functions in vitro greatly enhances our understanding of extrinsic niche factors in vivo and provides a bridge to identify intrinsic molecular mechanisms regulating SSC fate decisions.

INTRINSIC MOLECULAR MECHANISMS REGULATING SPERMATOGENIAL STEM CELL SELF-RENEWAL

Disruption of *Plzf* and *Taf4b* Expression Impairs Spermatogonia Activity in Mice

Loss-of-function studies provide a strong approach to examine the importance of specific molecules in the function of particular cell types. Over the past four years, studies involving the assessment of impaired spermatogenesis in mice with inactivating disruption of a specific molecule through either natural mutation or experimental targeting have been used to make several discoveries of transcription regulators potentially involved in SSC functions. Disrupted expression of the transcriptional repressor *Plzf* (promyelocytic leukemia zinc finger protein) in male mice results in impaired spermatogenesis and infertility, which become progressively more pronounced with advancing age (Buaas et al. 2004, Costoya et al. 2004). Testes of these males contain varying percentages of seminiferous tubules with a Sertoli cell-only phenotype, which lack developing germ cells with observable spermatogonia populations, suggesting that SSC functions are impaired. Inactivation of *Taf4b* [TATA box-binding protein (TBP)-associated factor 4b] expression results in a similar phenotype in which Sertoli cell-only tubules are observed and males become infertile by three months of age (Falender et al. 2005). In both types of mutant animals, multiple factors may contribute to the phenotypes, and thus transplantation analyses are the only means to determine whether SSC functions are impaired. Transplantation of germ cells from targeted *Plzf*^{-/-} or homozygous luxoid mutant male mice, which contain an inactivating polymorphism in *plzf* loci, failed to restore spermatogenesis in recipient testes, indicating that SSC functions are impaired in mice lacking *Plzf* expression. Similar

transplant experiments in which Taf4b-deficient germ cells are transplanted into recipient testes have not been reported; however, Taf4b null testes do harbor reestablishment of spermatogenesis from transplanted wild-type SSCs (Falender et al. 2005), indicating that the impaired spermatogenesis in TAF4b null mice is due to germ cell defects. These in vivo-based experiments can be difficult to interpret with regard to SSC self-renewal because disrupted spermatogenesis may be due to a variety of factors in mutant or null animals. Impaired SSC self-renewal or differentiation will result in an identical phenotype of diminishing sperm production and in fertility as a male ages. Additionally, disruption of the hypothalamic-pituitary-gonadal axis will also produce a similar phenotype. Even though transplantation experiments provide a direct assessment of the activity of SSCs lacking expression of specific molecules, it is challenging to make distinctions between effects on SSC self-renewal and differentiation because both impairments will result in a lack of donor-derived spermatogenesis within recipient testes following transplantation. Whether disrupted spermatogenesis in mice lacking Plzf or Taf4b expression or an inability of Plzf-deficient SSCs to reform spermatogenesis following transplantation is due to SSC self-renewal or differentiation is undetermined because impairment of either function would produce an identical result in vivo.

GDNF-Regulated Transcription Factors Are Important for Mouse SSC Self-Renewal

Rarity of SSCs in the testis is a major reason for the limitations of in vivo experiments in examining self-renewal and differentiation. The use of an in vitro system that supports SSC self-renewal provides a means to examine directly the effects from loss of function of a specific molecule on SSC activities. In this experimental condition, self-renewal and differentiation can be distinguished, and secondary factors that may affect SSC functions in vivo (e.g., endocrine disruption) are removed. Combining culture systems with functional SSC transplantation provides an assay system to examine SSC self-renewal specifically. Because GDNF is essential for self-renewal of rodent SSCs, microarray-based gene expression profiling was used to identify genes regulated by GDNF stimulation in cultures proven to contain SSCs by functional transplantation (Oatley et al. 2006). These studies identified the upregulation of several transcription factor-encoding genes, including dynamic regulation of *bcl6b* (B cell CLL/lymphoma 6, member B; also termed *bazf*), *etv5* (Ets variant gene 5; also termed *erm*), and *lhx1* (Lim homeobox protein 1; also termed *lim1*). Each of these molecules has transcription factor activity and plays a role in the function of other cellular systems. Disruption of *Bcl6b* in mice results in impaired T lymphocyte proliferation (Manders et al. 2005), ablation of *Etv5* expression affects overall growth and development (Liu et al. 2003, Yang et al. 2003, Schlessner et al. 2007), and *Lhx1* inactivation results in craniofacial deformities in addition to inhibited gonadal morphogenesis (Kobayashi et al. 2005, Shawlot & Behringer 1995). To determine whether these GDNF-regulated transcription factors are biologically relevant to SSC functions, their expression was transiently reduced individually by RNAi in cultures of self-renewing mouse SSCs. Subsequent transplantation analyses demonstrated impairment of SSC expansion in vitro, strongly suggesting that *Bcl6b*, *Etv5*, and *Lhx1* are transcription factors important for SSC self-renewal (Oatley et al. 2006, 2007).

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GDNF stimulation of cultured SSCs resulted in activation of Src family kinase (SFK) signaling, and pharmacological impairment of SFK signaling completely blocked GDNF up-regulation of these genes without affecting cell survival (Oatley et al. 2007). These results demonstrate a specific pathway involved in SSC self-renewal in which extrinsic GDNF stimulation activates intercellular SFK signaling to stimulate the intrinsic regulation of *bcl6b*, *etv5*, and *lhx1* gene expression levels (Figure 3). In contrast, GDNF stimulation also activates Akt signaling in cultured SSCs (Oatley et al. 2007) and GS cells (Lee et al. 2007), but pharmacological inhibition of Akt in SSCs results in increased apoptosis and impaired expression of both GDNF-regulated and non-GDNF-regulated genes. These results indicate a role of Akt in general cell survival rather than SSC self-renewal (Oatley et al. 2007). Studies by Hofmann et al. (2005b) measured gene expression changes in Gfra1⁺ cells collected from mouse pups upon exposure to GDNF, and results revealed upregulated expression of several genes, including the transcription factor *n-myc*. Subsequent studies by Braydich-Stolle et al. (2007), using similar Gfra1⁺ testis cell populations, observed an important influence of SFK signaling in proliferation, including regulation of *n-myc* expression. Unfortunately, the SSC content of Gfra1⁺ cell populations used in both of those studies was not assessed with functional transplantation. Thus, interpreting whether GDNF affected SSCs as opposed to other Gfra1-expressing germ cell types is difficult (see explanation above). Additionally, in germ cell populations proven to be enriched for SSCs by functional transplantation, the expression of *n-myc* is not affected by GDNF stimulation (Oatley et al. 2006).

In vivo, targeted disruption of *Bcl6b* or *Etv5* expression results in impaired spermatogenesis; a Sertoli cell-only phenotype is observed in varying percentages of seminiferous tubules within adult testes (Chen et al. 2005, Oatley et al. 2006). With advancing age, *Etv5* null males become infertile, whereas *Bcl6b* null males exhibit subfertility at puberty, but effects on their fertility at older ages have not been assessed. These phenotypic observations suggest that SSC function is disrupted in vivo when *Bcl6b* or *Etv5* expression is ablated. Initial observations identified *Etv5* expression in Sertoli cells of adult males, leading some investigators to hypothesize that *Etv5* has an essential role in the maintenance of the SSC niche (Chen et al. 2005). Other studies revealed *Etv5* expression in cultured SSCs, in addition to spermatogonia and Sertoli cells within pup and adult testes (Oatley et al. 2007). Collectively, those observations indicate that *Etv5* may have dual roles in regulating SSC functions: intrinsically, via regulating the expression of specific gene networks, and extrinsically, via influencing niche factor output by Sertoli cells. This dynamic role of *Etv5* in the testis and growth deformities observed following its inactivation may explain the pronounced infertility of male *Etv5* null mice. In contrast, expression of *Bcl6b* and *Lhx1* has been observed only in individual spermatogonia within seminiferous tubules of pup and adult male mice, suggesting a germ cell-specific function in regard to male fertility (Oatley et al. 2007). *Bcl6b* and *Etv5* have been shown to have a role in male fertility, but assessment of *Lhx1* function in spermatogenesis has been challenging because targeted disruption results in neonatal lethality (Shawlot & Behringer 1995). Even though *Plzf* and *Taf4b* have been suggested as molecules important for SSC self-renewal, their expression is not regulated by GDNF in cultured SSCs (Oatley et al. 2006, 2007), and their importance in SSC self-renewal in vitro has not been assessed. Collectively, studies over the past four

years have shaped our current understanding of GDNF influence on SSC function (Figure 3), which involves activation of SFK signaling to regulate the expression of specific transcription factor–encoding genes, including *bcl6b*, *etv5*, and *lhx1*, which are important regulators of self-renewal.

Expression of Core Transcription Factors Regulating Self-Renewal of Pluripotent Stem Cells Is Altered in SSCs

The core transcription factors that regulate self-renewal and pluripotency of ES cells include the POU domain factor Oct3/4, Sox2, and Nanog (Boyer et al. 2005). In these cells, interaction between Oct3/4 and Sox2 controls *nanog* transcript expression (Boyer et al. 2005). Recently, several reports have described the conversion of adult somatic cells into pluripotent ES cell–like cells in vitro, referred to as induced pluripotent stem (iPS) cells (Takahashi & Yamanaka 2006, Takahashi et al. 2007, Wernig et al. 2007, Yu et al. 2007). Ectopic expression of the transcription factors Oct3/4, Sox2, Klf4, and c-Myc is adequate to induce a pluripotent ES-like state in fibroblasts of adult rodents and humans (Takahashi & Yamanaka 2006, Park et al. 2007, Takahashi et al. 2007, Wernig et al. 2007). In another report, forced expression of Oct3/4, Sox2, Nanog, and Lin28 produced similar results (Yu et al. 2007). Interestingly, Oct3/4, Sox2, Klf4, c-Myc, and Lin28 are all expressed by SSC-enriched germ cell populations in vitro (Figure 4), yet a pluripotent nature of these cells or tumor formation following their transplantation is not observed (Oatley et al. 2006; J.M. Oatley, M.J. Oatley, M.R. Avarbock & R.L. Brinster, unpublished data). However, expression of Nanog is not detected in these SSC cultures or similar GS cell cultures and may be the missing piece to the puzzle that would induce pluripotency in testicular stem cell populations (Kanatsu-Shinohara et al. 2005b, Oatley et al. 2006). In fact, the rare appearances of apparently multipotent stem cells in GS cultures are associated with Nanog expression (Kanatsu-Shinohara et al. 2004a). Constitutive expression of Nanog promotes autonomous self-renewal of ES cells (Chambers et al. 2003) but also appears to be dispensable for this fate, likely owing to compensation from other factors (Chambers et al. 2007). However, recent evidence indicates that Nanog expression is essential for PGC maturation in the genital ridge during embryonic development (Chambers et al. 2007). SSC maturation from PGCs or gonocytes is associated with the silencing of Nanog expression, and so induction of Nanog expression may result in a pluripotent state by SSCs (Figure 4). The progress with iPS cells is a major forefront in potential stem cell therapy because pluripotent cells can be generated from patient-specific adult fibroblasts that are immunologically compatible. Perhaps more importantly, iPS cells will be an important model to understand pluripotency, fate commitment, and genetic diseases. SSCs may provide unique insights in this context because they represent one of the earliest fate decisions of epiblast/ES cells and biologically SSCs are of fundamental importance to species continuity.

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Glossary

Spermatogenesis	the process by which mature male gametes or spermatozoa are generated, beginning with the differentiation of spermatogonial stem cells
Stem cells	cells capable of continual self-renewal and generation of progeny committed to differentiation into particular cell type(s). Generally classified as two types; embryonic and adult
Spermatogonial stem cell (SSC)	the adult tissue-specific stem cell population of postnatal mammalian testes. Self-renewal and differentiation of SSCs provide the foundation for spermatogenesis
Self-renewal	the unique ability of stem cells to replicate themselves continually without committing to differentiation
Gonocyte	precursor germ cell of spermatogonial stem cells in the postnatal mammalian testis. Derived from primordial germ cells of the embryonic gonad, which have very similar characteristics
PGC	primordial germ cell
Stem cell niche	a microenvironment providing external stimuli that are essential for stem cell maintenance
Spermatogonial stem cell transplantation	procedure in which a donor or experimental testis cell population is microinjected into recipient testes. Reestablishment of spermatogenesis from injected cells provides a direct measure of stem cell number and assessment of stem cell biological activity
Thy1	thymus cell antigen 1, theta; also termed CD90
Glial cell line-derived neurotrophic factor (GDNF)	a cytokine niche factor that has an essential role in regulating the self-renewal of rodent spermatogonial stem cells
Gfra1	GDNF family receptor alpha 1
Ep-CAM	epithelial cell adhesion molecule
Germline stem (GS) cell	a self-renewing germ cell population derived from culture of gonocytes collected from neonatal mouse testes. These cells have varying degrees of stem cell potential
Oct3/4	POU domain, class 5, transcription factor 1, Pou5f1
Plzf	promyelocytic leukemia zinc finger protein
Bcl6b	B cell CLL/lymphoma 6, member B; also termed Bazf
Etv5	Ets variant gene 5; also termed Erm

Lhx1

Lim homeobox protein 1; also termed Lim1

Induced pluripotent stem (iPS) cells

somatic fibroblasts induced to acquire a pluripotent state upon ectopic expression of specific transcription factors

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SUMMARY POINTS

1. Spermatogenesis and thus male fertility are dependent on the functions of SSCs, which are an adult stem cell population whose self-renewal and differentiation are supported extrinsically through niche stimuli and intrinsically through the regulation of specific gene expressions. Currently, the only unequivocal means to identify SSCs and study their biological activities is functional transplantation.
2. Currently, there are no described markers that specifically identify SSCs in mammals. All phenotypic and molecular markers described to date are also expressed by other spermatogonial subtypes or subpopulations of testis somatic cells. However, SSC-enriched cell populations have been isolated on the basis of expression of α_6 - or β_1 -integrins, Thy1, CD9, Ep-CAM, and Gfra1. In these populations, SSC content varies widely, with the greatest enrichment found in Thy1⁺ and Ep-CAM⁺ cells in mice and rats, respectively. Conversely, the lowest enrichment of SSCs is found in Gfra1⁺ cell populations, in which SSCs are even depleted in this cell fraction when isolated from adult testes.
3. GDNF is a niche factor essential for SSC self-renewal. Overexpression in mice results in the accumulation of undifferentiated spermatogonia, and targeted disruption causes impaired spermatogenesis, suggesting the involvement of GDNF in SSC functions in vivo. Importantly, inclusion of GDNF in culture media is essential for SSC and GS cell self-renewal in vitro, and additional supplementation with bFGF or EGF augments those effects. Currently, culture systems that support long-term SSC self-renewal are available only for mouse, rat, and hamster.
4. Expression of several transcription regulators has been associated with SSC functions. Both Plzf and Taf4b are expressed by multiple spermatogonia subtypes, including SSCs, and targeted disruption of either molecule results in impaired spermatogenesis in mice, with a progressive, age-dependent loss of all germ cells. In cultured SSCs, GDNF regulates the expression of Bcl6b, Etv5, and Lhx1, but not Plzf or Taf4b. Transiently reducing expression of these molecules by RNAi results in impaired SSC self-renewal in vitro. Additionally, inactivation of both Bcl6b and Etv5 expression in male mice results in impaired spermatogenesis, which is manifested as a Sertoli cell-only phenotype in varying percentages of seminiferous tubules.
5. GDNF regulation of the expression of self-renewal transcription factors involves activation of SFK signaling. In addition, GDNF activation of Akt signaling promotes SSC survival.

FUTURE ISSUES

1. Are there molecules solely expressed by SSCs in the mammalian testis, and can these molecules be used to isolate SSCs from a variety of species?
2. Can culture systems that support long-term proliferation of SSCs be developed for non-rodent mammals? Can rodent systems be used as a blueprint to achieve this goal?
3. Are there other niche factors besides GDNF, bFGF, and EGF that influence SSC functions? Are they needed to support in vitro expansion of SSC from nonrodent species?
4. What are the intrinsic gene regulatory networks that control SSC fate decisions, and are these networks disrupted in clinical cases of human male infertility?

Figure 1.

Characteristics of mammalian spermatogonial stem cell (SSC) functions and their associated niche microenvironment. (a) Two possible SSC fate decisions. Stem cells are defined by their ability to produce both more stem cells (self-renewal) and differentiating progeny (differentiation). In the mammalian testis, SSCs undergo either self-renewal, to maintain a pool of SSCs that support fertility for the majority of a male's life span, or differentiation, resulting in the formation of A_{pr} spermatogonia, which are committed to the terminal pathway of spermatozoa production. (b) Possible division pathways of SSCs. Symmetrical division dictates that an SSC divides to produce either two new SSCs (self-renewal) or A_{pr} spermatogonia (differentiation) with an interconnecting cytoplasmic bridge; A_{pr} spermatogonia are destined for differentiation into spermatozoa. The asymmetrical theory suggests that SSC division produces two daughter cells: one new SSC (self-renewal) and one committed differentiating cell that produces A_{pr} spermatogonia upon its next division. It is currently unknown whether one or both of these pathways occur in mammalian testes. (c) Differentiation and proliferation of the spermatogonia population in rodent testes.

Spermatogenesis is the sum of all germ cell divisions and transformations beginning with SSC differentiation and ending with the generation of spermatozoa. Following SSC differentiation is the formation of A_{pr} spermatogonia, which mitotically divide to produce $A_{al(4)}$ spermatogonia followed by $A_{al(8)}$ and $A_{al(16)}$ spermatogonia. More mature spermatogonia are then formed and undergo another series of mitotic amplifying divisions, beginning with A_1 spermatogonia and ending with type B spermatogonia. This stage is followed by meiosis and the formation of haploid spermatozoa. (d) Postulated SSC niche microenvironment in mammalian testes. SSCs reside in the basal compartment of the seminiferous tubule, surrounded by Sertoli cells but below their tight junctions, which separate the seminiferous epithelium into basal and adluminal compartments. Stem cell niches are formed on the basis of both architectural support and specific growth factors produced by so-called niche cells. In the mammalian testis, only Sertoli cells have currently been identified as a niche cell that produces growth factors influencing SSC functions. However, contributions from other somatic cell populations surrounding the seminiferous tubules, such as peritubular myoid cells and interstitial Leydig cells, are also possible.

Figure 2.

The spermatogonial stem cell (SSC) transplantation assay in mice. Stem cells are defined by their ability to reestablish the function of a tissue system from which they are derived. Spermatogenesis is a classic adult stem cell-dependent process in which SSCs continually produce terminally differentiated spermatozoa; thus, SSCs are defined by their functional ability to colonize a recipient testis and generate spermatogenesis. Currently, the only direct means to identify SSCs within a cell population and study their activity is the functional transplantation assay. (a) In this assay a testis cell population is collected fresh from a donor testis or following a culture period and microinjected into the testes of recipient males that have been depleted of germ cells with chemotoxic drugs or irradiation. Additionally, sterile mutant males that lack germ cells (e.g., W/W^v mice) can be effectively used as recipients. After a period of several months, colonies of donor-derived spermatogenesis can be detected in the recipient testes if SSCs were present in the injected cell suspension. When transgenic donors that express a marker gene (e.g., LacZ or GFP) are used, colonies of donor-derived spermatogenesis can easily be detected and quantified. Each colony is clonally derived from an individual SSC; therefore, this system provides a quantifiable measure of SSC number in an experimental cell population. In the schematic presented here, a mixed population of donor testis cells isolated from a male Rosa mouse that expresses LacZ in all germ cell types (represented as *blue* coloring) is microinjected into the seminiferous tubules of an immunologically compatible non-Rosa recipient testis that was pretreated with a chemotoxic drug (busulfan). SSCs in the injected cell suspension (distinguished from other Rosa testes cells by *dark blue* coloring) colonize the recipient seminiferous tubules and reestablish

spermatogenesis. These donor SSC-derived colonies of spermatogenesis can then be visualized several months later upon incubation with X-Gal, which results in blue staining. (b) Use of the SSC transplantation method to assay the SSC content of different testis cell populations. (*Left*) Recipient testis injected with a donor Rosa cell suspension in which no SSC colonization occurred, indicating a lack of SSCs. (*Middle*) Recipient testis injected with a donor Rosa cell suspension containing a small number of SSCs, which is representative of a typical result from transplanting an unselected testis cell suspension. (*Right*) Recipient testis with abundant donor SSC colonization, which is indicative of results obtained following injection of an SSC-enriched cell suspension.

Figure 3.

Current understanding of molecular mechanisms regulating spermatogonial stem cell (SSC) self-renewal in mice. Glial cell line–derived neurotrophic factor (GDNF) is the only growth factor demonstrated to have an essential role in regulating SSC self-renewal, and basic fibroblast growth factor (bFGF) or epidermal growth factor (EGF) enhances this influence. GDNF binds to a receptor complex consisting of c-Ret tyrosine kinase and the GPI (glycosyl phosphatidylinositol)-anchored binding molecule Gfra1 (GDNF family receptor alpha 1). This interaction activates PI3K (phosphoinositide 3-kinase) and Src family kinase (SFK) intercellular signaling mechanisms, leading to downstream activation of Akt signaling, which influences general cellular functions such as survival and proliferation. SFK signaling also elicits a second pathway leading to the regulation of specific gene expression levels that are important for SSC self-renewal. The transcription factor–encoding genes *bcl6b* (B cell CLL/lymphoma 6, member B; also termed *bazf*), *etv5* (Ets variant gene 5; also termed *erm*), and *lhx1* (Lim homeobox protein 1; also termed *lim1*) are regulated through the SFK-activated pathway and are important for the maintenance of self-renewing SSC cultures. Five of the eight known mammalian SFK isoforms—c-Src (Rous sarcoma oncogene), Yes (Yamaguchi sarcoma viral oncogene), Fyn (Fyn proto-oncogene), Lyn (Lyn tyrosine kinase), and Hck (hemopoietic cell kinase)—are expressed in mouse SSCs. Additionally, observations of disrupted spermatogenesis in null mutant mice have pointed to an essential role of the transcription factors Plzf (promyelocytic leukemia zinc finger protein) and Taf4b [TATA box–binding protein (TBP)-associated factor 4b] in mouse SSC self-renewal. However, GDNF does not influence the expression of either Plzf or Taf4b in cultured SSCs, and the importance of either molecule in SSC self-renewal in vitro has not been determined. To date, mechanisms by which bFGF or EGF influences the self-renewal and survival of SSCs have not been reported.

Figure 4.

Expression of transcription factors in nonpluripotent spermatogonial stem cells (SSCs) that are thought to be involved in regulating the pluripotent states of embryonic stem (ES) and induced pluripotent stem (iPS) cells. (a) Expression of Oct3/4 and Sox2 is essential for the maintenance of pluripotency in ES cells, in which these two molecules control the expression of Nanog. (b) Ectopic expression of Oct3/4, Sox2, Klf4, and Myc induces pluripotency in mouse and human fibroblasts (iPS cells). Similarly, ectopic expression of Lin28 and Nanog, in addition to expression of Oct3/4 and Sox2, also induces pluripotency of human fibroblasts. Additionally, Myc expression appears to be dispensable; iPS cells can also be generated by ectopic expression of Oct3/4, Sox2, and Klf4 alone. ES cells also express high levels of Klf4, Myc, and Lin28, but the importance of these three molecules in ES cell pluripotency has not been determined. (c) Cultured SSCs express nearly all the transcription factors regulating ES cell pluripotency and those that induce a similar potential in fibroblasts, including Oct3/4, Sox2, Klf4, Myc, and Lin28, but do not express Nanog. The absence of Nanog expression in SSCs may signify a distinct difference in the transcription factor milieu that regulates the function of an adult stem cell population such as SSCs and that of pluripotent ES and iPS cell populations. During embryo development, the first germ cells formed, primordial germ cells (PGCs), require the expression of Nanog, and these cells can become pluripotent under appropriate conditions. However, SSCs, the postnatal descendants of PGCs, do not express Nanog, and many researchers have found their conversion to pluripotency difficult. Thus, ectopic expression of Nanog may be a missing piece to the puzzle by which SSCs can be artificially transformed into a pluripotent state.

because they already express the array of other molecules that induce pluripotency in somatic cells.

Table 1

Relative spermatogonial stem cell enrichment in rodent testis cell fractions isolated on the basis of expression of specific surface antigens

Surface antigen	Mammalian species examined	Donor age	Relative SSC enrichment ^a	Reference(s)
α_6 -integrin	Mouse			
		Pup	?	-
		Adult	8×	Shinohara et al. 1999
β_1 -integrin	Mouse			
		Pup	?	-
		Adult	4×	Shinohara et al. 1999
Thy1	Mouse			
		Pup (6 dpp)	5×	Kubota et al. 2004a
		Adult	30×	Kubota et al. 2004a
CD9	Mouse			
		Pup	?	-
		Adult	7×	Kanatsu-Shinohara et al. 2004c
	Rat			
		Pup	?	-
Ep-CAM	Rat			
		Pup (8–14 dpp)	11×	Ryu et al. 2004
		Adult	?	-
Gfra1	Mouse			
		Pup (6–10 dpp)	1.8×, ^b 2.5×	Buageaw et al. 2005, Ebata et al. 2005
		Adult	0.13×	Ebata et al. 2005

^aDetermined by transplantation analyses. Values are approximate fold enrichment compared with unselected wild-type total testis cell populations.

^bCompared with a Gfra1-depleted testis cell population.